

Exosomal MicroRNA-10a Is Associated with Liver Regeneration in Rats through Downregulation of EphA4

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Abstract

Background: MicroRNAs (miRNAs) have been reported to play vital roles in liver regeneration. Previous studies mainly focused on the functions of intracellular miRNAs, while the functions of circulating exosomal miRNAs in liver regeneration remain largely unknown. The aim of this study was to identify the key exosomal miRNA that played vital roles in liver regeneration.

Methods: The Sprague–Dawley male rats were assigned to 70% partially hepatectomized group ($n = 6$) and sham surgery group ($n = 6$). The peripheral blood of both groups was collected 24 h after surgery. The exosomal miRNAs were extracted, and microarray was used to find out the key miRNA implicated in liver regeneration. Adenovirus was used to overexpress the key miRNA in rats, and proliferating cell nuclear antigen (PCNA) staining was applied to study the effect of key miRNA overexpression on liver regeneration. Western blotting was used to validate the predicted target of the key miRNA.

Results: Exosomal miR-10a was upregulated more than nine times in hepatectomized rats. The level of miR-10a was increased in the early phase of liver regeneration, reached the top at 72 h postsurgery, and decreased to perioperative level 168 h after surgery. Moreover, enforced expression of miR-10a by adenovirus facilitated the process of liver regeneration as evidenced by immunohistochemical staining of PCNA. Erythropoietin-producing hepatocellular receptor A4 (EphA4) has been predicted to be a target of miR-10a. The protein level of EphA4 was decreased in the early phase of liver regeneration, reached the bottom at 72 h postsurgery, and rose to perioperative level 168 h after surgery, which was negatively correlated with miR-10a, confirming that EphA4 served as a downstream target of miR-10a. Moreover, inhibition of EphA4 by rhynchophylline could promote the proliferation of hepatocytes by regulating the cell cycle.

Conclusion: Exosomal miR-10a might accelerate liver regeneration through downregulation of EphA4.

Key words: Cell Cycle; EphA4; Liver Regeneration; Microarray; miR-10a

INTRODUCTION

Liver is one of the most significant organs in the body, which participates in many biological activities including metabolism, detoxification, and defending the external injury.^[1,2] Many factors could damage liver such as tumor, infection, hepatolith, drugs, alcohol, and even surgery. When the liver suffers injuries, surgical resection is always chosen to remove the damaged liver, especially for hepatocellular carcinoma. After surgery, the remnant liver initiates the process of liver regeneration to restore the proper liver architecture and homeostasis. When the regenerative capacity of the remnant liver is not enough to restore the whole liver, the patient will die on account of liver failure. Therefore, it is of great importance to decode the underlying mechanisms of liver regeneration.

MicroRNAs (miRNAs) are about 18–24 nucleotide noncoding RNAs that are highly conservative. It can generate RNA-induced silencing complexes to inhibit the transcription or degrade the messenger RNA (mRNA) by binding to their 3'-untranslated regions (UTRs).^[3] More than 45,000 binding sites between mRNA's 3'-UTRs and miRNAs have been identified, and over 70% of

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the protein-coding genes in mammals are regulated by miRNAs.^[4] miRNAs play vital roles in many important physiological activities such as cell cycle, metabolism, immune response, and tumorigenesis. Recent studies also showed that miRNAs are also implicated in the process of liver regeneration.^[5,6] However, previous studies mainly focused on the functions of the intracellular miRNAs in liver regeneration. The studies on the function of circulatory miRNAs engaged in liver regeneration are rare.

Exosomes, which are secreted by many kinds of cells, are a kind of extracellular vesicles. Nowadays, exosomes are considered as a prominent and universal form of cell–cell communication. It is widely found in the body fluids such as blood, urine, and bile and has many cargos in it, including miRNAs, proteins, and mRNAs. Many researches have demonstrated that the exosomal miRNAs have important regulatory functions in the physiological and pathological processes of the liver^[7,8] influencing the process of the progression, invasion, and metastasis of hepatocellular carcinoma and taking part in the process of drug- and alcohol-induced liver damage.

In our study, we extracted exosomes from the peripheral blood of the partially hepatectomized rats and identified the differential expression miRNAs through miRNAs microarray. We found that miR-10a increased markedly during the process of liver regeneration. In addition, liver regeneration was enhanced when we increased the level of miR-10a by adenovirus. Moreover, we revealed that inhibition of erythropoietin-producing hepatocellular receptor A4 (EphA4), a target of miR-10a, stimulated the proliferation of hepatocytes through promotion of cell cycle. Taken together, these findings indicated that miR-10a could facilitate the process of liver regeneration by downregulate the level of EphA4.

METHODS

Animals and treatment

Twelve Sprague Dawley male rats at 7–8 weeks were assigned to experimental group (70% partial hepatectomy) ($n = 6$) and sham group ($n = 6$). The 70% partial hepatectomy was performed on the rats in the experimental group,^[9] while in the sham group, we just made a same incision in the abdomen and sutured it but did not cut the liver.

Exosome isolation from serum samples

Exosomes were isolated from serum samples following the manufacturer's protocol (System Biosciences, USA). Briefly, 2 ml of peripheral blood was collected from the tail veins of the rats in both groups 2 h after surgery and centrifuged at 3000 rpm for 15 min at 4°C to spin down the blood cells. 0.5 ml of ExoQuick isolation buffer was added to the pellet and mixed up. The pellet was centrifuged at 1500 rpm for 5 min at 4°C and the supernatant was removed twice. The exosome pellet was resuspended in 0.2 ml of nuclease-free water and stored at –80°C until use.

Transmission electron microscopy

A copper mesh was placed on a clean wax plate, and 30 μ l of the exosome suspension was added. After 4 min, the copper mesh was removed and placed in 3% phosphotungstic acid for 5 min. The mesh was laid on the filter paper for air-drying for 1 h. Transmission electron microscopy (TEM) was used to observe the morphological features of the exosome.

Western blot analysis

The exosome pellet was dissolved in the radio-immunoprecipitation assay lysis buffer supplemented with protease inhibitors for 15 min on ice and centrifuged at 10,000 for 6 min. The supernatant was collected, and the protein concentration was determined using a BCA Protein Assay Kit. The protein was separated on a polyacrylamide gel before transfer to a polyvinylidene difluoride membrane. The blotting membrane was blocked and incubated with CD9 antibody at 1:1000 and CD63 antibody at 1:1000 at 4°C overnight and incubated with secondary antibody for 1 h at 37°C. The proteins were detected using enhanced chemiluminescence.

RNA extraction from exosome

RNA was extracted from the exosome pellets using Plasma/Serum Exosome RNA Purification Kit according to the manufacturer's protocol. Briefly, PS solution A and PS solution B were added to the exosome pellets at the ratio of 5:1 and 9:10. They were mixed them up and incubated at 60°C for 10 min. Then, 96% ethyl alcohol was added to the mixture at the ratio of 1:3. They were mixed them up and transferred it to spin columns. They were centrifuged at 14,000 rpm for 1 min and the supernatant was removed. Wash solution was added to the pellets at the ratio of 5:2 and centrifuged at 14,000 rpm for 1 min. The washing process was repeated for three times. Elution solution was added to the spin columns at the ratio of 10:1 and centrifuged at 2000 rpm for 2 min. They were centrifuged again at 14,000 rpm for 3 min. The pellet was collected and stored at –80°C until use. The purity of the isolated RNA was determined according to the OD260/280 using a NanoDrop ND-1000 system (NanoDrop Technologies, USA).

Microarray analysis

Microarray assay was performed using a service provider (LC Sciences, China). Six micrograms of total RNA sample was 3'-extended with a poly(A) tail using poly(A) polymerase. An oligonucleotide tag was then ligated to the poly(A) tail for later fluorescent dye staining. Hybridization was performed overnight on a μ ParaFlo microfluidic chip using a micro-circulation pump (Atactic Technologies, USA).^[10,11] On the microfluidic chip, each detection probe consisted of a chemically modified nucleotide-coding segment complementary to target miRNA or other RNAs and a spacer segment of polyethylene glycol to extend the coding segment away from the substrate. The detection probes were made by *in situ* synthesis using photogenerated reagent chemistry. The hybridization melting temperatures were balanced by chemical modifications of the detection

probes. Hybridization used 6× SSPE buffer (0.90 mol/L NaCl, 60 mmol/L Na₂HPO₄, 6 mmol/L EDTA, and pH 6.8) containing 25% formamide at 34°C. After RNA hybridization, tag-conjugating Cy3 dye was circulated through the microfluidic chip for dye staining. Fluorescence images were collected and digitized using Array-Pro image analysis software (Media Cybernetics, USA). Data were analyzed by first subtracting the background and then normalizing the signals using a locally weighted scatterplot smoothing (LOWESS) regression.^[12]

Validation of real-time quantitative polymerase chain reaction

To determine the exactly changing curve of miR-10a, 12 rats were equally divided into experimental group and sham group. The surgery of both groups was described above. The peripheral blood was collected at 3, 24, 72, 120, and 168 h. The exosomes' isolation and RNAs extraction were described above. Quantitative reverse transcription-polymerase chain reaction (qRT-PCR) was performed to detect the level of miR-10a. First, the cDNAs were synthesized using TUREscript first-strand cDNA synthesis kit following the manufacturer's protocol. Briefly, each reaction was performed in a 10 µl volume system, containing 5 µl of total RNA, 2 µl of 5× RT reaction mix, 0.8 µl of oligo (dT) or random primer, 0.5 µl of TUREscript H⁺ RTase/RI mix, and 1.7 µl of RNase-Free dH₂O. After the cDNAs were synthesized, the cDNAs were used for qRT-PCR. Each reaction was performed in a 10 µl volume system containing 5 µl of 2× SYBR[®] Green Supermix, 0.2 µl of each primer, 1 µl of cDNA, and 3.6 µl of RNase-free water. The PCR program consisted of denaturation at 95°C for 3 min, followed by 39 cycles each of denaturation for 15 s at 95°C and annealing and extension for 30 s at 60°C. The U6 expression level was used as a stable endogenous control for normalization. All assays were conducted in triplicate.

Target prediction

The target genes of the candidate miRNAs were predicted by TargetScan 6.2 prediction software (<http://www.targetscan.org/>), PicTar prediction software (<http://pictar.mdc-berlin.de/>), and MiRanda prediction software (<http://www.microrna.org/>). The intersection of the predicted miRNAs in all these three software was chosen for target genes of candidate miRNAs.

Overexpression of miR-10a

Adenovirus purchased from Obio Technology (Shanghai) Corp., Ltd. was used to overexpress miR-10a. In the miR-10a overexpression group, the virus vector was pAdeno-EF1-rno-miR-10a-BGH-MCMV-EGFP-3FLAG. The total amount of the virus is 1.6×10^{12} PFU with the titer 8×10^{11} PFU/ml. Moreover, in the vector group, the virus vector is pAdeno-EF1-MCS-MCMV-EGFP-3FLAG. The total amount of the virus in the vector group is 1×10^{12} PFU with the titer 5×10^{11} PFU/ml. For each rat, 3×10^9 PFU of virus was injected which was diluted in 200 µl saline.

Immunohistochemistry

To test whether overexpression of miR-10a can accelerate the proliferation of hepatocytes, immunohistochemistry was performed to test the positive rate of proliferating cell nuclear antigen (PCNA) in the remnant liver after partial hepatectomy. Immunohistochemical staining for PCNA was performed on paraffin-embedded liver tissue with anti-PCNA antibody (GeneTex, USA) following the manufacturer's protocols. PCNA monoclonal antibody was used at a dilution of 1:100 overnight at 4°C. The sections were counterstained with hematoxylin. Evaluation of PCNA immunostaining was performed based on the percentage of positive nuclei of 400–600 hepatocytes from 4 to 6 highest positive fields.

Inhibition of EphA4 in L-02 *in vitro*

Normal hepatocytes' cell line L-02 was cultured in Dulbecco's Modified Eagle Medium (Invitrogen, USA) containing 10% fetal bovine serum, 10⁷ U/L of penicillin, and 10 mg/L of streptomycin. 300 mg/L of rhynchophylline was added to the medium to downregulate the level of EphA4. The morphological and growth condition of the cells was observed by microscope 48 h after adding rhynchophylline.

Flow cytometric analysis

Flow cytometric analyses were performed to define the cell cycle distribution for rhynchophylline-treated and untreated cells. Cells grown in 6-well plate were harvested by trypsinization and fixed with 70% ethanol 48 h after the rhynchophylline (300 mg/L) was added to the medium. Cells were stained for total DNA content with a solution containing 20 µg of propidium iodide, 200 µg of RNase, and 1 ml of Triton X-100. Cell cycle distribution was then analyzed at flow cytometer (BD Biosciences, USA).

Statistical analysis

Statistical analyses of experimental data were performed using a two-sided Student's *t*-test with STATA software version 13.0 (StataCorp, College Station, TX, USA). Significance was set at $P < 0.05$.

RESULTS

Identification of the key exosomal microRNA associated with liver regeneration

After extraction of exosomes from the serum of partially hepatectomized rats, we obtained transmission electron microscope photograph of exosomes [Figure 1a]. We found that the exosomes were 30–100 nm in diameter, which was consistent with the previous report.^[13] In addition, immunoblots detected the expressions of CD9 and CD63 in the exosomes [Figure 1b], which were regarded as the protein markers of exosomes.^[14]

To identify the exosomal miRNAs associated with liver regeneration, miRNA microarray was performed to find out the differentially expressed miRNAs after partial hepatectomy. The result showed that 17 kinds of miRNAs displayed significant changes (8 miRNAs were upregulated and 9 miRNAs were downregulated in partially

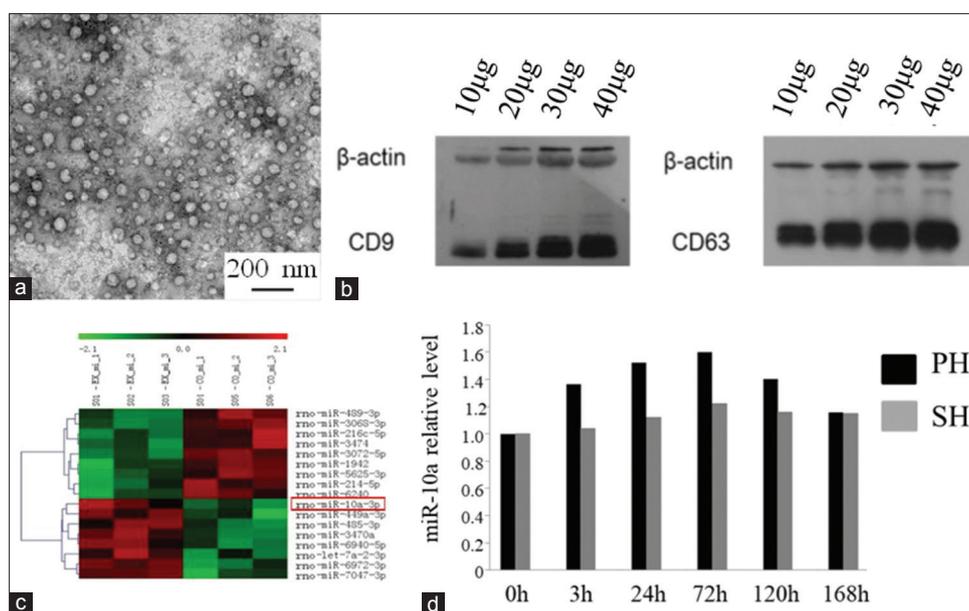


Figure 1: Identification of the exosomal key miRNA in liver regeneration. (a) Transmission electron microscopy of the exosomes extracted from the PB. (b) Western blot of exosome marker CD9 and CD63. (c) Microarray analysis results of key microRNA working in liver regeneration. (d) Quantitative polymerase chain reaction results of miR-10a levels in liver regeneration. PH: Partial hepatectomy; SH: Sham.

hepatectomized rats [experimental group] compared with rats received sham surgery [sham group]) after 70% partial hepatectomy [Figure 1c]. Among the differentially expressed miRNAs, miR-10a was significantly overexpressed in experimental group and has been demonstrated to promote cell proliferation,^[15] suggesting that miR-10a might stimulate the process of liver regeneration.

To confirm the expression change of miR-10a, we examined the levels of miR-10a in the serum of both experimental and control group at different time points [Figure 1d]. The qRT-PCR results showed that the level of miR-10a significantly increased 3 h after 70% hepatectomy and reached its peak point at 72 h. After that, the level of miR-10a began to decline. In the end, the level of miR-10a returned to preoperative level at 168 h. Collectively, these data indicated that miR-10a was upregulated during the process of liver regeneration.

Overexpression of miR-10a-accelerated liver regeneration

To determine whether miR-10a promotes liver regeneration, we constructed adenovirus to overexpress miR-10a in rats. After overexpression of miR-10a, 70% partial hepatectomy was performed on the rats. The remnant liver was obtained 24, 72, 120, and 168 h after surgery. The results showed that the liver weight/body weight ratio did not show any difference between miR-10a overexpression group and vector group at 24 and 168 h, while at 72 and 120 h, the ratio was significantly higher in miR-10a overexpression group than that in the vector group [Table 1]. In addition, the result of immunohistochemistry showed that the expression of PCNA in miR-10a overexpression group was higher than vector group at 24 and 72 h [Figure 2] after surgery, which was consistent with the results of miR-10a expression and liver weight/body weight ratio.

Table 1: The liver weight/body weight ratio of miR-10a overexpression group and vector group after surgery

Time after surgery	Overexpression group	Vector group	<i>t</i>	<i>P</i>
24 h	0.025 ± 0.002	0.024 ± 0.001	1.52	0.917
72 h	0.058 ± 0.002	0.046 ± 0.001	18.22	<0.05
120 h	0.068 ± 0.001	0.054 ± 0.002	19.74	<0.05
168 h	0.071 ± 0.003	0.069 ± 0.002	1.88	0.872

miR-10a promoted liver regeneration through downregulation of EphA4

To investigate the mechanism of miR-10a-mediated regulation of liver regeneration, we used TargetScan, miRanda, and PicTar to predict the putative target gene of miR-10a. EphA4, which was predicted by all three tools, was identified as a putative miR-10a target. Meanwhile, previous studies also have reported that miR-10a could bind the 3'-UTR of the EphA4 transcript.^[16] EphA4 belongs to the ephrin receptor subfamily of the protein-tyrosine kinase family. A recent study showed that upregulation of miR-10a could suppress the expression of EphA4 in liver tumor cells, thus facilitating the progression and metastasis of liver tumor.^[16] Therefore, we hypothesized that miR-10a could contribute to liver regeneration by suppressing the expression of EphA4 in normal liver cells. The immunoblot of EphA4 showed that the expression of EphA4 gradually declined in the first 72 h after partial hepatectomy and returned to preoperative level at 168 h after surgery [Figure 3a], which was negatively correlated with the level of miR-10a, suggesting that hepatectomy-induced miR-10a overexpression could downregulate the expression of EphA4.

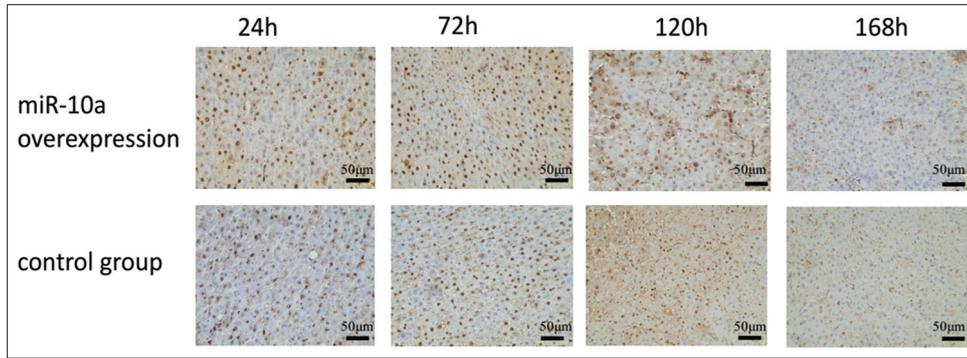


Figure 2: The expression of PCNA in the liver of miR-10a overexpression group was higher than that in control group. Adenovirus was used to overexpress miR-10a in rats, and PCNA staining was used to assess the ability of liver regeneration in both groups. PCNA: Proliferating cell nuclear antigen.

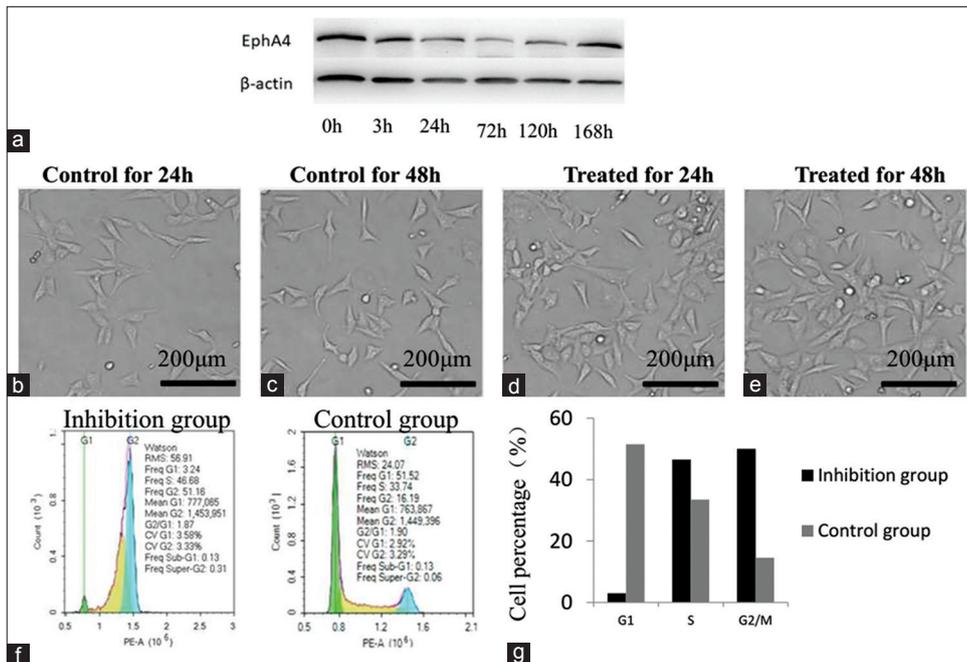


Figure 3: Inhibition of EphA4 accelerated the proliferation of hepatocytes through promotion of cell cycle. (a) Western blot of EphA4 showed that the level of EphA4 was in inverse correlation with miR-10a. (b–e) Representative images of untreated L-02 cells and cells treated with rhynchophylline (300 µg/L) for 24 h or 48 h. (g) The statistical data of the flow cytometry (f) showed that the percentage of cells in S stage and G2/M stage increased after inhibition of EphA4 by rhynchophylline.

Inhibition of EphA4 stimulated cell proliferation of hepatocytes through promotion of the cell cycle

To further determine the function of EphA4 on liver cells, we treated the normal liver L-02 cells with rhynchophylline, which was reported as an inhibitor of EphA4. The result showed that L-02 cells cultured with rhynchophylline for 24 h [Figure 3d] and 48 h [Figure 3e] showed higher cell density than that in control group cultured without rhynchophylline for 24 h [Figure 3b] and 48 h [Figure 3c], suggesting that the proliferation of hepatocytes was accelerated after rhynchophylline treatment. Moreover, the result of flow cytometry showed that the cell percentage of S stage and G2/M stage cells was higher in rhynchophylline-treated cells than that in the control group [Figure 3f and 3g]. Taken together, these results suggested that miR-10a might promote the proliferation of hepatocytes through inhibition of EphA4.

DISCUSSION

Liver is an important organ for metabolism and energy transformation that possesses the strong ability for regeneration. Once suffered physical, nutritional, vascular, chemical, or virus-triggered liver injury, the liver will turn up the process of regeneration quickly.^[17] Unlike the regeneration of skin and other organs, the regeneration of liver relies on the proliferation of mature hepatocytes but not the stem cells.^[18] The previous studies have demonstrated that the activation of intrahepatic urokinase and the reconstruction of extracellular matrix occur several minutes after 70% partial hepatectomy in rats, and the volume of the remnant liver will return to preoperative liver in 5–7 days.^[19] In addition, it has also been proved that, in human, the most rapid period for liver regeneration is the following week after

hepatectomy, and the liver can return to preoperative volume one year after surgery.^[20] Although the liver is so powerful to regenerate after injury, many patients suffered partial hepatectomy developed liver failure because the remnant liver is not enough to support the whole body. Therefore, understanding the mechanisms of liver regeneration is indispensable for solving this clinical problem.

Exosomes are a kind of extracellular vesicles that are spherical particles enclosed by a phospholipid bilayer. Exosomes are 40–100 nm in diameter and secreted in a variety of cells. Recently, it has been reported that exosomes can regulate cell–cell communication and many pathophysiological events through horizontal transfer of their cargoes.^[21] Exosomal cargoes include mRNAs, miRNAs, and proteins, which can be transferred from donor cells to recipient cells and can regulate cell activities such as cell proliferation, differentiation, and antiviral responses. In our study, we extracted the exosomes from the peripheral blood of the rats. miRNA microarray and quantitative PCR (qPCR) were performed and identified that exosomal miR-10a might play an important role in liver regeneration of rats. However, the detailed mechanism underlying exosomal miR-10a-mediated promotion of liver regeneration remains to be elucidated.

miR-10a has been reported to be aberrantly overexpressed in a series of human tumors.^[22,23] In addition, many previous studies have shown that miR-10a plays vital roles in tumor progression, maintenance, and metastasis.^[24] As for liver, the previous studies also showed that miR-10a can downregulate EphA4 and promote the progression and metastasis of hepatocellular carcinoma.^[16] In this study, to determine the function of miR-10a in liver regeneration, we overexpressed miR-10a in rats through adenovirus and 70% partial hepatectomy was performed. After that, we compared the liver weight/body weight ratio between miR-10a overexpression group and vector group to access the regenerative ability of the remnant liver. We found that the liver weight/body weight ratio of miR-10a overexpression group was significantly higher than vector group at 72 and 120 h, indicating that the upregulation of miR-10a could facilitate liver regeneration. On the other hand, no significant difference was observed at 24 and 168 h. This might be because the first 24 h after hepatectomy is the preparatory stage for liver regeneration. Many proliferation-related DNAs are synthesis in this period, while the proliferation of liver parenchyma occurs after that. Moreover, at 168 h after surgery, the main phase of liver regeneration of rats is almost finished, and the weight of the remnant liver has already returned to the preoperative level.^[25]

EphA4 has already been demonstrated to be implicated tumor progression and metastasis.^[26] In addition, downregulation of EphA4 can facilitate the regeneration of nerve axon.^[27] In our study, we showed that the expression of EphA4 was downregulated after hepatectomy and inversely correlated with the expression of miR-10a, suggesting that miR-10a could promote liver regeneration by downregulating the

expression of EphA4. Moreover, we proved that inhibition of EphA4 accelerated the proliferation of hepatocytes through the promotion of the cell cycle. Further studies are needed to investigate the regulatory mechanism of EphA4 in cell cycle.

In conclusion, our study showed that exosomal miR-10a was upregulated during the proliferation phase of liver regeneration. We also demonstrated that overexpression of miR-10a could facilitate liver regeneration. Moreover, we revealed that miR-10a might promote the process of liver regeneration through inhibition of the EphA4.

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Conflicts of interest

There are no conflicts of interest.

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外泌体中的miR-10a可以通过下调EphA4的方式促进肝再生

摘要

背景：大量的研究证据显示microRNAs在肝再生过程中发挥着重要的作用。然而之前的报道大都集中在关于细胞内microRNAs功能的研究，对于循环系统中外泌体内的microRNAs在肝再生中的作用却研究较少。本文的目的在于找到在肝再生过程中发挥重要作用的外泌体microRNA。

方法：以大鼠70%肝切除术后的肝再生模型作为实验组，以单纯开关腹的假手术模型作为对照组。术后24小时分别抽取实验组和对照组大鼠的外周血，并提取其中的外泌体microRNAs。通过microRNA芯片筛选出在对照组和实验组中显著差异性表达的microRNA。于70%肝切除术后各设定的时间点经尾静脉抽取外周血并提取外泌体，采用qPCR明确目标miRNA在术后的变化规律。对大鼠行70%肝切除术后经门静脉注射目标microRNA的过表达腺病毒载体和对照载体，于术后各设定时间点分别处死大鼠，完整切取肝脏组织，获得肝重/体重比；将所得肝组织行免疫组化检测PCNA (proliferating cell nuclear antigen)，明确增殖情况；通过生物信息学分析和文献及数据库检索等方式，筛选出目标miRNA的下游作用基因，确定结合位点，并利用WB检测所得肝脏标本中该下游蛋白表达量的差异。体外实验培养人L-02肝细胞，利用流式细胞术进一步验证下游基因的作用。

结果：实验结果显示，术后24小时实验组中的miR-10a比对照组增高9倍余。在肝再生早期，miR-10a的表达水平已经开始增高，在术后72小时达到峰值，在此后逐渐下降，直到术后168小时，恢复到了术前水平。PCNA免疫组化染色的结果也表明，过表达miR-10a可以促进大鼠的肝再生。我们通过软件预测和文献检索，确定了EphA4是miR-10a的下游靶标。同时，我们也发现EphA4在肝再生的早期聚在下降，在术后72小时达到最低值，并于术后168小时组件恢复到了术前水平与miR-10a的表达呈负相关，进一步证实了EphA4是miR-10a的下游靶标。除此之外，在体外实验中，我们也发现，运用钩藤碱抑制EphA4的表达后可以通过调节细胞周期的方式促进肝脏细胞的增殖。

结论：外泌体中的miR-10a可以通过下调EphA4的方式促进肝再生。