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miRNA Regulation of Liver Growth After 50% Partial Hepatectomy and Small Size Grafts in Rats

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Background. The molecular mechanisms underlying the growth of small size grafts and the remaining livers are poorly understood. MicroRNAs (miRNAs) negatively modulate expression of genes that are involved in cellular function and metabolism. The aim of this study is to identify critical miRNA species that modulate the growth of small grafts and the remaining livers after partial hepatectomy (PH).

Methods. Small size graft liver transplantation was performed in rats. Liver tissue was harvested after transplant or PH for the determination of miRNA expression profile, and the data were confirmed by quantitative reverse-transcriptase polymerase chain reaction. The genes involved in cell cycle and proliferation were analyzed by quantitative reverse-transcriptase polymerase chain reaction and immunohistochemical staining.

Results. Compared with control liver, miR_122a, Let_7b, and miR_26a were reduced by more than 90% in 45% volume grafts. In the remaining livers after 50% PH, 30 miRNAs were down-regulated by more than 50%, and among them, miR_22a, miR_26a, miR_30b, Let_7f, and Let_7g were markedly decreased. A negative correlation existed between down-regulated miRNAs and highly up-regulated genes involved in cell cycle and proliferation in the remaining livers. Moreover, overexpression of miR_26a markedly down-regulated cyclin E2 protein levels and significantly decreased proliferation of HepG2 cells.

Conclusion. Down-regulated miRNAs play a pivotal role in promoting the growth of small size grafts and the remaining livers. The negative correlation between down-regulated miRNAs and up-regulated genes suggests that these specific miRNAs participate in the modulation of a growth response in both living donors and small size graft recipients.

Keywords: miRNA, Living donor liver transplantation, Liver regeneration.

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Living donor liver transplantation (LDLT) is an alternative approach to ease the increasing donor organ demand, shorten the waiting time, or avoid death on the waiting list (1). LDLT takes advantage of the tremendous regenerative capacity of the liver and represents a major advance in transplant medicine. Hundreds of successful LDLTs were performed in the United States each year during the past decade (www.unos.org); and an increasing number of LDLTs have

been seen in Asian countries (2). However, the growth and function of small size grafts and the remaining livers are still key issues in recipients and living donors (1). Many factors may affect the growth of small size grafts in recipients and the remaining liver after living donation; these include graft conditions such as overly small grafts, marginal grafts with steatosis or cirrhosis plus hepatitis B or hepatitis C virus infection, the recipient's prior conditions, the use of immunosuppressive agents, etc (3). Factors that may affect the remaining liver largely involve the success of the operation,

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X.C. conducted the major work in experiments of miRNA RT-PCR, RT-PCR array, and miRNA transfection; M.M. participated in miRNA array analysis; Y.-Y.C. participated in small size graft liver transplantation and partial hepatectomy; L.-J.Y. participated in immunohistochemical staining of liver sections; S.K.V. participated in miRNA array and some data analysis; K.D. participated in miRNA data analysis by bioinformatic tools; and J.W. participated in conception and design, collection and analysis of data, and final approval of manuscript.

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occurrence of complications, and the general condition of the donors (4). Obviously, the identification of critical factors affecting the growth, and understanding the molecular mechanisms of growth control, is essential for improving the well being of both recipients and living donors.

MicroRNAs (miRNAs) are a family of small noncoding 21- to 23-nucleotide RNAs that negatively regulate gene expression by targeting mRNAs in a sequence-specific manner, inducing translational repression or mRNA degradation, depending on the degree of complementarity between miRNAs and their targets (5). miRNA biogenesis is highly regulated (6), and they seem to function through several mechanisms in repressing gene expression and regulating cellular activities, such as development, cell proliferation, differentiation, apoptosis, and cancer (6–8). An example of an miRNA that is abundant in the liver, and seems to affect hepatic function, is miR_122a (9). The administration of nucleic acid–modified oligonucleotide complementary to miR_122 led to long-lasting hepatitis C virus suppression in infected chimpanzees without resistance (10). In previous studies, we have characterized the signatures of miRNAs in acute liver injury and hepatic fibrosis and defined the molecular targets of significantly increased or suppressed miRNAs in these processes (11, 12). To our knowledge, no information regarding whether miRNAs participate in modulation of liver growth in LDLT is available in the literature. Thus, we hypothesized that miRNAs may play a fundamental role in modulating growth of liver grafts and the remaining liver after living donation, which is a critical process for the recovery after LDLT. In this study, we characterized miRNA signatures in the grafts using a rat model of small size graft liver transplantation (SSGLT) (13) and in the remaining livers after 50% hepatectomy, a model of living donation, and found a negative correlation between significantly suppressed miRNAs with highly activated genes involved in cell cycle and proliferation. Our study delineates for the first time that specific miRNA(s) modulate the expression of genes participating in the concerted wave of liver growth in both small size grafts and the remaining livers after living donation.

RESULTS

miRNA Signatures in Small Size Liver Grafts

To investigate the changes of the miRNA profile in small size grafts after transplant, we performed SSGLT in rats. Graft size at 45%, 75%, and 95% of native liver volume was prepared and transplanted in body weight-match inbred Lewis rats avoiding immune rejection. Two days after transplantation, miRNA-enriched total RNA was extracted in liver grafts for miRNA array analysis. miRNAs that were significantly increased (2-fold) or decreased (by more than 50%) in grafts are shown in Figure 1 (A and B). Only a few miRNAs seemed to be significantly up-regulated in 75% and 95% graft size, and miR_223 was increased approximately 6-fold compared with control liver, so were miR_514, miR_141, miR_350, and miR_224 up to 3- to 4-fold. The miRNA heatmap (Fig. 3A) confirmed the up-regulation of miR_223 in 45% and 75% graft size.

Seven miRNAs were markedly down-regulated in 45%, 75%, and 95% graft volume (Fig. 1B), and the levels of Let_7b, Let_7f, miR_21, miR_26a, and miR122a were decreased to less than 20% of the controls. The changes in

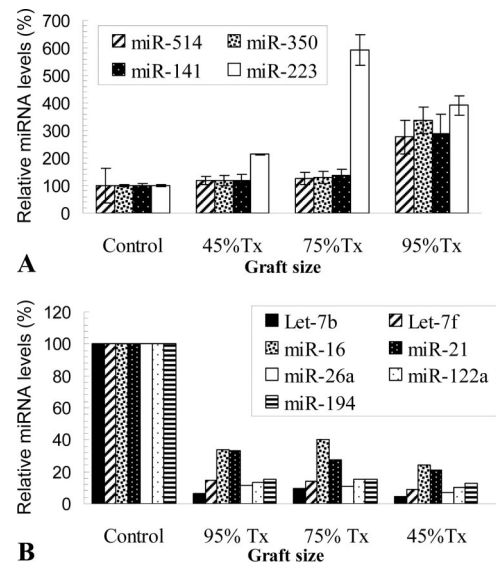


FIGURE 1. Increased (A) or decreased (B) microRNAs (miRNAs) in small size liver grafts. Small size graft liver transplantation was performed in Lewis inbred rats at 45%, 75%, and 95% graft volume, and graft specimens were collected 2 days after transplant. miRNA-enriched total RNA was extracted for miRNA array analysis. Each bar represents the average of two recipient rats. Controls were resected tissue from donor livers.

miRNA levels are reflected in the heatmap (Fig. 3A) and largely overlapped with decreased miRNAs after partial hepatectomy (PH) as described below.

miRNA Signature After PH

Many miRNAs were down-regulated in the first 3 days after 50% PH. As shown in Figure 2, most miRNAs were decreased on the first day and further decreased in the following 2 days. miRNAs with this changing pattern include miR_16, miR_22, miR_23, miR_24, miR_29, miR_30, miR_31, miR_122a, miR_126, miR_145, and miR_192. Among them, miR_22, miR_26a, miR_30, and miR_31 were reduced to less than 40%. A few miRNAs were first up-regulated and then down-regulated, including miR_21, miR_26b, miR_192, and miR_194, etc. In the Let-7 family (Fig. 2B), half of the miRNAs did not change during the first day but were reduced significantly on the second and third day. In contrast, the other members of the family were up-regulated during the first day and then decreased further. The changing pattern of the majority of these miRNAs was reflected in the heatmap (Fig. 3B).

It was evident that both miR_22 and miR_26a were markedly down-regulated in remaining liver after PH; hence, their changes were further verified in separate PH experiments. As shown in Figure 4, both miR_22 and miR_26a were decreased significantly 2 days after 50% PH and then gradually recovered to nearly normal levels.

Up-Regulation of Cell Cycle–Related Genes After PH

To further confirm that potential target genes were up-regulated in the remaining liver, a multiplex cell cycle reverse-transcriptase polymerase chain reaction (RT-PCR) array was

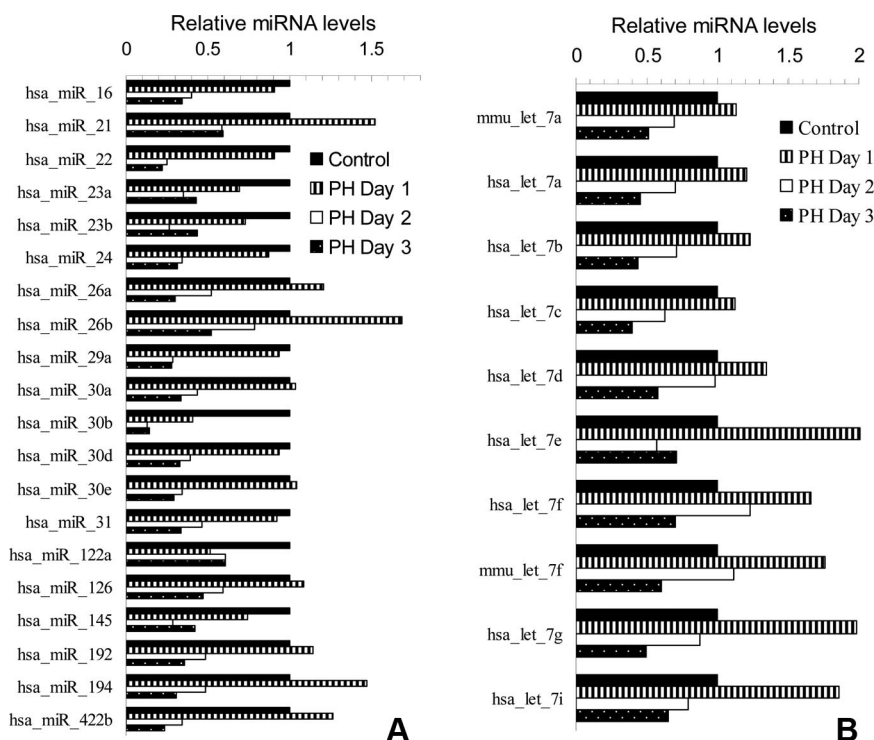


FIGURE 2. MicroRNA (miRNA) signature in remaining liver after partial hepatectomy (PH). Fifty percent PH was performed to mimic the living donor in rats, and hepatectomized rats were killed 1, 2, and 3 days after PH for collection of liver tissue. miRNA-enriched total RNA was extracted for miRNA analysis. (A) The miRNAs which displayed more than 2-fold increase or decreased by more than 50%, and each bar represents the average of two rats at each time points. (B) Let-7 family of miRNAs in remaining liver after PH. hsa, human; mmu, mouse.

used to evaluate mitosis-related genes. As shown in Figure 4(D), a number of cyclins (A, D, E, F, and G) and cyclin-dependent kinases (CDK), and E2F transcription factor were up-regulated more than 5-fold. A number of miRNAs, which were down-regulated in our study, such as miR_22, miR_26a, miR_30, miR122a, and Let_7 family, have been indicated by bioinformatics to negatively modulate these genes (Fig. 4D and **Table, Supplemental Digital Content 1**, <http://links.lww.com/TP/A334>). In addition, we performed Western blot analysis of cyclin G1, a critical molecule involved in the G1 to S phase transition, which was implicated as a target gene of miR_122a. As shown in Figure 4(C), cyclin G1 was clearly up-regulated 2 days after PH. The growth of both liver grafts and remaining liver was verified by immunohistochemical staining of Ki67 and proliferating cell nuclear antigen (PCNA; Fig. 3C–F) 2 days after transplant or PH. Therefore, it seems that most down-regulated miRNAs identified in liver grafts and the remaining livers correspond to up-regulated genes in a growing liver.

To Identify the Target Gene of Down-Regulated miR_26a

Because miR_26a was markedly decreased in both liver grafts and remaining livers, and bioinformatic analysis suggested that cyclin E2 is a potential gene target, which is highly up-regulated after PH, it is intriguing to investigate whether overexpressing miR_26a will lead to down-regulation of this gene target in cell culture. We transfected miR_26 precursor and negative pre-miRNA in HepG2 cells, and miRNA and total RNA were extracted 1 day after transfection. From Figure 5(A), it is clear that the transfection led to an approximately 100-fold increase in miR_26a levels in transfected cells 1 day after transfection, and this resulted in a significant decrease in cyclin E2 protein level (Fig. 5b). Moreover, there was

an approximately 20% decrease in cyclin E2 mRNA levels in miR_26-transfected cells; in contrast, both cyclin E2 mRNA and protein levels were not affected by transfection with the negative pre-miRNA (Fig. 5C). Transfection with miR_26a significantly inhibited [³H]-thymidine incorporation in HepG2 cells (Fig. 5D). These findings clearly demonstrate that cyclin E2 was negatively affected by overexpression of miR_26a, indicating cyclin E2 is a target gene of miR_26a, which was much down-regulated in the growing liver. Thus, when miR_26a was overexpressed, cell proliferation was consequently inhibited in vitro.

DISCUSSION

Orthotopic liver transplantation is the only established therapy available for fulminant liver failure or end-stage of liver diseases. Because of a severe shortage of donor livers, LDLT emerged as an alternative approach to ease the donor liver shortage. Recent reports demonstrated that living donors have an improved graft survival and function, and a better 5-year survival compared with cadaveric livers (1). However, a series of complications, morbidity, and mortality occur in both recipients and living donors (1), and these complications affect the general well being of recipients and living donors (14). Many factors that affect the growth of small grafts and the remaining livers have been identified, whereas the molecular mechanisms of growth regulation are largely unknown. Thus, it is highly relevant to delineate the molecules that are critical for modulating the growth process in both situations.

In this study, we used both SSGLT and 50% PH in rats to mimic LDLT and identified four miRNAs that were up-regulated in small size liver graft (SSLG) and seven miRNAs that were markedly down-regulated in recipients. Moreover, 30 miRNAs were down-regulated by more than 50% in PH

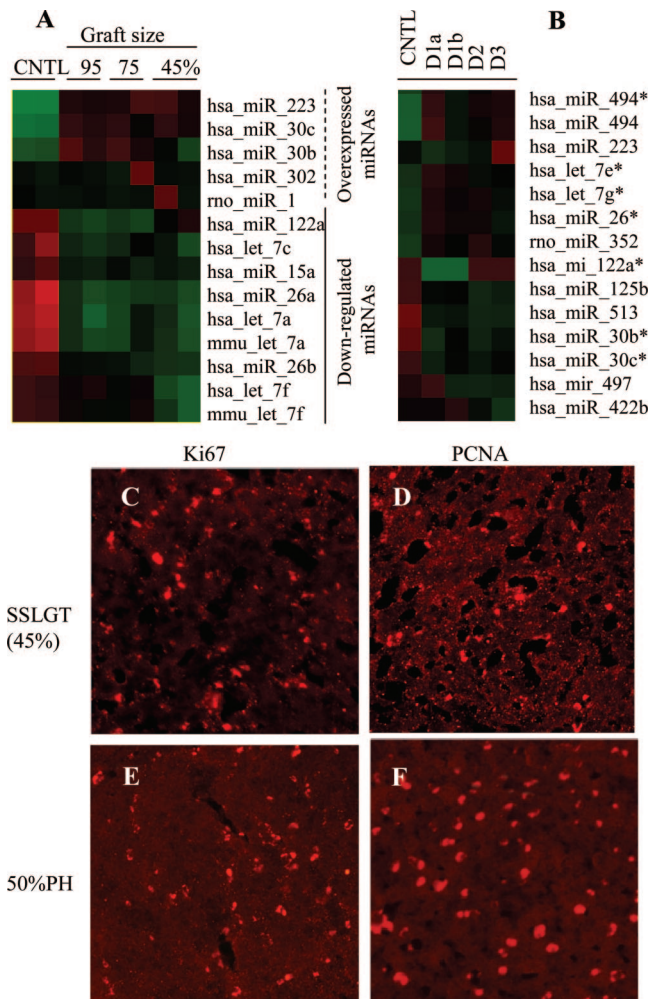


FIGURE 3. Heatmaps of microRNA (miRNA) profiles and immunohistochemical staining of Ki67 and proliferating cell nuclear antigen (PCNA). Heatmaps of miRNA profile of small size grafts (A) and remaining liver after partial hepatectomy (PH; B). The raw data of miRNAs from both liver grafts and remaining livers after PH were analyzed in R using the limma package. After baseline correction and normalization, significantly differentially regulated miRNAs were imported into gene cluster. The miRNA data were clustered using hierarchical clustering and self organizing maps and visualized as heatmaps with Java TreeView (Alok Saldanha, Open source software, Available at: <http://jtreeview.sourceforge.net/>). Overexpressed miRNAs and down-regulated miRNAs (*) were clearly indicated. Each condition represents one to two recipient rats or rats after PH. hsa, human; mmu, mouse; rno, rat. (C–F) Both liver graft and remaining liver tissues were collected 2 days after transplant or PH, snap-frozen, and cryosectioned. Formalin-fixed cryosections were stained with primary antibodies against ki67 or PCNA, and Cy5-conjugated second antibodies. Fluorescent images were recorded by a digital camera under a Nikon microscope (Nikon, Inc., Melville, NY). (C–F) Representative images of the staining for three 45% small size graft liver transplantations (SSGLT) and three 50% PH. CNTL, control.

during the first 3 days, and some of them were up-regulated during the first day and then down-regulated significantly afterward. Among down-regulated miRNAs, miR_16, miR_21, miR_26, miR_122a, Let_7b, and Let_7f were strik-

ingly reduced in both SSLGs and remaining livers. In 50% PH, miR_22, miR_30a, miR_30b, miR_30d, and miR_30e were reduced more than 50% in the remaining livers. This changing profile of miRNAs was reflected in the heatmaps of miRNA array data. However, variations existed in specific miRNAs between each animal with the same treatment. To avoid variations among animals, we performed 50% PH and collected liver specimens within 3 consecutive days after PH in the same animals. As shown in Figure 4 (A and B), miR_22 was decreased in all three rats during the first day and then gradually recovered to nearly normal levels in days 2 to 3. miR_26a was decreased during the first 2 days in all rats and then was recovered on the third day. Thus, the quantitative RT-PCR analysis partially verified the miRNA array data shown in Figures 1 to 3 and confirmed that these two miRNAs were indeed decreased at the early stage of PH.

Liver regeneration is a concerted response to mass loss or damage, and hormones, growth factors, transcription factors, and genes regulating the growth process have been well characterized most in 75% PH (15, 16). Three separate studies with mRNA array analysis have shown that genes involved in liver regeneration change in groups following different dynamic patterns (17–19). Most up-regulated genes are involved in stress responses, nutrition and energy metabolism, cell cycle, and proliferation (18, 20). We used a quantitative RT-PCR array to verify up-regulation of genes involved in cell cycle and proliferation and found that many cyclins and CDKs were up-regulated 2 days after 50% PH, which was in accordance with positive staining of PCNA and Ki67 in both SSLG and remaining livers, and up-regulated cyclin G1 expression. Bioinformatic analysis indicated that these up-regulated genes have sequence homology with decreased miRNAs, such as miR_Let_7f, miR_22, miR_26a, miR_30, and miR_122a. Moreover, potential target genes of these miRNAs include insulin-like growth factor receptor, platelet-derived growth factor receptor- α , fibroblast growth factor, growth differentiation factor, erythropoietin, hepatocyte growth factor, STAT3, glycogen synthase, cyclins, mitogen-activated protein kinases, E2F, and calmodulin, etc (Table, Supplemental Digital Content 1, <http://links.lww.com/TP/A334>). Most of them are highly activated, at least more than 2-fold, during liver regeneration after PH (Fig. 4D) (18). Among them, *cyclin A2* is the target of Let_7, which down regulates caspase-3 expression and inhibits ras (21). Hence, the down-regulation of Let_7 family members promotes cell proliferation and differentiation. *Cyclin G1* has been confirmed as a target of miR_122a in HepG2 cells (8). Therefore, there exists a negative correlation between down-regulated miRNAs and up-regulated genes in the remaining livers, which suggests that the down-regulated miRNAs are required for up-regulation of genes contributing to the concerted wave of liver regeneration.

To further identify the gene targets of miR_26a, we transfected HepG2 cells with miR_26a precursor. One day after transfection, miR_26a levels in transfected cells were increased up to approximately 2 log levels. This overexpression of miR_26a resulted in a marked decrease in cyclin E2 protein levels, a moderate but statistically significant decrease in *cyclin E2* mRNA levels, and reduced cell proliferation. But transfection with negative pre-miRNA did not exhibit any effects on *cyclin E2* at the mRNA or protein levels. These data suggest that *cyclin E2* is a gene target of miR_26a, and its inhibition on cyclin E2 expression is pre-

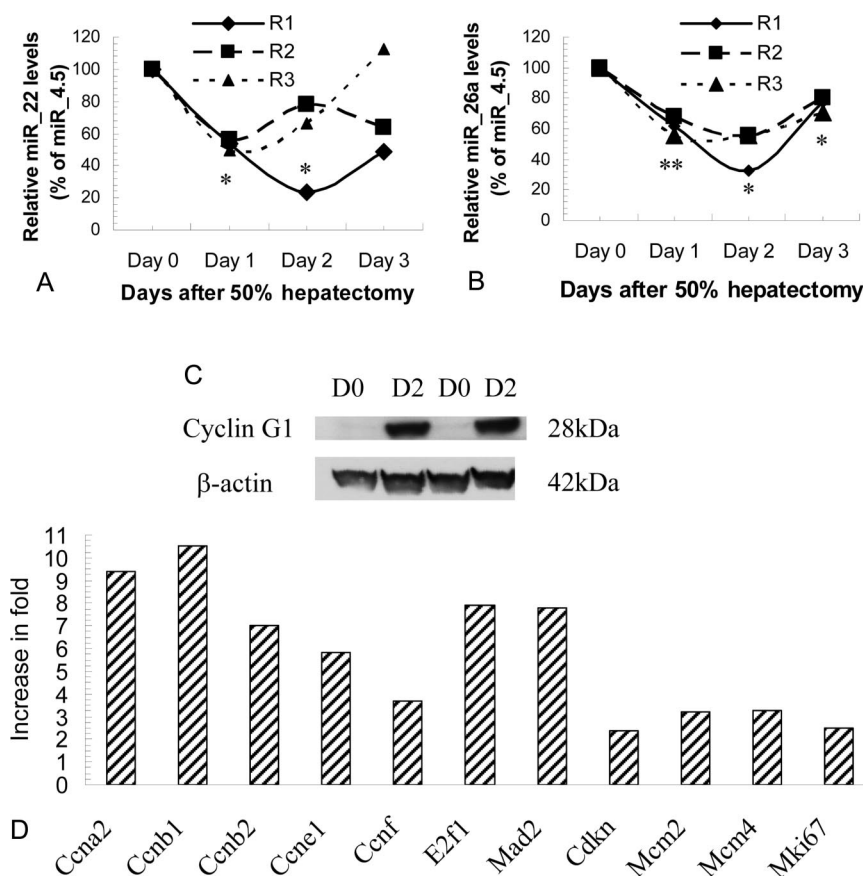


FIGURE 4. Verification of microRNA (miRNA) changes by stem-loop quantitative reverse-transcriptase polymerase chain reaction (RT-PCR) analysis, Western blot analysis of cyclin G1, and RT-PCR array of cell cycle genes. Partial hepatectomy (PH) was performed in separate rats, and liver specimens were collected every day surgically under anesthesia for 3 consecutive days after PH. miRNAs were extracted for stem-loop quantitative RT-PCR analysis of specific miRNAs (miR_22 in A and miR_26a in B). Each line represents specific miRNA change in an individual rat. **P* less than 0.05; ***P* less than 0.01 compared with day 0. (C) Western blot analysis of cyclin G1 expression from two rat livers 2 days after 50% PH. β -actin was used as a loading control. (D) Multiplex RT-PCR analysis of genes modulating cell proliferation after 50% PH. The genes regulate cell cycle and proliferation were determined by quantitative RT-PCR array and expressed by fold of increase. The abbreviations of the gene names are as follows: *Ccna2*, cyclin A2 (Let_7, miR_26a); *Ccnb1*, cyclin B1 (miR_31, 142); *Ccnb2*, cyclin B2 (miR_19, 23, 29); *Ccne1*, cyclin E1 (Let_7i, miR_16, 29a, b, c, 122); *Ccnf*, Cyclin F (Let_7a, b, c, d, e, miR_98, 202); *E2f1*, E2F transcription factor 1 (Let_7f-2, miR_17, 148a, 205); *Mad2*, MAD2 (mitotic arrest deficient, homolog)-like 1; *Cdkn*, cyclin-dependent kinase inhibitor 1A (p21, Cip1) (miR_22); *Mcm2*, minichromosome maintenance complex component 2; *Mcm4*, minichromosome maintenance complex component 4; and *Mki67*, antigen identified by monoclonal antibody Ki-67. The miRNAs that have been shown sequence homology with each gene are indicated in parentheses.

dominantly through translational repression rather than transcriptional inhibition. Cyclin E2, a main subunit of cyclin E that regulates CDK2, is considered as a requisite regulator of G1 progression. The G1 to S phase transition is coordinated by the sequential activities of CDK and the cyclin E/CDK2 complex (22, 23). Therefore, it is conceivable that the down-regulation of miR_26a during LDLT is crucial for the initiation of the regenerative wave, and many other miRNAs may also contribute to this process, leading to a concerted gene expression profile seen in the mRNA array at mRNA levels (17, 18), and true liver growth in animal models (13, 16) and clinical settings (4). Thus, this study seems to reveal a critical molecular mechanism of miRNA modulation of liver growth in models of SSGLT and 50% PH in rats.

In conclusion, the findings in this study demonstrate that down-regulated miRNAs play a pivotal role in promot-

ing the growth of small size grafts and the remaining liver after PH. The negative correlation between down-regulated miRNAs and up-regulated genes suggests that many miRNAs participate in the modulation of a growth response in both living donors and small size graft recipients. *Cyclin E2* is a target gene of miR_26a that was much down-regulated in growing liver and is a critical molecule for G1 to S phase transition in cell cycle progression.

MATERIALS AND METHODS

SSGLT and 50% PH in Rats

The animal experimental protocol was approved by the Institutional Animal Care and Use Committee, according to guidelines of the National Institutes of Health. Inbred Lewis rats and outbred Sprague-Dawley rats, provided by Harlan Laboratory, Indianapolis, IN, were fed a pellet diet and water

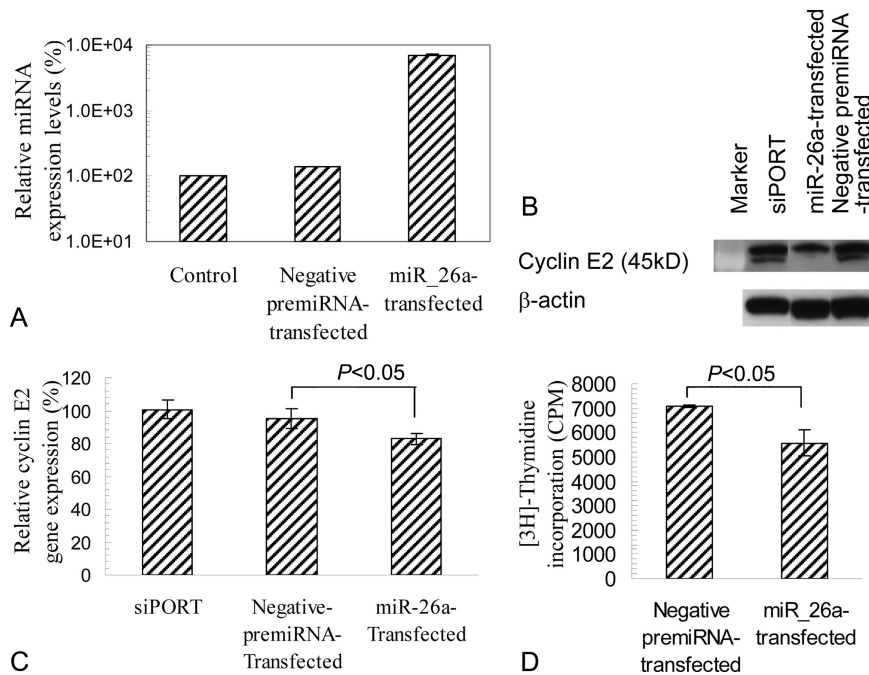


FIGURE 5. Identification of target gene of miR_26a by overexpression. (A) HepG2 cells were transfected with miR_26a precursor or nonspecific control microRNA (miRNA). One day after transfection, miRNA was extracted for stem-loop quantitative reverse-transcriptase polymerase chain reaction (RT-PCR) to determine the transfection efficiency. Each bar represents the mean of triplicates. The experiments were repeated three times. (B) Two days after miR_26a precursor transfection, total protein was extracted for Western blot analysis of cyclin E2 levels in transfected cells. The representative ECL X-ray image was shown, and β -actin was used as a loading control. (C) In a separate experiment, total RNA was extracted 1 day after miR_26a precursor transfection, and *cyclin E2* gene expression was determined by real-time RT-PCR. There was a moderate but statistically significant decrease in *cyclin E2* gene mRNA levels in miR_26a precursor-transfected cells in comparison with transfection reagent (siPORT) controls or nonspecific miRNA controls. (D) In separate experiments, [3 H]-thymidine DNA incorporation assay was performed to evaluate the DNA synthesis 20 hr after transfection with miR_26a or nonspecific control miRNA. The experiments were repeated twice.

ad libitum and kept on a 12-hr light/dark cycle. Male inbred Lewis rats were used as both donors and recipients. The donor liver harvest, cuff preparation, and size reduction were performed as described previously by us (13). Graft tissue was collected 2 days after transplant for miRNA profiling analysis. Sprague-Dawley rats were used for 50% PH following a procedure similar to a report (16) by us with minor modification. Liver specimens were collected with surgical procedures under anesthesia within 3 consecutive days after PH from the same rats for miRNA RT-PCR quantitation and multiplex RT-PCR analysis of gene expression, for immunohistochemical staining of PCNA and Ki67, and for Western blot analysis of cyclin G1.

miRNA Isolation, Labeling, Purification, and Microarray

miRNA isolation, labeling, and purification were performed as we reported previously (12). Hybridized miRNA slides were scanned with a Genepix 4000B scanner (Agilent Technologies Inc., Santa Clara, CA) (12). Raw data were analyzed using Genepix pro version 6 software (Molecular Devices, Sunnyvale, CA). Normalization was performed by expressing each miRNA replicate relative to a control miRNA (provided in the Bioarray essentials kit [Applied Biosystems, Foster City, CA]).

Immunohistochemical Staining of Liver Tissue

Two days after transplant or PH, specimens of liver grafts or remaining livers were harvested, snap-frozen in liquid nitrogen in tissue freezing medium, cryosectioned at 10- μ m thickness, and fixed in 10% formalin. Cryosections were stained with primary antibodies against PCNA and Ki67 according to the method we described previously (24), and fluorescent images were recorded with a digital camera.

Stem-Loop Quantitative RT-PCR of Specific miRNAs

To confirm miRNA changes in the remaining liver after 50% PH, two-step stem-loop quantitative RT-PCR was performed as we described previously (12, 25). The relative expression of miR_26a or miR_22 was calculated based on rat miR_4.5s levels (12). miR_26a expression after transfection in HepG2 cells was also quantitated by the stem-loop RT-PCR analysis with human RNU-44 as an endogenous RNA control.

Real-Time RT-PCR Array

Two days after PH, total RNA was extracted with RNeasy kit from Qiagen (Valencia, CA). Total RNA was subjected to first-strand cDNA synthesis using the first strand cDNA synthesis kit (Invitrogen, San Diego, CA). The cDNA was amplified in rat cell cycle multiplex PCR plates in an ABI 7300 PCR machine (Superarray Biosciences, Frederick, MD). PCR data were analyzed with online software (www.sabiosciences.com).

Effects of Overexpression of miR_26a on Cell Proliferation in HepG2 Cells

To study the functional role of miR_26a, miR_26a precursor molecules and nonspecific miRNA (used as negative control) were obtained from Applied Biosystems (Foster City, CA). HepG2 cells were cultured in minimum essential medium containing 10% fetal bovine serum, antibiotics, and sodium pyruvate. HepG2 cells were transfected with miR_26a or negative premiRNA oligos by SiPORT neoFX transfection reagent as previously reported by us (11, 12). Transfected cells were harvested for RNA extraction with an RNeasy kit 1 day after transfection. The inhibition of *cyclin E2* by overexpres-

sion of miR_26a in HepG2 cells was determined by real-time RT-PCR using human β -actin as a house-keeping gene control as we described previously (25, 26). [3 H]-thymidine DNA incorporation was performed 20 hr after miR_26a or negative pre-miRNA transfection according to our previous report (27).

Western Blot Analysis of Cyclin G1 and Cyclin E2

Two days after 50% PH or after transfection, total protein in liver specimens or HepG2 cells was extracted with lysing buffer containing protein inhibitor cocktail from Sigma-Aldrich Co. (St. Louis, MO) and quantitated spectrophotometrically with a BCA protein assay kit from Forma Scientific (Asheville, NC). Western blot analysis was performed as described previously by us (26), and primary antibodies against cyclin E2 and G1 were from Santa Cruz Biotechnology Inc (Santa Cruz, CA).

Bioinformatics and Statistical Analysis

miRNA target gene prediction was performed with three public tools: Targetscan (www.targetscan.org), Human miRNA Targets—Search & View (<http://cbio.mskcc.org/cgi-bin/mirnaviewer/mirnaviewer.pl>) and Microcosm (www.ebi.ac.uk/enright-srv/microcosm/htdocs/targets/v5/). Individual miRNA changes over time after PH was analyzed by paired Student's *t* test, and data from miR_26a transfection were first analyzed by one way variance test following Newman-Keuls test for multiple comparisons among groups. *P* less than 0.05 was considered as statistically significant.

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