Genomewide MicroRNA Down-Regulation as a Negative Feedback Mechanism in the Early Phases of Liver Regeneration

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The liver is one of the few organs that have the capacity to regenerate in response to injury. We carried out genomewide microRNA (miRNA) microarray studies during liver regeneration in rats after 70% partial hepatectomy (PH) at early and mid time points to more thoroughly understand their role. At 3, 12, and 18 hours post-PH ~40% of the miRNAs tested were up-regulated. Conversely, at 24 hours post-PH, ~70% of miRNAs were down-regulated. Furthermore, we established that the genomewide down-regulation of miRNA expression at 24 hours was also correlated with decreased expression of genes, such as *Rnasen*, *Dgcr8*, *Dicer*, *Tarbp2*, and *Prkra*, associated with miRNA biogenesis. To determine whether a potential negative feedback loop between miRNAs and their regulatory genes exists, 11 candidate miRNAs predicted to target the above-mentioned genes were examined and found to be up-regulated at 3 hours post-PH. Using reporter and functional assays, we determined that expression of these miRNA-processing genes could be regulated by a subset of miRNAs and that some miRNAs could target multiple miRNA biogenesis genes simultaneously. We also demonstrated that overexpression of these miRNAs inhibited cell proliferation and modulated cell cycle in both Huh-7 human hepatoma cells and primary rat hepatocytes. From these observations, we postulated that selective up-regulation of miRNAs in the early phase after PH was involved in the priming and commitment to liver regeneration, whereas the subsequent genomewide down-regulation of miRNAs was required for efficient recovery of liver cell mass. *Conclusion:* Our data suggest that miRNA changes are regulated by negative feedback loops between miRNAs and their regulatory genes that may play an important role in the steady-state regulation of liver regeneration. (HEPATOLOGY 2011;54:609-619)

The liver has the remarkable ability to regenerate to its original size after injury. As such, liver regeneration after 70% partial hepatectomy (PH) is a unique model system for the study of *in vivo* regulation of cell proliferation and gene expression.[1] In the rat, the entire liver mass can be restored within 7-10 days after PH.[2] However, in the first 4-5 hours after PH, the liver remains refractory to the stimulation of growth factors and is believed to be in a so-called "priming" phase, in which the cells undergo necessary modifications in preparation for the regenerative process. Priming might be critically related to the

Abbreviations: bp, base pair; Dgcr8, DiGeorge syndrome critical region gene 8; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; LR, liver regeneration; miRNA, microRNA; NF-κB, nuclear factor kappa B; PH, partial hepatectomy; Prkra, interferon-inducible double-stranded RNA-dependent protein kinase activator A; qRT-PCR, quantitative reverse-transcriptase polymerase chain reaction; RISC, RNA-induced silencing complex; Rnasen, ribonuclease III, isoform 2; Tarbp2, TAR RNA binding protein 2; 3′ UTR, 3′ untranslated region.

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Received December 7, 2010; accepted May 2, 2011.

This work was supported by the National Institutes of Health (Bethesda, MD) ARRA Grant R01 DK081865-01 (to C.J.S.).

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View this article online at wileyonlinelibrary.com.

DOI 10.1002/hep.24421

Potential conflicts of interest: Nothing to report.

Additional Supporting Information may be found in the online version of this article.

liver's extraordinary ability to accurately restore its original size.[3] Cell-cycle–related genes, such as *p21*, *p53*, and *Mdm2*, are not expressed until 8 hours after PH, whereas the expression of most other genes remain repressed until 24 hours, after which many are expressed in a predictable fashion.[4] The physiological role of this priming period and its underlying mechanisms remain under investigation. After priming, DNA synthesis for hepatocytes begins at ~12 hours and peaks at 24 hours.[5]

It has recently become apparent that microRNAs (miRNAs) might be important players involved in the steady-state regulation of many organ systems. miRNAs are 18-24-bp (base pair) small noncoding RNAs that can bind to the 3′ untranslated region (3′UTR) of mRNAs and regulate their expression and translation. The majority of miRNAs are transcribed by polymerase II[6] and are processed by a protein complex, including Drosha (RNASEN) and Pasha (DGCR8),[7] among others, to form pre-miRNAs in the nucleus. The 70-bp pre-miRNAs are exported into the cytoplasm and further cleaved by the RNase III enzyme Dicer, which interacts with TRBP and PACT to become mature miRNAs. The TRBP/Dicer complex can further recruit Ago proteins to form RNA-induced silencing complex (RISC), which, when directed to target mRNA by the miRNA, can degrade mRNA and/or inhibit its translation.[8]

miRNAs have been implicated in many biological processes, such as tumorigenesis,[9] stem cell differentiation,[10] and organ development.[11] Functions of miRNAs in the physiology and pathology of the liver have also been studied. For example, miR-122, which is one of the most abundant miRNAs in the adult liver, can regulate hepatic lipid metabolism,[12-14] control bile acid synthesis,[15] and is associated with hepatocellular carcinoma, among other functions.[16] The liver-specific conditional Dicer deletion can cause hepatic steatosis, impaired regulation of blood glucose, and promote hepatocellular carcinoma.[17] It is, therefore, highly likely that miRNAs might also play an important role in the process of liver regeneration. For example, it has been reported that miR-21 is up-regulated during the proliferative phase of liver regeneration, targets *Pellino-1*, and could provide a negative feedback mechanism to inhibit nuclear factor kappa B (NF-κB) signaling.[18]

Under this assumption, we carried out a study for the expression pattern of miRNAs during liver regeneration, using miRNA microarrays. We discovered a biphasic expression pattern for most of the miRNAs, including an early overexpression that coincides with the priming period and a subsequent reduction that superimposes on the later phases of cell-cycle– and growth-regulated genes in this model. This was most likely mediated by a negative feedback between certain miRNAs and the proteins involved in miRNA maturation and function, such as Dicer and Drosha, among others, allowing cell proliferation and restoration of liver mass. We, therefore, concluded that miRNAs play an important role in regulating the homeostasis of cell growth and organ size in liver regeneration after 70% PH.

Materials and Methods

Partial Hepatectomies. Male Sprague-Dawley rats (Harlan Sprague-Dawley, Indianapolis, IN), ~175 g, were subjected to sham surgery or 70% PH as originally described.[2] All animal work was approved by the Institutional Animal Care and Use Committee at the University of Minnesota (Minneapolis, MN) and received humane care according to the criteria outlined in the "Guide for the Care and Use of Laboratory Animals" prepared by the National Academy of Sciences and published by the National Institutes of Health (NIH publication 86-23; revised 1985).

Global miRNA Expression Profiling. Genomewide miRNA changes were studied in both sham and PH samples at the indicated time points by a custom microarray platform,[19] as described in Supporting Information. A minimum of 2-3 replicates were studied in each group. Array data for each of the different time points have been deposited in the Gene Expression Omnibus under accession number GSE28404.

Quantitative Reverse-Transcriptase Polymerase Chain Reaction, Immunofluorescence, and Western Blot Analyses. Quantitative reverse-transcriptase polymerase chain reaction (qRT-PCR), Western blots, and immunoflurescence were performed, following the manufacturer's instructions. Please refer to Supporting Information for additional details.

Plasmids, Cloning, and anti-miR. Human *RNASEN* (Drosha), *TARBP2* (TRBP), and *PRKRA* (PACT)-3′UTRs were amplified and cloned into pSGG prom 3′UTR reporter plasmid (SwitchGear Genomics, Menlo Park, CA) by *Nhe*I and *Xho*I. *DICER* and *DGCR8* 3′UTR reporters were purchased from SwitchGear Genomics. Ten individual miRNAs or miRNA clusters were cloned into pcDNA3.1 (Invitrogen, Carlsbad, CA) by *Hind*III/*Xba*I or *Nhe*I/*Xho*I. The miR-17-92 expression construct was kindly provided by Dr. He Lin (University of California, Berkeley, CA). The miRNAs included in the

Table 1. miRNA Changes at Different Time Points During Liver Regeneration

Post-PH (hours)	Up-regulated	No Change	Down-regulated	Total
3	143 (41%)	178 (52%)	24 (7%)	345
6	92 (25%)	160 (43%)	121 (32%)	373
12	84 (30%)	162 (58%)	31 (11%)	277
18	141 (37%)	181 (47%)	61 (16%)	383
24	29 (10%)	55 (20%)	198 (70%)	282
36	62 (19%)	179 (55%)	83 (26%)	324
48	31 (12%)	131 (50%)	98 (38%)	260
72	48 (15%)	133 (41%)	145 (44%)	326

constructs and primers used in cloning are shown in Supporting Tables 2 and 3, respectively. Anti-miR-107, anti-miR-424, and anti-let-7a were purchased from Qiagen (Hilden, Germany).

Cell Culture. Human hepatoma Huh-7 cells were cultured in high-glucose Dulbecco's modified Eagle's medium (DMEM) as previously described.[20] Cells were plated at 70% density 24 hours before transfection. Primary rat hepatocytes were obtained from male (225-250 g) Sprague-Dawley rats via collagenase perfusion, as previously outlined.[21] Please refer to Supporting Information for additional details.

3′UTR Luciferase Reporter Assay. Different 3′UTR reporter constructs were cotransfected with miRNA constructs (or anti-miRs) and the SV40-RL internal control plasmid (Promega, Madison, WI) by Lipofectamine 2000 into Huh-7 cells. Cells were harvested 24 hours after transfection, and luciferase activity was determined by the Dual-Glo Luciferase Assay System (Promega), using a Synergy 2 microplate reader (BioTek, Winooski, VT).

Cell-Proliferation, Cell-Cycle, and DNA-Synthesis Analyses. A number of different miRNA expression constructs were transfected into Huh-7 cells, and cells were harvested after 24 hours. For cell-cycle and cell-death studies of both Huh-7 cells and primary hepatocytes, please refer to Supporting Information.

Results

Biphasic Genomewide miRNA Changes during Liver Regeneration. We analyzed hepatic miRNA expression profiles from both sham and 70% hepatectomized rats from 3 to 72 hours after surgery. Between 300 and 400 miRNAs were expressed at these various time points (Table 1). Comparing sham and PH groups, 208 miRNAs could be detected at all indicated times. Based on their expression levels, we grouped these miRNAs into three sets and classified them as down-regulated (<0.8-fold), unchanged (0.8- to 1.2-fold), and up-regulated (>1.2-fold). To more clearly elucidate the pattern of miRNA changes, 181 miRNAs with expression level changes up to 2-fold at all eight time points were selected to generate the heat map and corresponding histogram (Fig. 1A,B). At time points between 3-18 hours, ~50% of miRNA expression remained unchanged, and 25%-40% were up-regulated (Table 1). However, at 24 hours and later, we detected a significant reduction in expression levels in up to 70% of the miRNAs (Fig. 1A), with a later trend to normal expression. The distribution of miRNA changes at 3, 24, and 72 hours showed a significant shift in expression levels (Fig. 1C).

Next, we determined miRNA distribution at the three time points (3, 24, and 72 hours) that showed the greatest change by microarray (Fig. 1D; Table 1). By Venn diagram, only a small subset of miRNAs exhibited the same expression patterns at 3, 24, or 72 hours post-PH, with 7 up-regulated miRNAs, 21 miRNAs showing no change, and 4 miRNAs that were down-regulated. Taken together, the microarray data suggested that miRNA levels undergo dynamic changes during different stages of liver regeneration after 70% PH and clearly display a biphasic expression pattern, reflecting their key role in regulating the regenerative process.[18,22-24]

Besides the mouse and rat miRNA results described above, we also found that some human miRNAs could also hybridize to the rat liver samples in the microarray study, and determined that the expression changes during the process of liver regeneration displayed similar patterns (Supporting Table 1).

Confirmation of the Biphasic Expression Pattern of miRNAs During Liver Regeneration. To validate the microarray results, qRT-PCR was performed for 20 miRNAs, representing all three expression patterns (i.e., up-regulated, unchanged, and down-regulated). The correlation between microarray and qRT-PCR results was ~80% at both 3 and 24 hours, with the best fit observed in the down-regulated miRNAs (Fig. 2A,B; Supporting Table 2). We also verified the time course of expression of miRNAs, let-7, miR-21, miR-29, and miR-30 at 3, 24, and 72 hours postsurgery (Fig. 2C). The qRT-PCR data confirmed the microarray results supporting the biphasic genomewide changes observed in the miRNA expression patterns at the various times post-PH.

Down-Regulation of miRNA Processing Genes Are Correlated with Repression of miRNAs. We postulated that the regulatory mechanism(s) involved in miRNA processing were responsible for this genomewide miRNA down-regulation at 24 hours post-PH.[4,25] To test this hypothesis, we studied the expression

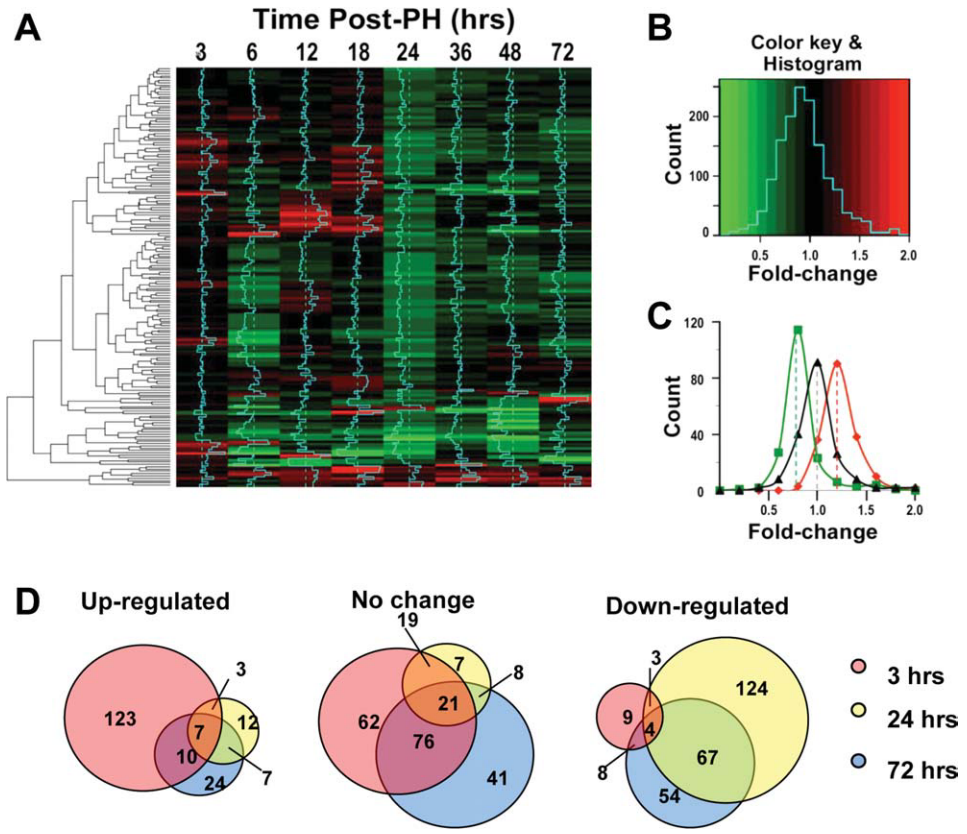

Fig. 1. Biphasic changes in genomewide miRNA steady-state levels during liver regeneration by miRNA microarray. (A) Heat map of miRNA changes between sham and PH samples at the eight different time points postsurgery. The miRNA clustering tree is shown on the left side, and the time post-PH is indicated above. Increases in miRNA are indicated by red, green denotes a decrease, and no change in miRNA level is indicated by black. The fold-change in a specific miRNA is represented by the blue curve in each column, with changes to the left of the center dotted line representing loss, whereas changes to the right of the dotted line represent increases in steady-state miRNA levels. (B) Color key and histogram showing the color assigned for the change in steady-state levels of all miRNAs at the indicated time points. The *x*-axis represents the fold-change in the miRNA steady-state levels in PH samples, compared to sham, whereas the *y*-axis represents the number of miRNAs that demonstrated fold-change in steady-state levels during LR. (C) Histograms representing the distribution of all miRNAs at 3 (red), 24 (green), and 72 hours (black) postsurgery. (D) Venn diagrams showing the analysis of individual miRNAs at 3, 24, and 72 hours post-PH in the up-regulated (left), no-change (center), and down-regulated (right) groups. Red circles represent miRNAs detected at 3 hours, yellow circles those at 24 hours, and blue circles miRNAs 72 hours post-PH. The number of specific miRNAs, detected and/or common to all three groups at the different times, are indicated by the numbers in their corresponding portions of the Venn diagrams.

patterns of miRNA-processing genes *Rnasen* (Drosha) and *Dgcr8* (Pasha), *Dicer*, *Tarbp2* (TRBP), and *Prkra* (PACT) during liver regeneration (LR). Our results indicated that gene expression was not stable in sham controls, suggesting some modulation of gene expression associated with the stress of the sham procedure (Supporting Fig. 1). To obviate effects from the stress, we normalized the results of treated sample to that of sham controls, as previously reported.[26-28] The qRT-PCR results of sham and PH samples revealed that miRNA-processing gene transcripts were significantly down-regulated between the 3- and 24-hour time points (Fig. 3A). We also examined the expression level of *Eif2c2* (Ago2), one of the four argonaute genes well characterized for their critical role in the RISC complex. In contrast to the miRNA-processing genes, Ago2 showed a significant increase (40%) at 3 hours. Because of their critical role in miRNA processing, protein levels of both Dicer and Drosha were studied by Western blot (Fig. 3B) and immunofluorescence in 3-, 18-, and 72-hour samples (Fig. 3C). Expression of both proteins was decreased in PH samples, compared with sham, and correlated with changes in mRNA levels. There were no detectable differences in immunofluorescence, however, between PH and sham for Dicer at 3 and 72 hours and for Drosha at 72 hours (data not shown). These data support the notion that the genomewide miRNA down-regulation occurring at times later than 3 hours post-PH is likely the result of an early repression of genes responsible for processing miRNAs.

Negative Feedback Mechanism Between miRNAs and Their Processing Genes in the Regenerating Liver. The above studies indicated that the miRNA-processing gene *Rnasen, Dgcr8, Dicer, Tarbp2*, and *Prkra*

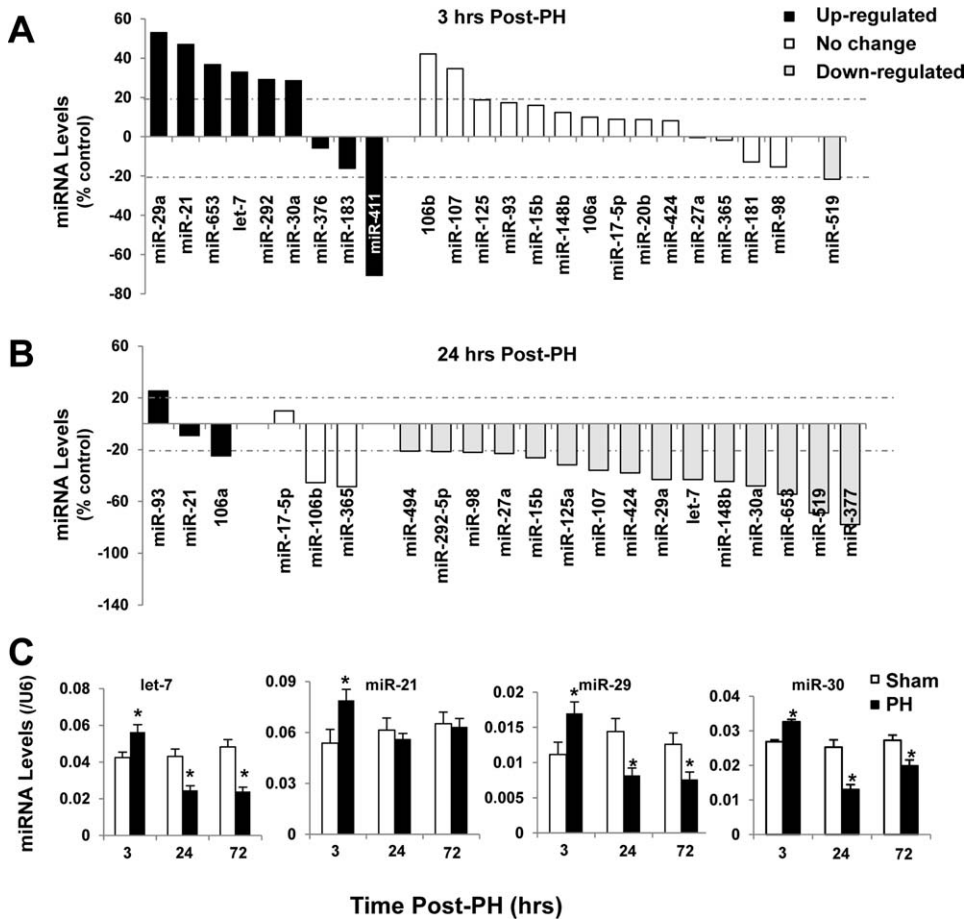

Fig. 2. Confirmation of microarray results by qRT-PCR. Changes of selected miRNAs between PH and sham controls at 3 (A) and 24 hours (B) post-PH by real-time PCR. miRNAs that showed increased expression by microarray analysis are indicated in black; those with no change in expression are white, whereas gray represents miRNAs that were decreased post-PH. The *y*-axis indicates percent change in PH samples determined by qRT-PCR; 20% or greater changes were defined as increased or decreased for both the microarray and qRT-PCR analyses. (C) Steady-state levels of miRNAs in both sham and PH samples at 3, 24, and 72 hours post-surgery determined by real-time PCR. U6 was used as the internal control. $^*P < 0.05$, from sham-operated control.

transcripts were down-regulated at 3 and/or 24 hours in hepatectomized animals. This occurred concurrently with the genomewide down-regulation in the majority of miRNAs at 24 hours post-PH. However, let-7 was up-regulated at 3 hours (Fig. 2A), and it was previously reported that the let-7 family of miRNAs can target and reduce Dicer expression.[29,30] Therefore, we hypothesized that a negative feedback loop, mediated by the up-regulated miRNAs at 3 hours, was a potential mechanism involved in the down-regulation of these miRNA-processing genes.

To test our hypothesis, the complete 3′UTRs of human *RNASEN, DGCR8, DICER, PRKRA,* and *TARBP2* were inserted after a luciferase reporter cDNA to monitor miRNA activities. Based on TargetScan predictions, we selected 11 candidate miRNAs or miRNA clusters, which were also up-regulated at 3 hours post-PH and could potentially target the 3′UTRs of the five miRNA-processing genes for further studies (Supporting Table 4). The targeting sites of these miRNAs on the 3′UTRs of the five miRNA-processing genes are conserved between humans and rats. All 11 miRNAs or miRNA clusters were cloned into the pcDNA3.1 vector, and constructs of pcDNA3.1-miR and luciferase-3′UTR reporter were cotransfected into human hepatoma Huh-7 cells. Using this luciferase reporter system, with Dicer1 and let-7a as positive controls, we found that expression of all five genes could be regulated by a subset of these miRNAs or clusters (Fig. 4A). With *Dicer1* as an example, we selected nine miRNAs, including let-7, miR-17-92 cluster, and miR-21, which were overexpressed at 3 hours and could potentially target *Dicer* mRNA. We found that overexpression of seven of these nine candidate miRNAs could target the *Dicer* 3′UTR, resulting in a significant decrease in luciferase expression, including let-7, consistent with previous reports.[29,30]

To confirm the effects of these miRNAs on the processing genes, we also attempted to inhibit them with miRNA antagonists. Among the miRNAs we cloned, only miR-107 and miR-424 are single miRNAs, whereas others are clustered, and let-7a has been reported to function in the regulation of Dicer. Therefore, we selected antagonists for these three miRNAs for further studies. The antagonists were cotransfected with *Dicer* and *TRBP* 3′UTR reporter vectors (Table 2). The empty pcDNA-3.1 vector was used as a

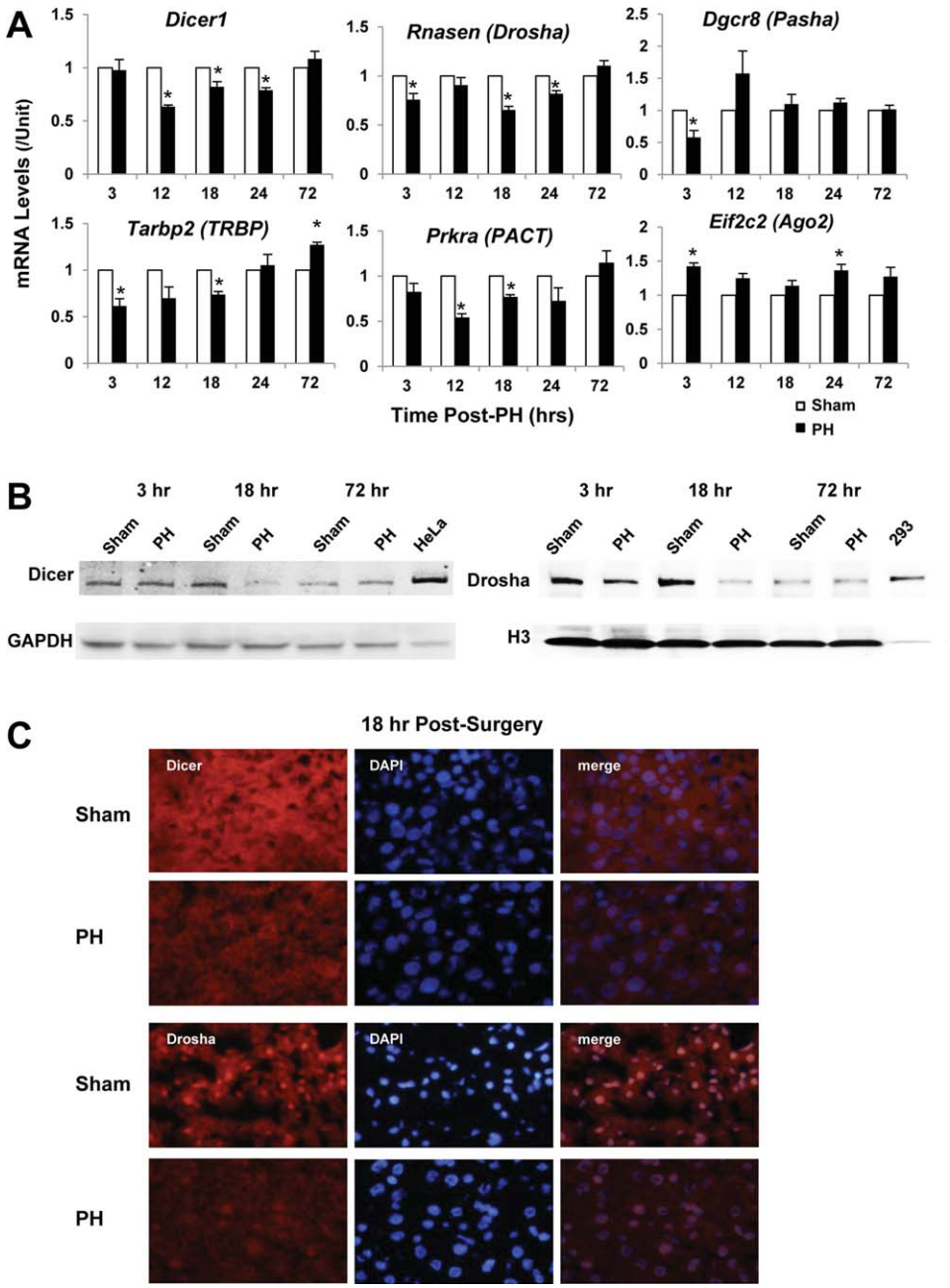

Fig. 3. Changes in key proteins involved in miRNA processing and function during liver regeneration. (A) mRNA levels of the miRNA-processing proteins. RNA from both sham-operated and hepatectomized animals from 3, 12, 18, 24, and 72 hours postsurgery was examined by qRT-PCR, using 18S rRNA as the internal control, and sham was used as the unit for normalization. Open bar represents sham control, and black bar represents PH samples, with the time point postsurgery below and the gene/protein designation above. $*P < 0.05$, from sham-operated control. (B) Protein levels of miRNA-processing genes by Western blot analysis. Liver tissue from both sham and PH samples at 3, 18, and 72 hours post-PH was processed as described in Materials and Methods. Dicer expression was tested in the cytoplasmic fraction, using glyceraldehyde 3-phosphate dehydrogenase (GAPDH) as an internal control and HeLa cells as a positive control. Drosha expression was detected in the nuclear fraction, with H3 as an internal control and HEK293 cells as a positive control. (C) Protein levels of miRNA-processing genes by immunofluorescence. Liver tissue from both sham and PH samples at 18 hours post-PH was studied by immunofluorescence with anti-Dicer and anti-Drosha (red). Immunoglobulin G was used as a negative control (data not shown), and 4′,6-diamidino-2-phenylindole was used for nuclear staining (blue).

negative control. Our results indicated that the antagonist was able to reverse the inhibitory effects of endogenous miRNAs, as luciferase activity was increased by approximately 20% (Fig. 4B). From these studies, we concluded that a negative feedback loop exists between the miRNAs and their processing proteins.

To validate data from the 3′UTR luciferase reporter assays, endogenous mRNA levels of *Drosha*, *Pasha*,

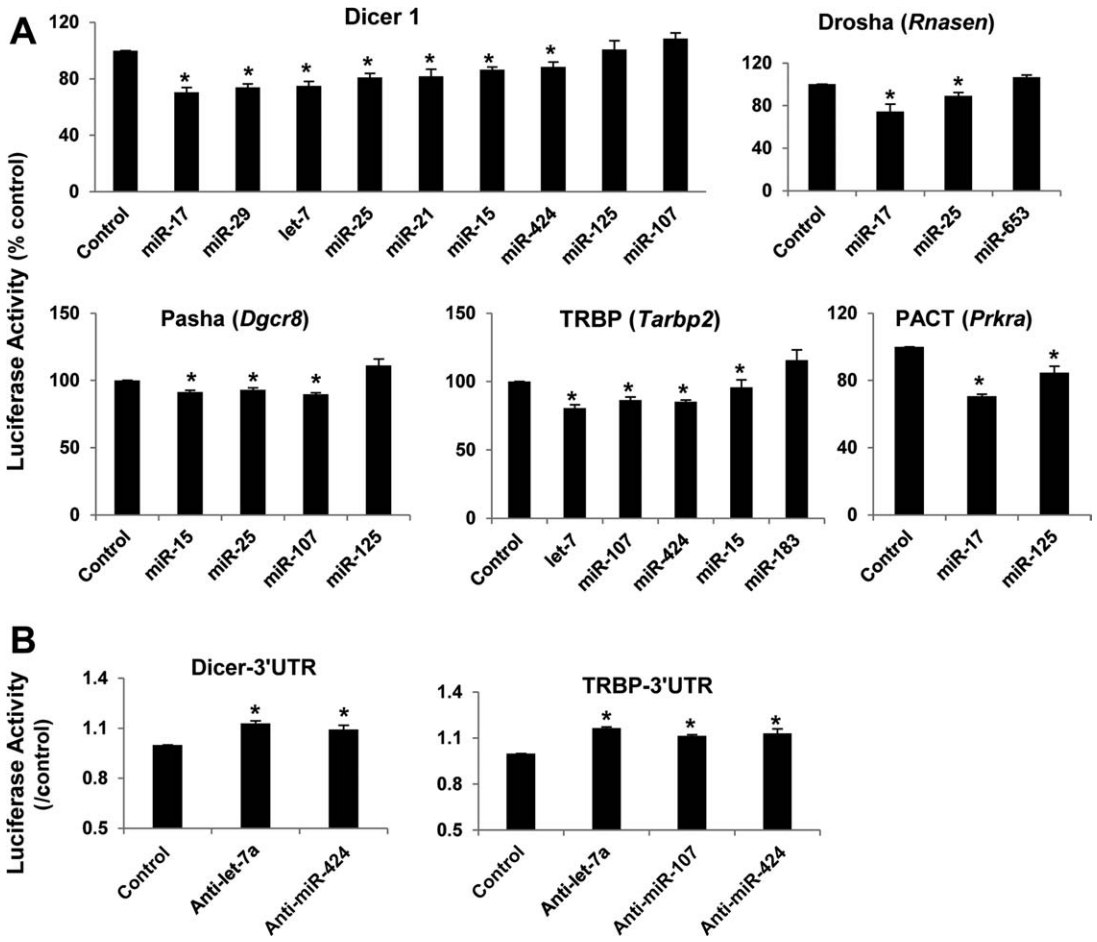

Fig. 4. Regulation of miRNA-processing proteins by miRNAs and miRNA antagonists. (A) Overexpression of miRNA-targeting, miRNA-processing protein 3′UTRs down-regulate luciferase reporter constructs. Huh-7 human hepatoma cells were cotransfected with the different 3′UTR luciferase reporter plasmids carrying the full-length human 3′UTR of the five miRNA processing genes, along with the indicated miRNA or miRNA cluster overexpression construct. Cells cotransfected with the same 3′UTR luciferase reporter and the pcDNA 3.1 empty vectors were used as control, and their luciferase activity was defined as 100%. Changes in luciferase activity in cells transfected with individual miRNAs, compared to control, were displayed. $*P < 0.05$, from respective 3′UTR control. (B) Overexpression of miRNA antagonist inhibit the repression function of miRNAs on their target 3′UTR reporters.

Dicer, and *TRBP* were determined by qRT-PCR and the protein levels of Dicer and Drosha were studied by Western blot in Huh-7 cells. mRNA and/or protein levels of these genes were also decreased with overexpression of the miRNAs that targeted their 3′UTRs, consistent with the luciferase reporter assay results (Fig. 5; Supporting Fig. 3). Interestingly, we found that each of these five miRNA-processing genes could be regulated by a group of miRNAs, and, as an example, *Dicer* could be targeted by seven of these miRNAs. A similar phenomenon was also observed with *Drosha*, *Pasha*, *TRBP*, and *PACT* (Fig. 4). We also found that six of the individual miRNAs could simultaneously target multiple processing genes (Table 2). For example, miR-17-92 cluster could target *Drosha*, *Dicer*, and *PACT*, all of which are involved in different stages of miRNA processing.

In addition to the 11 miRNAs, many other candidate miRNAs, which could potentially target the miRNA-processing genes, were identified by TargetScan software analysis. We analyzed the expression pattern of the predicted miRNAs that potentially target *Drosha*, *Pasha*, *Dicer*, *PACT*, and *TRBP* at 3 hours post-PH. TargetScan

Table 2. miRNAs and Their Validated Target Genes

Pasha	*Dicer*	*Drosha*	*PACT*	*TRBP*
	let-7			let-7
miR-15				
	miR-17	miR-17	miR-17	
	miR-21			
miR-25	miR-25	miR-25		
	miR-29			
miR-107				miR-107
			miR-125	
	miR-424			miR-424

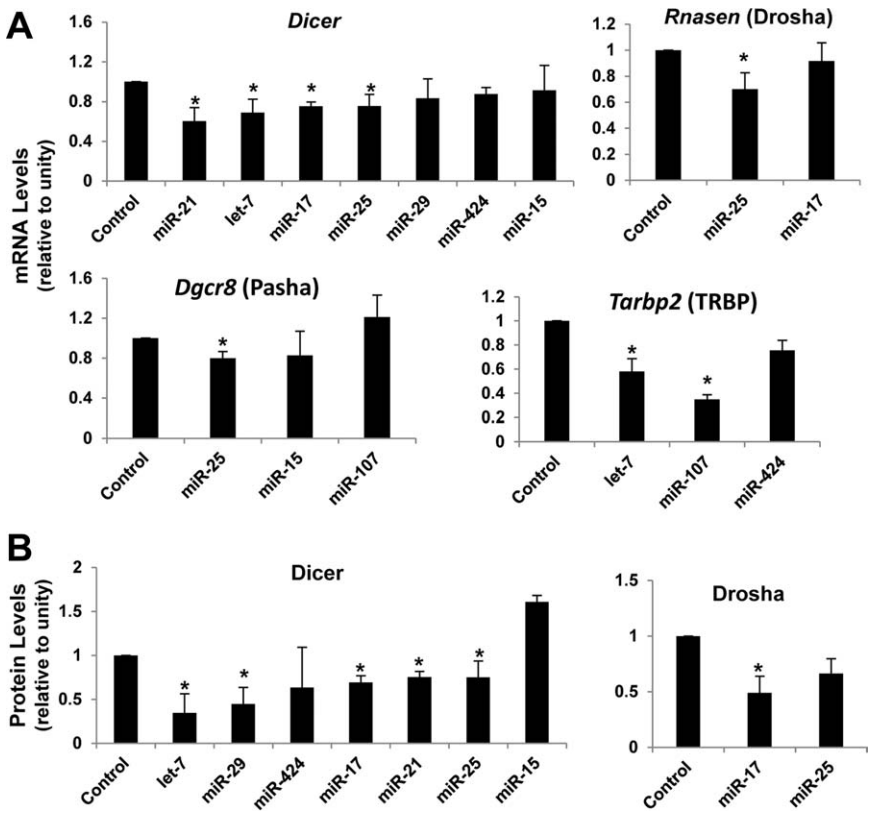

Fig. 5. Endogenous miRNA-processing genes are down-regulated with overexpression of candidate miRNAs in Huh-7 cells. (A) mRNA levels of four of the processing genes that showed significant changes from controls were examined after overexpressing the relevant candidate miRNAs in Huh-7 cells by qRT-PCR, using GAPDH as the internal normalization control. (B) Protein levels of Dicer and Drosha were determined after candidate miRNA overexpression in Huh-7 cells by Western blot, using tubulin as the internal control. Huh-7 cells transfected with the pcDNA 3.1 empty vector were used as control, and the mRNA or protein levels for the different miRNA-processing genes were defined as 100%. Changes in the target gene expression in cells transfected with the miRNA overexpression constructs, compared to control, are shown. $*P < 0.05$.

predicted that rat *Dicer* was targeted by 131 miRNAs, of which 83 could be detected by microarray analysis. Among the miRNA candidates, the majority (55 of 83; 66%) did not change after PH; 34% (28 of 83) were up-regulated, and none were down-regulated (Supporting Table 5). Thus, based on these results, *Dicer* could be down-regulated at 3 hours post-PH by increased expression of, potentially, 28 miRNAs targeting its 3′UTR. Similar results were also observed for *Drosha*, *Pasha*, *TRBP*, and *PACT*.

miRNAs Expressed Early in LR Regulate Cell Proliferation and Cell Cycle in Huh-7 Cells. To elucidate the biological relevance of miRNAs that target their own processing genes to mediate a negative feedback mechanism, we used the Huh-7 human hepatoma cell line as an *in vitro* model. We studied the role of these 10 miRNAs or clusters in cell proliferation after transfecting the Huh-7 cells with each of the pcDNA3.1 miRNA overexpression constructs. We found that overexpression of 8 of 10 miRNAs, except for the miR-25a and miR-125a clusters, reduced total cell number by 10%-30% ($P < 0.05$) (Fig. 6A). This decrease in total cell number was not the result of cell death, as indicated by propidium iodide staining with flow cytometry (Fig. 6B).

To elucidate the mechanism by which the miRNAs might regulate cell proliferation, we examined whether their overexpression arrested cells in specific stages of the cell cycle in Huh-7 cells. Interestingly, we found that overexpression of 7 of the 10 miRNA constructs dramatically decreased cell number in the S-phase (Fig. 6C, left panel). We also consistently observed minor increases in cell number, both in the G_1 and G_2-M phases (Fig. 6C, middle and right panels). The results suggested that these miRNAs, in some manner, either inhibited DNA synthesis or blocked cell-cycle progression at the G_1/S-phase check point.

miRNA-mediated Regulation of Cell Proliferation in Primary Rat Hepatocytes. To validate these results in nontransformed hepatocytes, we carried out miRNA overexpression studies in rat primary hepatocytes induced to proliferate under cell-culture conditions. We found that overexpression of several of the miRNAs, including let-7a, miR-17-92 cluster, miR-29, miR-30, and miR-424, in rat hepatocytes caused a decrease in number of viable cells by ~10% (Fig. 6D). Interestingly, when DNA synthesis was examined in cells overexpressing miRNAs identified as reducing the number of viable cells, a corresponding decrease of 10%-20% was observed (Fig. 6E). Taken together, the results suggested that these miRNAs play a key role in modulating the proliferative capacity of hepatocytes mediated, in part, by directly targeting the 3′UTRs of the miRNA-processing pathway genes.

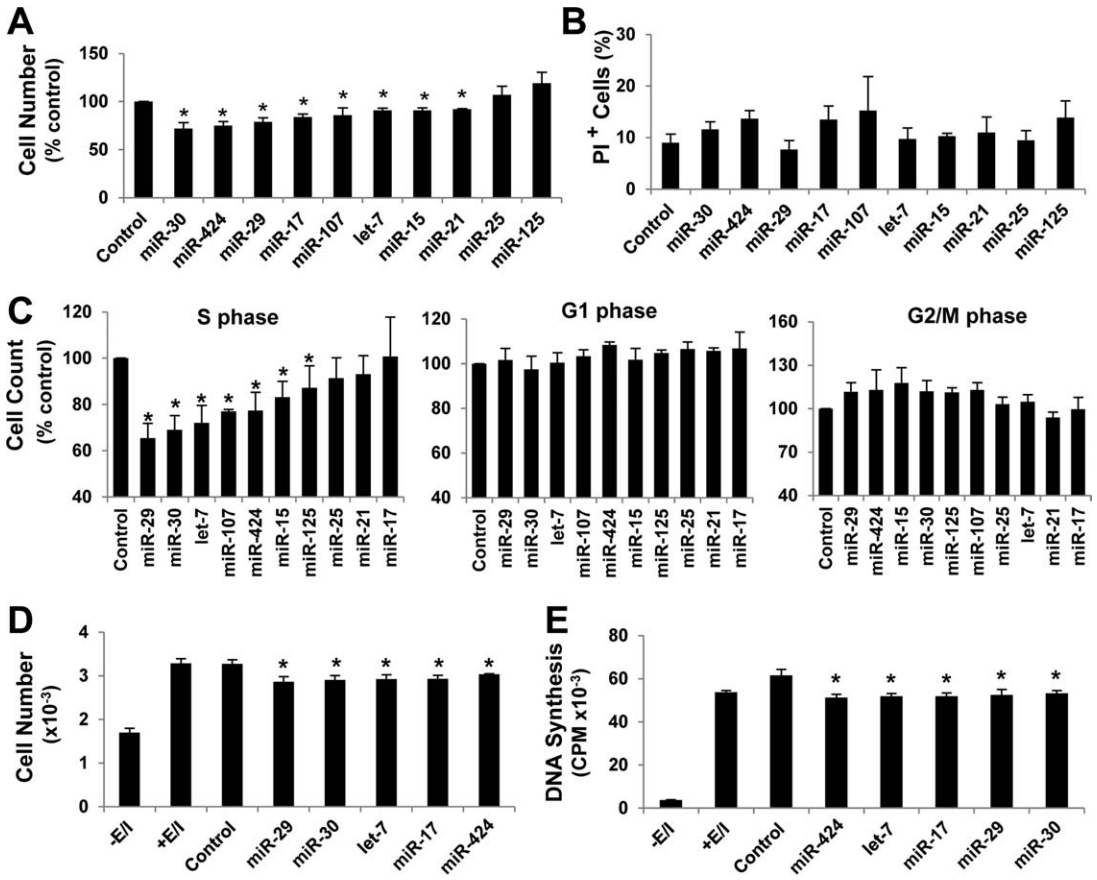

Fig. 6. Cell proliferation and DNA synthesis are regulated by overexpression of candidate miRNAs in Huh-7 cells and primary rat hepatocytes. (A) Cell proliferation (left panel) was determined by CellTiter-Blue assay after 24 hours of overexpression of the various candidate miRNAs in Huh-7 cells. (B) Cell death was analyzed by propidum iodide staining and fluorescence-activated cell sorting analysis in Huh-7 cells transfected in parallel with the miRNA overexpression constructs. (C) Number of cells in each cell-cycle phase was determined by propidium iodide staining and flow cytometry in cells transfected with the candidate miRNA overexpression construct, which is indicated below the graph. In (A), (B), and (C), Huh-7 cells transfected with the pcDNA 3.1 empty vector was used as control and defined as 100%. $*P < 0.05$, from control. (D) After plating and transfection with the candidate miRNA overexpression constructs, primary hepatocytes were stimulated to replicate and cell viability was determined using the CellTiter-Blue assay. (E) DNA synthesis was determined by [^{3}H]thymidine incorporation in epidermial growth-factor–stimulated primary rat hepatocytes transfected in parallel with the same miRNA overexpression constructs. The pcDNA 3.1 empty-vector–transfected cells were used as control, and the data shown represent the mean ± standard error of the mean from six replicates. The overexpression construct is indicated below the graph, with untransfected primary hepatocytes (I°) cultured in the presence (+E) or absence of epidermal growth factor (−E) serving as the cell-proliferation induction controls. $*P < 0.05$, from the pcDNA3.1 transfected controls.

Discussion

We have characterized the levels of miRNAs during liver regeneration and documented a biphasic expression pattern for miRNAs characterized by an early up-regulation and late down-regulation. This biphasic change was most likely caused, in part, by a negative feedback mechanism mediated by miRNA-processing genes. The early up-regulation of specific miRNAs might have been responsible for the priming phase of LR by inhibiting cell proliferation and DNA synthesis, and their later down-regulation eventually allowed the liver to fully regenerate.

Given the important regulatory roles miRNAs play in diverse biological processes, it is very likely that those miRNAs also participate actively in coordinating the events of LR.[8] It is of particular interest to note that this early activation of miRNAs coincides with a period initially termed the priming period of LR (i.e., the first 4-5 hours after PH), in which the hepatocytes are refractory to growth signals. It is tempting to speculate that the up-regulation of miRNAs is a critical mechanism that contributes to the priming phase of LR.

Considering the broad spectrum of down-regulation of miRNAs identified in this screen after the initial priming period (i.e., 70% of all miRNAs at 24 hours), it suggested that miRNA processing was potentially involved in expression changes. It is possible, but unlikely, that the transcription of these miRNAs is

decreased, because miRNAs are mostly transcribed via RNA polymerase II (Pol II) promoters and a global decrease in RNA Pol II activity during the later phases LR was not observed. Thus, we examined the transcript levels of several miRNA-processing genes, including *Rnasen* (Drosha), *Dgcr8* (Pasha), Dicer, *Tarbp2* (TRBP), and *Prkra* (PACT), and showed that the mRNA steady-state levels of these genes at 24 hours were, indeed, decreased. Earlier studies had shown that Dicer was subject to post-translational regulation by miRNAs, such as by let-7,[29,30] suggesting that early up-regulation of miRNAs might, in part, be responsible for down-regulating these miRNA-processing genes, which, in turn, promotes the global decrease of miRNAs observed in the later stages of LR.

Sequence analysis of the 3′UTRs of the miRNA-processing genes predicted that many miRNAs could potentially target these genes. These included 11 miRNAs that were significantly up-regulated at 3 hours post-PH, concurrent with the first observed decrease in miRNA-processing gene transcript levels. Using overexpression of a select group of these miRNA and luciferase reporter constructs with the 3′UTRs of the miRNA-processing genes in Huh-7 cells, we established that select miRNAs targeted and down-regulated the expression of these genes. This work also extended the target spectrum of the candidate miRNAs.

Early up-regluation of miRNA expression coincides with the priming period after PH, which is characterized by refractory response to growth signals and decrease in DNA synthesis. Some miRNAs have previously been reported to function as tumor-related genes, such as let-7 and miR-17-92.[9,30,31] We found that overexpression of several miRNAs that target miRNA-processing genes, including let-7, miR-17, miR-29, miR-30, and miR-424, decreased cell proliferation and DNA synthesis in Huh-7 cells and primary hepatocytes and were up-regulated during early LR. Based on the data, it is likely that they, in fact, contribute to the priming phase of LR. Finally, the pattern of miRNA expression in the later phases of LR suggests that their down-regulation is also essential for the termination of replication, consistent with the majority of the hepatocytes completing DNA synthesis followed by cell division by 30 hours post-PH. Genomewide miRNA down-regulation at 24 hours may contribute to the S-phase peak at 24 hours post-PH. If correct, down-regulation of miRNAs should begin earlier than 24 hours, which is consistent with the microarray results that found down-regulation begins from 6 hours post-PH. We examined expression levels of the nine miRNAs, which can target the miRNA-processing genes at 18 hours by qRT-PCR, and found that most were down-regulated, but not as dramatically as at 24 hours (Supporting Fig. 2). So, we believe the peak of the down-regulation is between 18 and 36 hours post-PH, which may be related to the S phase at 24 hours.

Based on the above findings, our results provide an additional temporal course for miRNA expression between proliferation and return to quiescence in the 70% PH liver (Supporting Fig. 4).[4] Its biphasic nature appears to define the temporal boundaries between the induction of growth- and cell-cycle–regulated gene expression and those activated after the major growth phase has occurred. The major portion of liver mass is reconstituted within 72-84 hours, and the entire process is complete within 7-10 days. Several patterns of immediate-early, delayed-early, and liver-specific genes have been defined during the 10-day period post-PH. The orchestration may be mediated by a negative feedback between up-regulated miRNAs and target mRNAs involved in miRNA maturation and function, such as Dicer, Drosha, Pasha, Ago2, PACT, and TRBP, allowing cell proliferation, and restoration of the liver mass.

Overall, this study has documented genomewide miRNA changes during liver regeneration after 70% PH. We also described a negative feedback loop between miRNAs and their processing genes, which appears to be an efficient mechanism for the homeostatic regulation of miRNAs. The early up-regulation of miRNAs might contribute to the priming period of LR, whereas the later normalization of these miRNAs might allow the later accurate cell growth and restoration of liver size. In conclusion, the synchronous model of cell replication of ~95% of hepatocytes after 70% liver resection provides a novel model, with dynamic flux of the miRNAs affecting their biogenesis, and provides a much-needed resource for studying both mechanisms controlling their synthesis, but also their degradation and loss, of which little is known.

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