Systematic analysis of the molecular mechanism of microRNA-124 in hepatoblastoma cells

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Abstract. The present study aimed to identify the molecular mechanisms of microRNA-124 (miRNA-124/miR-124) in hepatoblastoma. The GSE6207 microarray dataset, obtained from the Gene Expression Omnibus database, included samples extracted from HepG2 cells transfected with miR-124 duplex (the experimental group) or negative control (the control group) at 4, 8, 16, 24, 32, 72 and 120 h after transfection. Differentially expressed genes (DEGs) were screened between the two groups. miR-124 activity was inferred based on the expression of its target genes. The mRNAs targeted by miR-124 were predicted and a miR-124-target mRNA network was constructed. Gene ontology (GO) and Kyoto Encyclopedia of Genes and Genomes pathway enrichment analyses were performed for the target genes. The number of DEGs was highest at 72 h. The experimental group had higher miR-124 activity than that of the control group at 4, 8, 16, 24 and 120 h. Small GTPase-mediated signal transduction and Ras protein signal transduction were significant GO terms enriched with syndecan binding protein (SDCBP), Ras homolog family member G (RHOG) and Rh GDP dissociation inhibitor-a (ARHGDIA). Regulation of actin cytoskeleton, D-glutamine and D-glutamate metabolism, and axon guidance were significant pathways. Axon guidance pathway was associated with neuropilin (NRP1), MET proto-oncogene, receptor tyrosine kinase (MET) and semaphorin 7A, GPI membrane anchor (SEMA7A). Small GTPase-mediated signal transduction, Ras protein signal transduction, regulation of actin cytoskeleton pathway, D-glutamine and D-glutamate metabolism pathway, axon guidance pathway, SDCBP, RHOG, ARHGDIA, NRP1, SEMA7A, and MET may be implicated in the underlying mechanisms of miR-124 overexpression in hepatoblastoma.

Introduction

Liver cancer is the sixth-leading cause of cancer and the second-leading cause of cancer-associated mortality worldwide (1), leading to 746,000 mortalities in 2012 (1). Hepatoblastoma, an embryonal malignancy originated in hepatocytes, is the most common primary liver tumor in children, and is usually diagnosed in the first 3 years of life (2). The incidence of hepatoblastoma in children has gradually increased, and the overall 5-year survival rate in children is ~70% (3,4). The most common manifestation of hepatoblastoma is abdominal distension or presence of a mass (2). Hepatoblastoma can generally be cured via tumor resection if the tumor is single, smaller and non-metastatic, but a number of children cannot be cured due to a late diagnosis, multiple subtypes and metastases, which cause a poor prognosis (5). Therefore, it is necessary to investigate the molecular mechanisms of hepatoblastoma and lay the basis for refining the current diagnosis or treatment interventions. The HepG2 cell line is originated from hepatoblastoma (6); thus, HepG2 cells were selected in the present study to investigate the molecular mechanisms of hepatoblastoma.

MicroRNAs (miRNAs/miRs) are small non-coding RNA molecules involved in diverse cell activities, including regulation of cell proliferation, differentiation, apoptosis and carcinogenesis (5,6). miR-124 is the most abundant miRNA in the adult brain and serves a tumor-suppressive role in diverse types of cancer, including those of the breast and prostate (7,8). miR-124 is potentially involved in liver cancer: miR-124 suppresses aggressive hepatocellular carcinoma by repressing Rho-associated, coiled-coil containing protein kinase 2 and enhancer of zeste 2 polycomb repressive complex 2 subunit (9). miR-124 overexpression appears to repress the migration and invasion of intrahepatic cholangiocarcinoma cells in vitro (10). This evidence indicates that miR-124 may serve a complicated tumor-suppressive role in hepatoblastoma.; however, its underlying molecular mechanism remains elusive.

The activity of miRNAs is negatively associated with the expression of their target mRNAs (11-13). On this basis, miR-124 transfection has been used to identify its downregulated target mRNAs by microarray time-course
experiments (14). These target genes may be implicated in various biological functions and signaling pathways, thereby contributing to the progression of hepatoblastoma. To further investigate the underlying mechanisms of miR-124 overexpression in hepatoblastoma, the present study applied a range of bioinformatics approaches to a microarray dataset (GSE6207). Differentially expressed genes (DEGs) between human hepatoblastoma HepG2 cells transfected with a miR-124 duplex or a negative control RNA duplex at different time points (4, 8, 16, 24, 32, 72 and 120 h) were screened, followed by Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analysis for the screened DEGs. Changes in miR-124 activity over the time course were also analyzed. miR-124-target mRNA pairs were then selected to construct miR-124-target mRNA networks for each time point. 

Gene ontology (GO) function and KEGG pathway enrichment analysis were performed for the targets genes of miR-124. The present study therefore provides additional data concerning the molecular mechanisms of miR-124 in hepatoblastoma.

Materials and methods

Microarray data and preprocessing. The present study used the GSE6207 microarray dataset, downloaded from the National Center for Biotechnology Information Gene Expression Omnibus (GEO; https://www.ncbi.nlm.nih.gov/geo/) repository based on the Affymetrix Human Genome U133 Plus 2.0 Array platform (14). It included RNA samples extracted from HepG2 cells transfected with a miRNA-124 duplex or a negative control, with one sample for each time point (4, 8, 16, 24, 32, 72 and 120 h). The gene expression data were sequentially preprocessed with background correction, quantile normalization and probe summarization using robust multiarray analysis (RMA) of the affy package in R (15). It included RNA samples extracted from HepG2 cells transfected with a miRNA-124 duplex or a negative control, with one sample for each time point (4, 8, 16, 24, 32, 72 and 120 h). The gene expression data were sequentially preprocessed with background correction, quantile normalization and probe summarization using robust multiarray analysis (RMA) of the affy package in R (15). The expression values of several probes that correspond to a certain gene were averaged, and the averaged expression value was defined as the expression value of the gene.

DEG screening. DEGs at each time point were screened in the HepG2 cells transfected with the miRNA-124 duplex and those transfected with the negative control RNA duplex. As there was only one sample for each experimental condition at each time point, log₂ fold change ≥0.58 was set as the strict cutoff (16). The expression values of DEGs at different time points were hierarchically clustered using Cluster software. Furthermore, GO function (24) and KEGG pathway enrichment analysis were performed for functional annotation of the obtained target genes in the network, with P<0.05 as the strict threshold.

Results

Screening of DEGs at different time points. DEGs in HepG2 cells transfected with the miRNA-124 duplex and negative control were screened at 4, 8, 16, 24, 32, 72 and 120 h. As shown in Fig. 1, the total count of DEGs increased following transfection, reaching its peak at 72 h and then decreasing. Of the DEGs, there were more downregulated genes than upregulated genes at 4, 8, 16, 24, 32 and 72 h after transfection. The exception was 120 h, when there were more upregulated genes than downregulated genes. The expression data of all DEGs at seven time points is displayed in Fig. 2.

KEGG pathway enrichment analysis for DEGs. Pathways significantly enriched for upregulated DEGs at different time points are shown in Table I. No significant pathways were detected at 4, 8 or 16 h; genes involved in the tumor protein p53 signaling and retinol metabolism pathways were significantly associated with upregulated genes at 24 h after transfection. Expression of genes involved in the p53, systemic lupus erythematosus and mitogen-activated protein kinase (MAPK) signaling pathways were significantly upregulated at 32 h; those involved in axon guidance and adherens junction pathways were significantly upregulated at 72 h; those involved in glycerolipid metabolism and p53 signaling pathways were significantly upregulated at 120 h. With respect to downregulated DEGs (Table II), no significant pathway was detected at 4 or 16 h; those involved in the ribosome pathway (hsa03010: ribosome) were significantly downregulated at 8 h; those involved in small cell lung cancer, sphingolipid metabolism and cell cycle were significantly downregulated at 24 h; those involved in small cell lung cancer, the cell cycle and
pathways in cancer were significantly downregulated at 32 h; those involved in the cell cycle and DNA replication pathways were significantly associated with downregulated genes at 72 h; and those involved in the complement and coagulation cascades, cell cycle, and valine, leucine and isoleucine degradation pathways were significantly downregulated at 120 h.

Activity changes of miRNA-124. The changes in activity of miR-124 following transfection are shown in Fig. 3. The activity of miR-124 varied with time after transfection: It was highest at 4 h after transfection. The activity of miR-124 was higher in the experimental group than that in the control group at 4, 8, 16 and 24 h after transfection. By contrast, the experimental group exhibited decreased miR-124 activity compared with that of the control group at 72 h after transfection. Notably, at 32 h, the activity of miR-124 in the experimental group was similar to that in the control group.

miR-124-target gene network analysis. The target mRNAs of miRNA-124 at each time point were predicted based on the aforementioned five repositories (miRanda, MirTarget2, PicTar, PITA and TargetScan). To investigate the association between miR-124 and its target genes, the miR-124-target gene pairs validated in at least three out of these five databases were used to construct miR-124-target gene networks for each time point. The expression values of all the predicted target genes at different time points are shown in a heat map (Fig. 4).

The miR-124-target gene networks are shown in Fig. 5. At 4 and 8 h after transfection, all miR-124-target genes were downregulated, indicating high miR-124 activity. At 16, 24 and 32 h, a small proportion of the target genes were upregulated, whereas the majority of the target genes were downregulated. By contrast, more upregulated genes were observed at 72 and 120 h when compared to that at 16, 24 and 32 h, indicating a reduction in miR-124 activity.

Functional annotation for target genes of miR-124. GO functional enrichment analysis was performed for miR-124-target genes at different time points. No significant GO term was enriched at 4 or 8 h. As shown in Table III, Ras protein signal transduction was a significant GO term at 16 h and was enriched with syndecan binding protein (SDCBP), Ras homolog family member G (RHOG) and Rho GDP dissociation inhibitor-α (ARHGDIA). At 24 h, small GTPase-mediated signal transduction and Ras protein signal transduction were primary GO terms, and the two were significantly enriched with SDCBP, RHOG and ARHGDIA, Rho-guanine nucleotide exchange factor 3 (ARHGEF3), GTPase activating protein (SH3 domain) binding protein 1 (G3BP1) and V-Ral simian leukemia viral oncogene homolog A (RALA). Similarly, at 32 h, the two GO terms were also enriched with the above six genes. Cell cycle and intracellular transport were significant GO terms at 72 h. A total of 44 genes were significantly associated with the cell cycle, such as kinesin family member 23 (KIF23), KIF15, cell division cycle 27 (CDC27) and CDC25A. A total of 38 genes were significantly involved in intracellular transport, such as karyopherin-α (KPNA)5, KPNA3 and KPNA1.

The potential signaling pathways and target genes that may be involved in these signaling pathways were also
Table I. KEGG pathways enriched with upregulated differentially expressed genes at different time points.

| Time after transfection, h | KEGG pathway identity | Enriched genes, n | P-value         |
|----------------------------|------------------------|-------------------|-----------------|
| 24                         | hsa04115:p53 signaling pathway | 5                | 1.00x10^-3     |
|                            | hsa00830:Retinol metabolism         | 3                | 5.00x10^-2     |
| 32                         | hsa04115:p53 signaling pathway | 5                | 1.02x10^-2     |
|                            | hsa05322:Systemic lupus erythematosus | 5                | 3.53x10^-2     |
|                            | hsa04010:MAPK signaling pathway | 8                | 4.90x10^-2     |
| 72                         | hsa04360:Axon guidance         | 17               | 4.41x10^-3     |
|                            | hsa04520:Adherens junction      | 12               | 6.03x10^-3     |
|                            | hsa04144:Endocytosis            | 20               | 1.44x10^-2     |
|                            | hsa00051:Fructose and mannose metabolism | 7                | 1.48x10^-2     |
|                            | hsa05130:Pathogenic *Escherichia coli* infection | 9                | 2.00x10^-2     |
|                            | hsa04115:p53 signaling pathway | 10               | 2.00x10^-2     |
|                            | hsa05410:Hypertrophic cardiomyopathy (HCM) | 11               | 3.09x10^-2     |
|                            | hsa05412:Arrhythmogenic right ventricular cardiomyopathy | 10               | 3.81x10^-2     |
|                            | hsa00983:Drug metabolism        | 7                | 4.26x10^-2     |
|                            | hsa05414:Dilated cardiomyopathy  | 11               | 4.93x10^-2     |
| 120                        | hsa00561:Glycerolipid metabolism | 8                | 4.35x10^-3     |
|                            | hsa04115:p53 signaling pathway | 9                | 1.30x10^-2     |
|                            | hsa00140:Steroid hormone biosynthesis | 7                | 1.89x10^-2     |
|                            | hsa04070:Phosphatidylinositol signaling system | 9                | 2.09x10^-2     |
|                            | hsa05120:Epithelial cell signaling in *Helicobacter pylori* infection | 8                | 3.77x10^-2     |
|                            | hsa00564:Glycerophospholipid metabolism | 8                | 3.77x10^-2     |
|                            | hsa05211:Renal cell carcinoma    | 8                | 4.32x10^-2     |
|                            | hsa04650:Natural killer cell-mediated cytotoxicity | 12               | 4.49x10^-2     |
|                            | hsa00983:Drug metabolism        | 6                | 4.80x10^-2     |

KEGG, Kyoto Encyclopedia of Genes and Genomes; MAPK, mitogen-activated protein kinase; hsa, *Homo sapiens*.

analyzed (Table IV). No significant pathways were enriched with target genes at 4, 8 or 16 h. Regulation of the actin cytoskeleton pathway was a significant pathway at 24 h, and was enriched with several target genes, including coflin 2 (*CFL2*) and WAS protein family, member 1 (*WASF1*). The target genes were predominantly associated with small cell lung cancer and ether lipid metabolism pathways at 32 h. At 72 h, the D-glutamine and D-glutamate metabolism pathway was significantly enriched with glutamate dehydrogenase 1 (*GLUD1*) and *GLUD2*. At 120 h, axon guidance, adherens junction and the transforming growth factor β signaling pathway were enriched. The axon guidance pathway was significant at 72 and 120 h, and was enriched with a number of target genes, such as neuropilin 1 (*NRP1*), MET proto-oncogene, receptor tyrosine kinase (*MET*) and semaphorin 7A, GPI membrane anchor (*SEMA7A*).

Discussion

microRNAs participate in a variety of cell activities via suppression of the expression of their target genes (25). The molecular mechanism by which miR-124 results in suppression of hepatoblastoma cells increased with time following miR-124 transfection, reached a peak at 72 h and then decreased, compared with the findings in cells treated with a negative control. miR-124 overexpression led to more downregulated genes than upregulated genes at 4, 8, 16, 24, 32 and 72 h, which was partly consistent with the fact that miR-124 inhibits the expression of its target genes (26). However, a proportion of DEGs were upregulated following miR-124 transfection. An explanation for this observation is that the upregulated DEGs may be the result of downregulation of DEGs by miR-124 overexpression. miRNAs may regulate the expression of other miRNAs (12,27), which could therefore strengthen or inhibit the activity of other endogenous miRNAs, thus contributing to differential expression of their target genes. These inhibited miRNAs may cause upregulation of their target genes.

Small GTPases are intracellular G proteins and hydrolase enzymes that hydrolyze guanosine triphosphate. They serve a key role in diverse cell activities, including cell growth, differentiation, movement and lipid vesicle transport (28,29). Ras protein family members belong to the small GTPase family. Ras is an intracellular anti-apoptotic protein that is closely associated with the phosphoinositide 3-kinase/AKT and MAPK signaling pathways, regulating various activities,
Table II. KEGG pathways enriched with downregulated differentially expressed genes at different time points.

| Time after transfection, h | Term                                               | Enriched genes, n | P-value       |
|----------------------------|----------------------------------------------------|-------------------|---------------|
| 8                          | has03010:Ribosome                                  | 3                 | 2.30x10^{-2}  |
| 24                         | has05222:Small cell lung cancer                    | 9                 | 7.02x10^{-4}  |
|                             | has00600:Sphingolipid metabolism                   | 5                 | 1.25x10^{-2}  |
|                             | has04110:Cell cycle                                | 8                 | 2.65x10^{-2}  |
|                             | has00565:Ether lipid metabolism                    | 4                 | 4.75x10^{-2}  |
| 32                         | has05222:Small cell lung cancer                    | 11                | 6.88x10^{-3}  |
|                             | has04110:Cell cycle                                | 10                | 5.95x10^{-3}  |
|                             | has05212:Pancreatic cancer                         | 7                 | 1.21x10^{-2}  |
|                             | has05200:Pathways in cancer                        | 16                | 2.79x10^{-2}  |
|                             | has00480:Glutathione metabolism                    | 5                 | 4.37x10^{-2}  |
| 72                         | has04110:Cell cycle                                | 46                | 7.63x10^{-20} |
|                             | has03030:DNA replication                           | 20                | 1.43x10^{-12} |
|                             | has04914:Progesterone-mediated oocyte maturation    | 19                | 8.17x10^{-5}  |
|                             | has03040:Spliceosome                               | 24                | 8.48x10^{-5}  |
|                             | has00240:Pyrimidine metabolism                     | 20                | 9.97x10^{-5}  |
|                             | has03430:Mismatch repair                           | 9                 | 2.18x10^{-4}  |
| 120                        | has04610:Complement and coagulation cascades       | 16                | 1.39x10^{-9}  |
|                             | has04110:Cell cycle                                | 11                | 4.75x10^{-3}  |
|                             | has00280:Valine, leucine and isoleucine degradation| 6                 | 1.05x10^{-2}  |
|                             | has00260:Glycine, serine and threonine metabolism  | 5                 | 1.41x10^{-2}  |
|                             | has05020:Prion diseases                            | 5                 | 2.13x10^{-2}  |
|                             | has00910:Nitrogen metabolism                       | 4                 | 3.20x10^{-2}  |

KEGG, Kyoto Encyclopedia of Genes and Genomes; has, Homo sapiens.

Figure 3. Changes in miR-124 activity across the time course following miR-124 transfection. The activity score of miR-124 in the miR-124 transfection group was highest at 4 h after transfection. miR-124, microRNA-124.

Figure 4. Heat map for the expression of the target genes of miR-124 in the miR-124 transfection and negative control groups at various time points. Red area, upregulated genes; black area, genes with unaltered expression; green area, downregulated genes. miR-124, microRNA-124.
including cell apoptosis and growth (30,31). Several lines of evidence have suggested that small GTPases have a regulatory role in hepatoblastoma (32,33). Consistent with this, the present study revealed that small GTPase-mediated signal transduction and Ras protein signal transduction were significant GO categories, enriched with SDCBP, RHOG and ARHG DIA in hepatoblastoma at 24 and 32 h. SDCBP encodes syntenin-1, which is a tandem PDZ domain-containing protein involved in trafficking, signaling and cancer metastasis (34). Syntenin-1 is implicated in regulating colon cancer cell migration (35). However, the role of SDCBP in hepatoblastoma has not been previously reported. RHOG encodes the Ras
Table III. GO terms enriched with target genes of microRNA-124 at different time points.

| Time after transfection, h | Term                                           | Enriched genes, n | P-value     | Genes                                                                 |
|---------------------------|------------------------------------------------|-------------------|-------------|----------------------------------------------------------------------|
| 16                        | GO:0007265~Ras protein signal transduction      | 3                 | 4.38x10^{-2} | SDCBP, RHOG, ARHGdia                                                  |
|                           | GO:0007264~small GTPase mediated-signal transduction | 10               | 1.47x10^{-4} | ARHGEF3, RAP2C, G3BP1, RALA, SDCBP, RAB6A, ARL3B, IQGAP1, RHOG, ARHGdia |
|                           | GO:0007265~Ras protein signal transduction      | 6                 | 5.71x10^{-4} | ARHGEF3, G3BP1, RALA, SDCBP, RHOG, ARHGdia                          |
|                           | GO:0046907~intracellular transport              | 11                | 9.37x10^{-3} | SLC25A20, SLC25A14, NRBP1, SCAMP2, TOM1L1, IPO4, SDCBP, SEC13, SLC25A1, ARFIP1, YWHAE, ENPP2, WASF1, KATNA1, SDCBP, LAMC1, PPAP2B, ITGB1, YWHAE, ARHGdia |
|                           | GO:0006928~cell motion                          | 9                 | 1.13x10^{-2} | SDCBP, RHOG, ARHGdia                                                  |
|                           | GO:0015031~protein transport                    | 11                | 2.41x10^{-2} | SDCBP, RHOG, ARHGdia                                                  |
| 32                        | GO:0007264~small GTPase mediated-signal transduction | 11               | 1.61x10^{-4} | ARHGEF3, RAP2C, G3BP1, RALA, SDCBP, RAB6A, ARL3B, IQGAP1, RHOG, ARHGdia, RGL1 |
|                           | GO:0007265~Ras protein signal transduction      | 6                 | 1.52x10^{-3} | ARHGEF3, G3BP1, RALA, SDCBP, RHOG, ARHGdia                          |
|                           | GO:0006928~cell motion                          | 11                | 4.64x10^{-3} | SLC25A20, SLC25A14, NRBP1, SCAMP2, TOM1L1, IPO4, SDCBP, SEC13, TRAPPc4, SLC25A1 |
|                           | GO:0046907~intracellular transport              | 13                | 6.11x10^{-3} | SDCBP, RHOG, ARHGdia                                                  |
|                           | GO:0051329~interphase of mitotic cell cycle      | 5                 | 9.28x10^{-3} | PPM1D, KATNA1, CDK4, ITGB1, CDC25A                                 |
| 72                        | GO:0007049~cell cycle                           | 44                | 3.69x10^{-7} | KIF23, CKS1B, E2F3, TUBB2B, CDC16, ITGB1, SESN1, CTNNB1, CCNE2, APP, NDE1, RAD21, DUSP13, SEH1L, CENPA, KATNA1, RB1CC1, TFDP2, VPS4A, PPP3CA, HELLS, CSRBP2P, NASP, KIF15, SUV39H1, SMAD3, MND1, ILF3, GAS2, MCM2, CDK4, CDC27, CDC25A, SMC4, MCM6, PPM1G, PA2G4, PPM1D, FANCD2, PLK1, GAS2L1, SETDB8, SIAH2, ACVR1 |
|                           | GO:0046907~intracellular transport              | 38                | 1.79x10^{-6} | PPM1D, KATNA1, CDK4, ITGB1, CDC25A                                 |
homology growth-related (RhoG) protein, which is a small GTP-binding protein and a member of the Rac subgroup of the Rho family (36). The Rac signaling pathways serve an essential role in the motility of hepatocellular carcinoma cells (37). ARHGDIA encodes Rho GDP-dissociation inhibitor 1 protein, which participates in regulating Rho activity. The absence of ARHGDIA has been reported to enhance the invasion and metastasis of hepatocellular carcinoma cells (37). However, the role of these genes in hepatoblastoma has not been reported thus far. Thus, the present study speculated that miR-124 overexpression may lead to the inhibition of small GTPase-mediated signal transduction and Ras protein signal transduction by regulating SDCBP, RHOG and ARHGDIA, thereby repressing hepatoblastoma development.

The present study unraveled that regulation of the actin cytoskeleton pathway was significantly enriched with several target genes such as CFL2 at 24 h after transfection. Regulation of the actin cytoskeleton pathway is involved in cancer cell migration, tumor invasion and metastasis (38). CFL2 is an intracellular actin-modulating protein that regulates actin-filament

| Time after transfection, h | Term | Enriched genes, n | P-value | Genes |
|---------------------------|------|-------------------|---------|-------|
| GO:002402–cell cycle process | 33 | 8.30x10^{-6} | KIF23, TUBB2B, CDC16, ITGB1, SESN1, CTNNB1, APP, NDE1, RAD21, DUSP13, SEH1L, CENPA, KATNA1, PPP3CA, HELLS, CSRP2BP, KIF15, SMAD3, MND1, ILF3, GAS2, CDK4, CDC27, CDC25A, SMC4, PPM1G, PA2G4, PPM1D, FANC2D, PLK1, GAS2L1, SETD8, ACVR1 |
| GO:0048193–Golgi vesicle transport | 13 | 8.58x10^{-5} | GABARAPL2, AP1M1, SCAMP2, NRPB1, SEC24A, AP1M2, VPS41, LMAN1, AP1S1, SEC13, TRAPPCC4, TRAPPCC1, SAR1B |
| GO:0070727–cellular macromolecule localization | 24 | 2.12x10^{-4} | KDELRE2, AP1M1, NUP98, SEC24A, AP1M2, EIF5A, VPS41, ARFIP1, CASC3, CTNNB1, AP1S1, AP2B1, CSE1L, TOMIL1, IPO4, ZFYVE9, SEC13, SDCBP, KPNAP5, PPP3CA, SAR1B, KPNAP3, KDELRE1, SSR2 |
| 120 | GO:0006928–cell migration | 17 | 1.86x10^{-4} | TLN1, NRP1, PTRPRM, ENPP2, WASF1, MET, LICAM, NRCAM, APP, CCR6, TNS1, KATNA1, SDCBP, NOS3, PAFAH1B1, RELN, ACVR1 |
| GO:0046907–intracellular transport | 18 | 2.27x10^{-3} | AP1M1, SEC24A, NRPB1, YWHAB, VPS41, LMAN1, SLC25A20, APP, SLC25A14, VPS4A, SDCBP, SEC13, SLC25A1, PAFAH1B1, KPNAP5, KPNAP3, KPNAP1, SEC61A2 |
| GO:0032355–response to estradiol stimulus | 5 | 3.94x10^{-3} | BMP4, SOCS2, ENO2, NOS3, IGFBP2 |
| GO:0016477–cell migration | 10 | 6.03x10^{-3} | NRCAM, TNS1, NRP1, KATNA1, MET, SDCBP, NOS3, PAFAH1B1, RELN, ACVR1 |
| GO:0006796–phosphate metabolic process | 22 | 6.12x10^{-3} | ATP6V0E1, NRPB1, PTRPRM, PTPN3, SLC20A1, ENPP2, ERBB3, SMAD7, TBK1, STK36, MET, DUSP10, OXSR1, PPM1B, CDK4, MAST3, PPM1D, APP, RELN, PAK1, ATP6V0D1, ACVR1 |

GO, gene ontology.
dynamics (39); it indicates that miR-124 overexpression may inhibit cancer cell migration, tumor invasion and metastasis by regulating actin cytoskeleton pathway genes such as CFL2. Furthermore, the present study revealed that the D-glutamine and D-glutamate metabolism pathways were enriched (GLUD1 and GLUD2) at 72 h. The D-glutamine and D-glutamate metabolic pathways are critical components of protein synthesis and cellular energy production (40,41). GLUD1 and GLUD2 are two mitochondrial matrix enzymes that serve a key role in glutamate metabolism and energy homeostasis (42). The findings of the present study revealed that miR-124 transfection may affect glutamate metabolism and energy homeostasis.

Notably, the expression of NRP1, SEMA7A and MET, which are involved in the axon guidance signaling pathway, was significantly increased at 72 and 120 h. An aberrant axon guidance pathway is involved in pancreatic carcinogenesis (43). Nevertheless, the role of the axon guidance pathway in hepatoblastoma has not been clearly characterized. Neuropilin-1, encoded by NRP1, is a co-receptor member for members of the semaphorin family, and is involved in several cellular processes, including angiogenesis and cell survival (44). A previous study revealed that the expression of NRP1 is higher in HepG2 cell lines than that in a human normal hepatic cell line, indicating the effect of NRP1 on hepatoblastoma (45). SEMA7A, which is encoded by SEMA7A, is a membrane-bound semaphorin and promotes axon outgrowth (46). MET encodes hepatocyte growth factor receptor (HGFR), which has tyrosine kinase activity and binds to HGF. It has been reported that invasion of hepatocellular carcinoma cells is dependent on HGF (47). Furthermore MET/HGF inhibitors have been increasingly recognized as anticancer therapies for various types of cancer, including hepatoma (48). These observations indicate that axon guidance pathway genes may be abnormally regulated due to miR-124 overexpression, thereby suppressing hepatoblastoma cell growth and invasion. The present study indicates the presence of an as-yet-undescribed role of the axon guidance pathway in hepatoblastoma.

The current study demonstrated that miR-124 overexpression may perturb small GTPase-mediated signal transduction and Ras protein signal transduction, and abnormally affect the regulation of the actin cytoskeleton, D-glutamine and D-glutamate metabolic and axon guidance pathways in hepatoblastoma. SDCBP, RHOG, ARHGDIA, NRP1, SEMA7A and MET may be promising molecular targets for therapies against hepatoblastoma. These pathways are not specific to hepatoblastoma, but may also be involved in other types of cancer. These findings provide useful information concerning the molecular mechanisms that underlie the tumor-suppressive role of miR-124 in hepatoblastoma. Further experimental studies are warranted to validate the findings of the present study.

Table IV. Kyoto Encyclopedia of Genes and Genomes pathways enriched with target genes of microRNA-124 at different time points.

| Time after transfection, h | Term | Enriched genes, n | P-value | Genes |
|---------------------------|------|-------------------|---------|-------|
| 24                        | hsa04810:Regulation of actin cytoskeleton | 5 | 4.87x10^{-2} | CFL2, WASP1, ITGB1, IQGAP1, NCKAP1 |
| 32                        | hsa05222:Small cell lung cancer | 4 | 3.14x10^{-2} | E2F3, LAMC1, CDK4, ITGB1 |
| 72                        | hsa00565:Ether lipid metabolism | 3 | 3.34x10^{-2} | ENPP2, PPAP2B, AGPAT1 |
|                           | hsa04360:Axon guidance | 15 | 6.34x10^{-6} | NRP1, MET, DPYSL2, ITGB1, EPHA2, SEMA6A, ROBO1, SEMA7A, SEMA3F, CFL2, NFAT5, PAK1, PPP3CA, NFATC3, RASA1 |
| 120                       | hsa04110:Cell cycle | 14 | 2.20x10^{-5} | E2F3, E2F5, SMAD3, MCM2, CDC16, CDK4, CDC27, CDC25A, MCM5, MCM6, CCNE2, RAD21, PLK1, TFDP2 |
|                           | hsa05212:Pancreatic cancer | 9 | 5.74x10^{-4} | E2F3, RALBP1, RELA, ARAF, PIK3CD, SMAD3, RALA, BCL2L1, CDK4 |
|                           | hsa05222:Small cell lung cancer | 9 | 1.60x10^{-3} | CCNE2, CKS1B, E2F3, RELA, PIK3CD, LAMC1, BCL2L1, CDK4, ITGB1 |
|                           | hsa00471:D-Glutamine and D-glutamate metabolism | 3 | 4.05x10^{-3} | GLS2, GLUD2, GLUD1 |
|                           | hsa04360:Axon guidance | 7 | 1.29x10^{-2} | NRP1, CFL2, SEMA7A, MET, NFAT5, L1CAM, PAK1 |
|                           | hsa04520:Adherens junction | 5 | 2.86x10^{-2} | PTPRM, WASP1, MET, PVRL2, IQGAP1 |
|                           | hsa04350:TGF-β signaling pathway | 5 | 4.21x10^{-2} | BMP4, ID2, SP1, SMAD7, ACVR1 |

hsa, Homo sapiens; TGF, transforming growth factor.
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