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Original Paper

Downregulation of IL6 Targeted MiR-376b May Contribute to a Positive IL6 Feedback Loop During Early Liver Regeneration in Mice

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Key Words
MiR-376b • Liver regeneration • Signal transducer and transcription activators 3 • NF-kappa-B inhibitor zeta

Abstract
Background/Aims: MicroRNAs (miRNAs) are a group of endogenous, small, noncoding RNAs implicated in a variety of biological processes, including cell proliferation, apoptosis, differentiation and metabolism. The present study aims to explore the potential role and molecular mechanism of miR-376b during the early phase of liver regeneration. ***Methods:*** MiRNA profiling microarrays were used to assess the changes in miRNA expression. For functional analysis, cell proliferation, apoptosis assays, real time quantitative PCR and westernblot analysis were performed. ***Results:*** The comprehensive miRNA expression profiling assays on regenerating liver tissues 4 h after partial hepatectomy (PH) showed that three miRNAs (miR-127, miR-376b and miR-494) located in the Dlk1-Gtl2 miRNA cluster were significantly downregulated. *In vitro* functional studies demonstrated that high-level interleukin 6 (IL6) inhibited the expression of miR-376b, and miR-376b mimics treatment decreased cell proliferation and increased apoptosis. Further target analysis showed that miR-376b reduced the mRNA and protein expression levels of NF-kappa-B inhibitor zeta (NFKBIZ) and signal transducers and transcription activators 3 (STAT3). Additionally, IL6-induced miR-376b downregulation would, in turn, increase the expression of IL-6 possibly via a feedback loop involving NFKBIZ or/and STAT3. ***Conclusion:*** During the early phase of liver regeneration, miR-376b expression was significantly decreased. Our findings reveal that a regulatory circuitry between miR-376b and IL-6 may exist, which trigger the initiation of liver regeneration.

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Introduction

MicroRNAs (miRNAs) are a class of post-transcriptional regulators that play a critical role in liver development and the fine-tuning of fundamental biological processes in the liver, including liver regeneration, metabolic function and hepatocellular carcinoma (HCC) development [1, 2]. Recent studies on liver regeneration have identified a number of miRNAs that show a regulated expression pattern [3-6]. Liver regeneration and hepatocyte proliferation is an orchestrated process involving myriads of signals including cytokines, growth factors, transcription factors and cell cycle regulators [7-10]. When there is a massive loss of hepatocytes, activation of hepatic progenitor cells (HPCs) also occurs [11, 12]. Therefore, miRNAs involved in the above signaling pathways are potential important regulators during hepatocyte proliferation. Array-based assays have been widely used in functional study of some miRNAs during liver regeneration. In situ hybridization (ISH) or qPCR showed that the expression of miR-21 was up-regulated hours post hepatectomy, which inhibited Peli1 expression and potentially regulate NF-κB signaling [13], while miR-34c*, miR-144, and miR-451 were markedly downregulated in the liver tissues after partial hepatectomy (PH) [14]. However, identification of the functional miRNAs in the regenerating liver remains incomplete, partly due to the obscure mechanism underlying liver regeneration in different stages and the large number of possible miRNA:mRNA targeting relationships.

In the present study, we used an array-based assay to screen the deregulated miRNA in regenerating liver tissues (4h) correspond to the G1 phase, which identified that miR-376b, miR-494 and miR-127 was significantly down-regulated 4 h post-surgery, as compared to the control. Interestingly, the three miRNAs are located in the imprinted Dlk1-Gtl2 imprinted region 12qF1 [15], map to the human Dlk1-Dio3 region on chromosome 14q32.2, which exert inhibitory effect in HCC malignancy *in vitro* [16]. The Dlk1-Dio3 region is also a cancer susceptibility locus and dysregulation of the miRNAs in this region has been found in liver tumors [17-19]. The above researches, to date, did not focus on the potential role of the Dlk1-Dio3 miRNA clusters during liver regeneration.

Interestingly, enforced interleukin 6 (IL6) production in cholangiocarcinoma cells is able to induce Dlk1-Dio3 domain hypermethylation, which in turn inhibits the expression of Dlk1-Dio3 region-embedded miRNA [20, 21].Since IL6 has been shown to play a major role in the acute phase response after liver injury, we hypothesized that the miR-376b is involved in the IL6 signaling transduction system and hence contribute to the priming phase of liver regeneration.

Materials and Methods

Ethics Statement

All C57BL/6 mice were appropriately cared and all animal experiments were performed according to the Institutional Animal Care Instructions approved by the Ethics Committee of the Second Military Medical University (Shanghai, China; Approval ID: SCXK 2007-0003). All animals were fed on a standard laboratory chow with free access to water.

Animals and Surgery

Male C57BL/6 mice aged 8-10 week were randomly assigned to partial hepatectomy (PH) and a sham operation (SH) group (3~6 animals in each group). After an overnight fast, the mice were injected intraperitoneally (i.p.) with sodium pentobarbital (30 mg/kg) and underwent 70% PH. Four hours after surgery, the animals were sacrificed to collect the remaining liver tissue for subsequent examination.

Microarrays

Total RNA samples from SH and PH mice were analyzed by CapitalBio Corporation (CapitalBio, Beijing, China) for miRNA microarray experiments as described before [4]. MiRNAs were separated from 20–30 ug total RNA using the Ambion miRNA Isolation Kit (including small RNAs). Fluorescein-labeled miRNAs

were used for hybridization on each miRNA microarray chip containing 1320 probes in triplicate. The differentially expressed miRNAs were chosen by using the Significance Analysis of Microarrays program (version 2.1). The alterations were defined as those with either <0.5 or >2-fold changes with a p value of less than 0.05. For mRNA expression profiling and data analysis, the mouse genome 35K oligonucleotide microarray was constructed and applied at CapitalBio Corporation (Beijing, China), as previously reported.

Cell culture and transfection

Murine hepatoma Hepa1-6 cells (Institute of Biochemistry and Cell Biology, Shanghai, China) were cultured in DMEM (Biowest, Loire, France) with 10% fetal bovine serum (FBS, Biowest, Loire, France) in a humidified atmosphere containing 5% CO_2 at 37°C. MiR-376b mimics and negative control RNA were obtained from GenePharma (Shanghai, China). Transfection was performed using Lipofectamine 2000 (Invitrogen, CA, USA) according to the manufacturer's protocols. Cells were cultured in 6-well plates and treated with IL-6 (10nM) for 4h or 8h. The cells were then collected to analyze the expression of NFKBIZ and STAT3.

Cell proliferation assay

Hepa1-6 cells were transfected with the miR-376b mimics or the negative control in the 24-well plate. After 24-h transfection, cells were reseeded in 96-well plates at a density of 4000 cells per well. At various time points (day 2 and 4), 20 μl methylthiazoletetrazolium (MTT) solution (5 mg/mL) was added into the culture medium for 4-h incubation. Then 150 μl dimethyl sulfoxide (DMSO) was added to each well to dissolve the crystals. After 10-min dissolution, the absorbance of each sample was recorded at 570 nm. The experiment was performed in triplicate.

Apoptosis assay

Hepa1-6 cells were plated in 6-well plates and transfected with the miR-376b mimics or the negative control. After 48-h incubation, the cells were collected for apoptosis analysis by flow cytometry. The number of apoptotic cells was determined with the annexin V/ FITC detection kit (Keygentec, Nanjing, China) according to the manufacturer's instructions. Data acquisition and analysis were performed using a FACSort Cytometer (FACSCA, New York, USA). The apoptotic cell rate was the sum of the early and the late apoptotic cells.

QRT-PCR

Total RNA was isolated from the prepared liver samples and cells using TRIzol reagent (Invitrogen, Carlsbad, CA, USA). cDNA was synthesized following the manufacturer' protocols (MBI Fermentas, Vilnius, Lithuania). QRT-PCR was performed with a standard SYBR-green PCR kit (TOYOBO, Osaka, Japan), and gene-specific PCR amplification was performed using the ABI 7300 (Applied Biosystems, Darmstadt, Germany). qRT-PCR reactions were performed in triplicate. Relative gene expression was calculated with the $2^{-\Delta\Delta Ct}$ method after normalization to the expression of β-actin or U6. The primers are listed as follows. U6 forward 5'- CTC GCT TCG GCA GCACA-3', U6 reverse 5'- AAC GCT TCA CGA ATT TGCGT-3'; β-actin forward 5'- TCG CAC AAT GAC TCT GGAAG-3', β-actin reverse 5'- CAG GGT CCA CAT TCA GGACT-3'; Stat3 forward 5'- GTT TAC CCA GTA TGC TTG TCG-3', Stat3 reverse 5'- CCC AGT AAG GCA CCC ACA-3'; Nfkbiz forward 5'- CCA GTT GCC TGT CTT TCG-3', Nfkbiz reverse 5'- CGG GCT GTT CAT TCT CCA-3'; IL6 forward 5'- TTG CCT TCT TGG GAC TGA-3', IL6 reverse 5'- TTG CCA TTG CAC AAC TCTT-3'.

Western blotting analysis

Cells treated with miR-376b were lysed in RIPA lysis buffer (Beyotime, Jiangsu, China). The lysates were centrifuged at 12,000 rpm at 4°C for 10 min. Equal amounts of protein were separated using 10-15% sodium dodecyl sulfonate-polyacrylamide gel electrophoresis and then transferred to nitrocellulose membranes (Boguang, Shanghai, China) subsequently. For immunoblotting, the membranes were respectively incubated with antibodies specific for NFKBIZ (Epitomics, Burlingame, CA), STAT3 (Abcam, Cambridge, UK), p-STAT3 (Epitomics, Burlingame, CA) and β-actin (Epitomics, Burlingame, CA). The immunoblotting sample was incubated with horseradish peroxidase (HRP)-coupled anti-rabbit secondary antibodies (ProteinTech, Chicago, USA) and visualized using enhanced chemiluminescence (Pierce, Rockford, USA).

Statistics

Statistical analysis was performed by using the SPSS statistics software package (SPSS). All results were expressed as mean ± SD, and $P < 0.05$ was used for significance.

Results

MiR-376b is downregulated in response to PH

To illustrate the miRNA expression pattern in the early phase of liver regeneration in mice, a comprehensive miRNA profiling microarray analysis was performed on regenerating liver tissues at 4 h post-surgery. It was found that four miRNAs were upregulated and three miRNAs were downregulated (Fig. 1A). Interestingly, all three downregulated miRNAs (miR-127, miR-376b and miR-494) were located in the mouse 12qF1 region, also known as Dlk1- Gtl2 region. This Dlk1- Gtl2 imprinted locus contains three protein-coding genes from the paternally inherited chromosome [delta-like homologue 1 (DLK1), retrotransposon-like gene 1 (RTL1), and the type 3 deiodinase (DIO3)], and several noncoding RNA genes including miRNAs, snoRNAs and a large noncoding RNA Gtl2 (Fig. 1B) [15]. Our QRT-PCR analysis further confirmed that the expression levels of miR-127, miR-376b and miR-494 were significantly decreased (<0.5 fold) in regenerating liver tissues.

Effect of IL6 regulated miR-376b on hepatocyte proliferation and apoptosis

It is known that IL6 can induce the hypermethylation of Dlk1-Dio3 domain and downregulate the expression of clustered miRNA [20, 21]. We speculated that IL6 may play a role in regulating miR-376b expression in hepatocytes. Our mRNA microarray analysis showed that 17 genes in the IL6/Jak/STAT signaling pathway were differentially expressed 4 h after PH (Table 1), suggesting that overexpressed IL6 may contribute to the downregulation of miR-376b expression during liver regeneration. Moreover, we confirmed that ectopic IL6

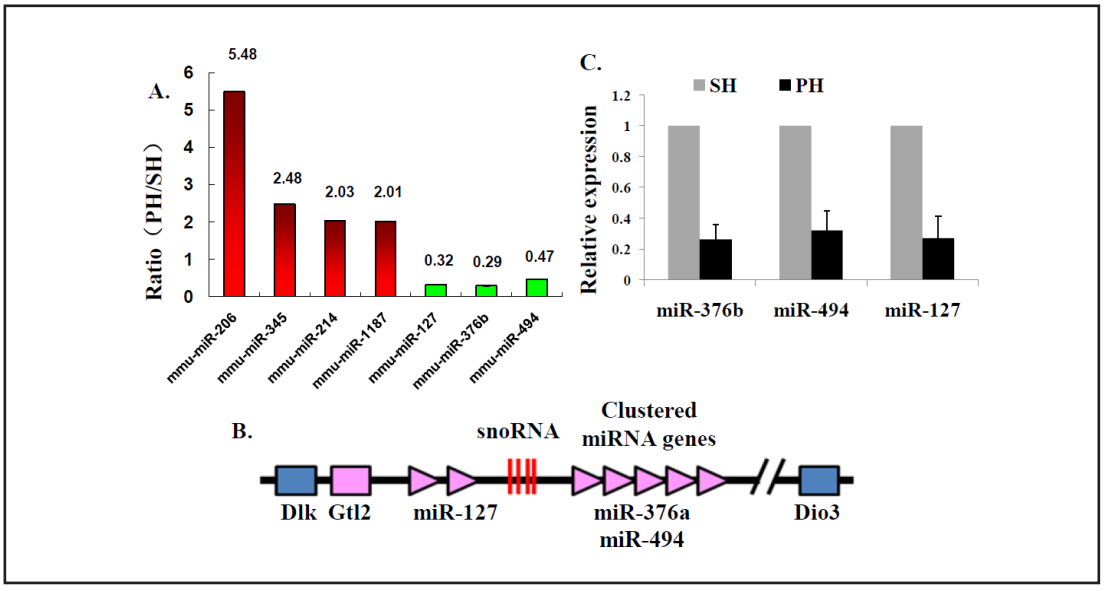


Fig. 1. Differentially expressed miRNAs during liver regeneration (LR). (A) miRNA expression profiling at 4h after partial hepatectomy (PH). Each data point represents the ratio of miRNA expression levels under PH to Sham operation (SH). Ratio values >1.5 or <0.67 were considered up-regulated (red) or down-regulated (green) in PH rats compared to SH rats. (B) The location of miR-127, miR-376b and miR-494 in Dlk1- Gtl2 region. (C) The expression pattern of miR-127, miR-376b and miR-494 during liver regeneration by quantitative real-time PCR (qRT-PCR) analysis. The data were obtained from at least three independent experiments and analyzed by t test.

significantly inhibited the expression of miR-376b in the murine hepatoma Hepa1-6 cell model, as well as two other miRNAs located in the Dlk-Dio3 domain (Fig. 2A).

To evaluate the biological effect of miR-376b in regulating murine liver cell proliferation, a MTT cell proliferation assay was employed in Hepa1-6 cells. After treatment with miR-376b mimics, cell growth was strongly repressed at day 4, as compared to the control group. In addition, the apoptotic effect was increased in the miR-376b treated cells (Fig. 1C). These data suggest thatmiR-376 working as a growth inhibitor may play an inhibitory role in the IL6 promitogenic axis in hepatocyte proliferation.

MiR-376b suppresses NFKBIZ and STAT3 expression

In order to identify the target genes and underlying molecular mechanism by which miR-376b regulates liver cell growth, we analyzed the intersection between differentially expressed mRNAs during early liver regeneration and bioinformatic target analysis (TargetScan and RNA22) on miR-376b. We identified two potential target genes, NF-kappa-B inhibitor zeta (NFKBIZ) and STAT3 (Fig. 3A), both of which have been reported to associate with the activation of IL6 signaling [22, 23]. We then carried QRT-PCR and Westernblot analysis to see whether miR-376b could regulate the expression of both genes. As shown in Fig. 3B-D, miR-376b mimics drastically inhibited the expression of NFKBIZ and STAT3 on both mRNA and protein levels. Conversely, miR-376b inhibitor significantly increased the protein levels of both genes. These data strongly

Table 1. Deregulated mRNA (in the IL6/Jak/STAT signaling pathway) at 4 h post PH

RefSeq	Name	Ratio
NM_009896	Socs1	7.1165
NM_009971	Csf3	6.9822
NM_009895	Cish	6.4476
NM_007707	Socs3	5.5829
NM_019667	Stam2	4.8267
NM_008842	Pim1	4.6271
NM_031168	Il6	4.0221
NM_010849	Myc	3.8774
NM_010557	Il4ra	3.0129
NM_007706	Socs2	2.8679
NM_008348	Il10ra	2.3978
NM_009743	Bcl2l1	2.3108
NM_007781	Csf2rb2	2.2999
NM_011486	Stat3	2.1033
NM_008358	Il15ra	0.4935
NM_011085	Pik3r1	0.4007
NM_008353	Il12rb1	0.2643

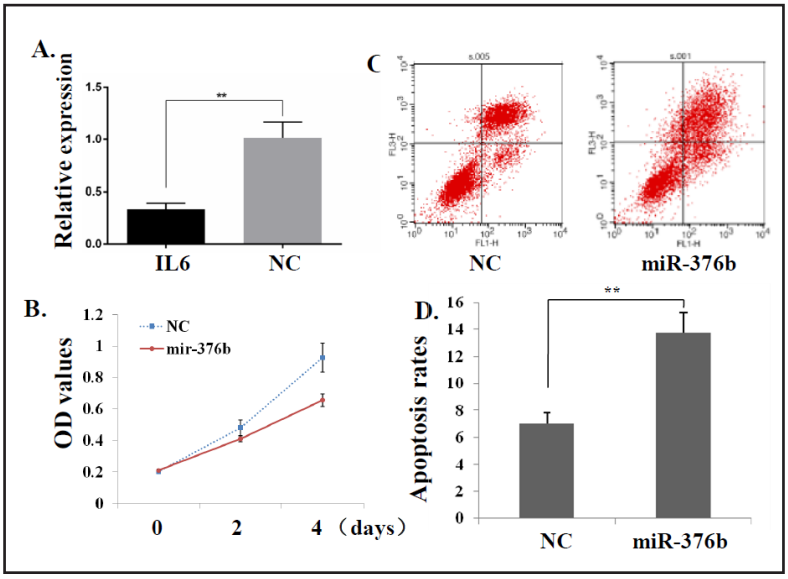


Fig. 2. IL6 regulated miR-376b inhibited cell proliferation and induced cell apoptosis in Hepa1-6 cell. (A) IL-6 treatment for 4 h decreased the expression of miR-376b, miR-127 and miR-494 in Hepa1-6 cell by qRT-PCR. (B) Hepa1-6 cell transfected with miR-376b or negative control (NC) were seeded in 96-well plates and examined at indicated time points. The absorbance of methylthiazoletetrazolium was recorded at 570 nm after staining. (C and D) Cells treated with miR-376b or NC were analyzed by flow cytometry. The data were obtained from at least three independent experiments and analyzed by t test (*P<0.05, **P<0.01).

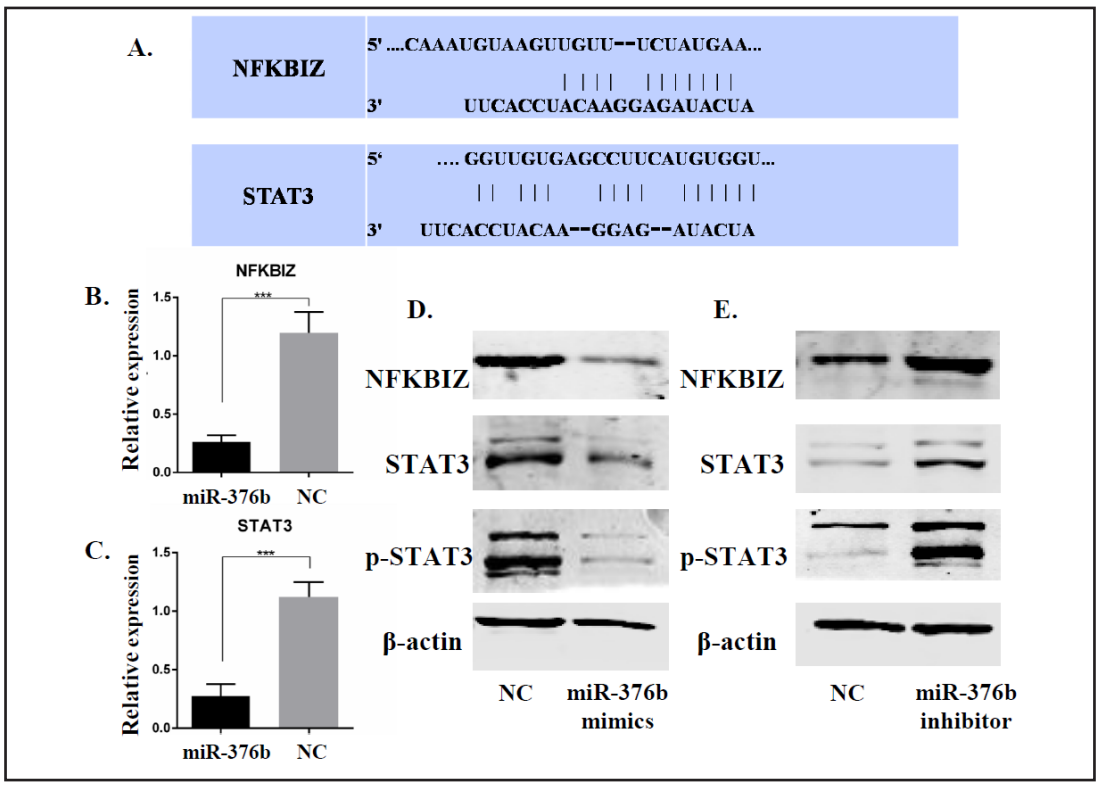


Fig. 3. Analyses of candidate target genes of miR-376b. (A) miR-376b-binding sites in NFKBIZ and STAT3 predicted by TargetScan and RNA22, respectively. (B) miR-376b decreased mRNA expression of NF-kappa-B inhibitor zeta (NFKBIZ) by qRT- PCR. (C) miR-376b decreased mRNA expression of signal transducers and transcription activators 3 (STAT3) by qRT- PCR. (D) miR-376b mimics decreased protein expression of NFKBIZ and STAT3 by western blot analysis. β-actin was used as sample control. (E) miR-376b inhibitor increased protein expression of NFKBIZ and STAT3 by western blot analysis. β-actin was used as sample control. NT stands for no treatment. The data were obtained from at least three independent experiments and analyzed by t test (***P<0.001).

suggest that miR-376b may target NFKBIZ and STAT3 in hepatocytes, and the downregulation of miR-376b in the regenerating liver tissue may lead to the increased expression of NFKBIZ and STAT3.

NFKBIZ and STAT3 may contribute to the miR-376b/IL6 positive feedback loop

STAT3 is a known target of IL6 signaling [22]. It was found in our study that ectopic IL6 treatment strongly increase the mRNA expression levels of STAT3, and miR-376b mimics largely blocked the effect of IL6 (Fig. 4A). The same effect of IL6 alone and its combination treatment with miR-376b mimics on NFKBIZ expression was observed in Hepa1-6 cell models (Fig. 4B), indicating that IL6 may upregulate both downstream genes through miR-376b dependent posttranscriptional mechanism.

Knowing that p-STAT3 is a positive regulator of IL6 expression [22] and the protein levels of p-STAT3 increases with miR-376b decreasing, we hypothesized that the suppression on miR-376b by IL6 overexpression would consequently upregulate p-STAT3 and IL6 expression. The hypothesis was confirmed by the data in Fig. 4C-E, showing that miR376b mimics inhibited the mRNA expression and supernatant levels of IL6 reciprocally. Our data also showed that overexpression of NFKBIZ could also abrogate the inhibitory effect of miR-376b on IL6 production, suggesting a miR-376b/NFKBIZ/IL6 feedback loop. Therefore, the present study revealed that both miR-376b downstream genes (NFKBIZ and STAT3) contributed to the IL6/miR-376B/IL6 feedback loop in hepatocytes.

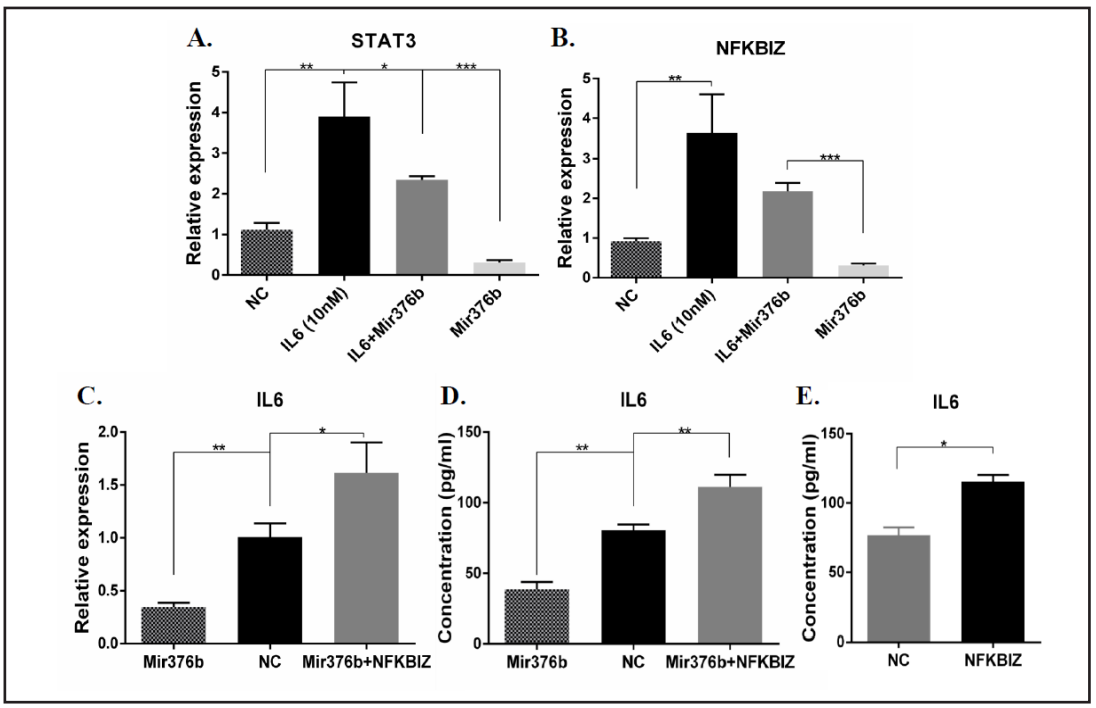
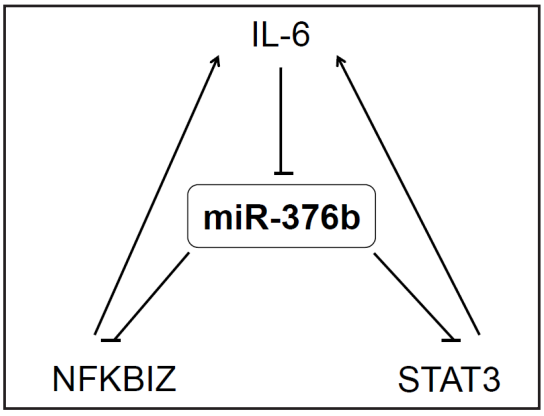


Fig. 4. Correlation between the expression levels of IL6, miR-376b, STAT3 and NFKBIZ. (A) IL6 treatment increased the mRNA expression levels of STAT3, and miR-376b mimics blocked the effect of IL6. (B) IL6 treatment increased the mRNA expression levels of NFKBIZ, and miR-376b mimics blocked the effect of IL6. (C, D) miR376b mimics inhibited the mRNA expression and IL6 levels in culture supernatant, overexpression of NFKBIZ abrogated the inhibitory effect of miR-376b on IL6 production. (E) NFKBIZ alone increases the expression of IL6. The data were obtained from at least three independent experiments and analyzed by t test (*P<0.05, **P<0.01, ***P<0.001).

Discussion

The mRNA array results in the present study showed that the Jak-STAT3 pathway was one of the essential signaling pathway during early liver regeneration (Table 1), which agrees with the previous finding that IL6 secreted by kupffer cells stimulated the initiation of liver regeneration [24, 25]. At the same time, microRNA array revealed that three Dlk-Dio3 region-imprinted miRNAs (miR-494, miR-376a and miR-127) were downregulated. IL6 has been shown to be a negative regulator of Dlk1-Dio3 cluster [20]. We therefore hypothesized that the downregulation of miR-376 and two other miRNAs may be attributed to the activation of IL6 during early liver regeneration. Of note, we confirmed that IL6 treatment could downregulate the expression of all three miRNAs on the cellular model (Fig. 2A). In human, miR-376, miR-127 and miR-494 have been reported to be downregulated in several cancers, including human hepatocellular carcinoma (HCC), breast cancer and oral cancers. Overexpression of these miRNAs in cancer cells may lead to suppressed cell growth or induced apoptosis [26-29]. As a promitogenic signal shortly activated after PH, IL6 plays an essential role in triggering the "initiation signal" for hepatocytes to reenter G1 phase and cell cycle progression.

Fig. 5. The feedforward loop comprised of IL-6, miR-376b, STAT3 and NFKBIZ.



Next, we confirmed that miR-376b had an anti-proliferative effect by increasing apoptosis and decreasing liver cell growth (Fig. 2). Thus, we assumed that when IL6 is induced post PH, the downstream pro-proliferative Jak/STAT pathway is activated and the anti-proliferative miR-376b signaling pathway is inhibited, which cooperatively contribute to liver regeneration progression.

Moreover, we identified that NFKBIZ and STAT3, the already known pro-inflammatory gene and oncogene respectively [30, 31], were the downstream target of miR-376b (Fig. 3). Thus, it is deemed that down-regulation of miR-376b during early phase triggers cell proliferation by upregulating the mRNA and protein levels of NFKBIZ and STAT3. Interestingly, we also observed a significant decrease in pSTAT3 expression by miR-376b overexpression. Since both the mRNA and protein expression levels of STAT3 were strongly repressed by miR-376b mimics treatment, we presumed that pSTAT3 level was changed accordingly when total STAT3 expression was decreased. Furthermore, accumulating evidence suggests that NFKBIZ and STAT3 can induce the expression of IL-6 as transcription factor [18, 23]. Then IL-6, miR-376b, STAT3 and NFKBIZ comprised a feedforward loop (Fig. 5), which links the output of a circuit back to its input. Namely, IL-6 secreted by kupffer cells may decrease the expression of miR-376b in hepatocyte cells, resulting in activation of NFKBIZ and STAT3, and promote the proliferation of hepatocytes.

Feed-forward regulation expands the set of possible biological properties and allows the system to convert graded inputs into decisive, all-or-none outputs [32]; while the feature of positive feedback loops cover signal amplification, bistability and memory module [33-35]. Hence, the positive feedback loop mediated by miR-376b may significantly amplify the IL-6 signaling and reinforce the proliferation caused by IL-6 activation. Our hypothesis could explain, as least in part, why the liver responds to injury so rapidly and robustly. Altogether, IL-6/miR-376b/STAT3 and IL-6/miR-376b/NFKBIZ feedback loop may cooperatively contribute to the initiation stage of liver regeneration. In summary, our findings reveal a regulatory circuitry between miR-376b and IL-6 during the early phase of liver regeneration.

Abbreviations

PH (partial hepatectomy); IL6 (interleukin 6); NFKBIZ (NF-kappa-B inhibitor zeta); STAT3 (signal transducers and transcription activators 3); SH (sham operation); miRNAs (microRNAs).

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Disclosure Statement

None.

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