Previous studies have confirmed that interfering with the expression of these miRNA in the liver can significantly inhibit liver cell regeneration after hepatic resection, while the overexpression of these miRNA through different means can significantly stimulate liver cell regeneration. However, after major hepatic resection, in addition to the miRNA with significantly increased expression, there are some miRNA with significantly decreased expression. The decrease in the expression of these miRNA increases the expression of molecules in liver cells that promote cell proliferation or cell growth cycles, thereby accelerating liver regeneration. In this study, we found that expression of miRNA-20a in mice and rat livers significantly decreased after hepatic resection. Further research confirmed that at 0, 24, 48, and 168 hours after hepatic resection, the expression of miRNA-20a is significantly negatively correlated with Ki67, the marker protein of liver cell proliferation. We were still unable to clarify, however, whether miRNA-20a regulates liver regeneration after hepatic resection. First, we confirmed that inhibiting the expression of miR-20a stimulates the proliferation of PHC and AML12 cells and the proportion of cells in S phase in vitro, and that overexpression inhibits the proliferation of AML12 cells and cell cycle progression. Next, we discovered that the overexpression of miR-20a in mice liver significantly interferes with the speed of liver regeneration. Yet, although the mice that received tail vein injections of adenovirus carrying the miRNA-20a interference sequence had greater liver regeneration compared to the control group at the early stage, there was no significant difference at the late stage. Studies have shown that the change in liver volume during liver regeneration is precisely regulated to prevent the liver from becoming too large and to prevent the development of liver tumors, and one of the most crucial signaling pathways for this liver volume control is the Hippo-YAP signaling pathway. This signaling pathway is activated by mechanical sensing, meaning a change in liver volume. At the late stage of liver regeneration, when the volume of the liver is almost normal, the activation of this pathway inhibits various pathways and molecules that promote cell proliferation, preventing the over-proliferation of liver cells. We know that the regeneration of the liver after hepatic resection is a complex physiological process involving many types of cells in the liver, including liver sinusoidal endothelial cells, liver Kupffer cells, hepatic stellate cells, and so forth. These interstitial cells secrete multiple types of cytokines, such as HGF, EGF, IL6, and TNFa, to activate signaling pathways such as PI3K/akt, MAPK/ERK, and NK-kB in the liver cells to together regulate the liver regeneration. Although we can confirm that interfering with the expression of miR-20a in liver cells accelerates liver cell proliferation and promotes regeneration at the early stage, at the late stage of liver regeneration the compensatory effect of the Hippo-YAP signaling pathway and the other molecules antagonizes the excessive growth of the liver and the risk of tumor development that miR-20a would cause by default. However, if the liver is in a pathological state—for example, in the case NAFLD, liver fibrosis, liver cirrhosis, or similar—this self-regulating mechanism of the liver may be damaged, and liver cells may over-proliferate and even become cancerous.

To sum up, we confirmed through experiments at the cell and animal levels that miRNA-20a has an important regulatory function in liver regeneration in mice after hepatic resection. We know that miRNA regulates the expression of target genes at the transcriptional and post-transcriptional levels, with the latter being primary. A given miRNA can usually regulate up to hundreds of target genes. However, the genes targeted by the miRNA can differ significantly for different cells and disease processes. In this study, we needed to clearly identify miRNA-20a’s target gene in the liver cell proliferation process. To this end, we began by isolating primary hepatocytes in mice and processed them with an miRNA-20a mimic and an miRNA-20a inhibitor. First, we found that the mimic inhibited PHC proliferation and cell cycles, while the inhibitor treatment significantly increased PHC proliferation and cell cycles. Next, we did a PCR array analysis for the miRNA-20a target gene for these two groups of PHC. The chip result showed that in the mimic-treated group, the expression of TCF4 was significantly lower than in the control cells. Meanwhile, the TCF4 expression in the inhibitor-treated group was significantly higher than in the control cells. These results suggest that TCF4 is probably the target gene regulated by miRNA-20a. In addition, the results of further precise target verification confirm that miRNA-20a binds to the 3’UTR region of TCF4, dialing down its expression. Moreover, we reanalyzed the correlation between miRNA-20a expression and TCF4 expression at different time points after the rat and mice hepatic resections. The results are shown in the figure. The expression of miRNA-20a in the liver was significantly negatively correlated with TCF4 at the different time points, which suggests that miRNA-20a inhibits the expression of TCF4 in the liver regeneration process.

Studies have shown that TCF4 is an important transcription factor in the Wnt signaling pathway. It participates in the proliferation of many types of cells and the regulation of cell cycles, including the development and growth of tumors. To verify that it is indeed through the target cell TCF4 that miRNA-20a regulates liver cell regeneration after hepatic resection, we separately inhibited TCF4 in mice liver and then observed the post-hepatic-resection liver regeneration in these mice. The results show that inhibiting the expression of TCF4 slows liver regeneration. In addition, we interfered with both the expression of miRNA-20a and TCF4 in PHC, and the results suggest that miRNA-20a’s regulation of liver cell proliferation is dependent on TCF4.

Because TCF4 is a transcription factor, it mainly binds to a gene’s promoter region to regulate a downstream gene, and in this way takes part in stimulating cell cycle progression. Our cell-level research confirms that TCF4 promotes the progression of liver cells from G2-M phase to S phase, thus regulating liver cell proliferation. However, TCF4 is just a transcription factor and is unable to act directly on proteins related to cell cycles. It mainly regulates cell cycles by regulating downstream genes at the transcription level. We know that many cell-cycle-regulating proteins take part in the process of liver cell proliferation. Therefore, we tested the TCF4-treated PHC using a cell-cycle-protein PCR array. The results show that when TCF4 expression in the PHC is inhibited, the cell cycle proteins CDC2 and CDC6 decrease in expression the most significantly, while they increase dramatically in expression when TCF4 is overexpressed.

Previous research has shown that CDC2 and CDC6 play an important role in promoting cell cycle progression. We further discovered using promoter binding site analysis software (JASPAR and PROMO) that the promoter region of CDC2 has two TCF4 binding sites, and that of CDC6 has four TCF4 binding sites. This suggests that TCF4 may ratchet up the expressions of CDC2 and CDC6 by binding to their promoter regions, and thus stimulate cell cycle progression. We then used promoter activity analysis and immunoprecipitation analysis to confirm that TCF4 is able to bind to the promoter regions of CDC2 and CDC6 and intensify their expressions.

Past research has shown that miRNA-20 is a microRNA with a tumor-inhibiting function. Researchers have discovered that its expression is decreased in multiple types of tumors, including liver cancer tumors. This means that continuously inhibiting its expression can potentially cause the development of hepatocellular carcinoma. Our study found that in normal mice or rats, its expression drops dramatically at 48 hours after hepatic resection, and then returns basically to normal by day 7 as the liver increases in volume. This indicates that a normal liver self-adjusts its miRNA-20a expression to prevent the risk of hepatocellular carcinoma caused by the over-inhibition of miRNA-20a.

In conclusion, this study confirms that the miRNA20a–TCF4–CDC2/6 control axis plays an important role in liver regeneration in mice after hepatic resection.