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# Control of mitogenic and motogenic pathways by miR-198, diminishing hepatoma cell growth and migration

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ABSTRACT

Hepatocellular carcinoma (HCC) is one of the leading causes of cancer deaths, worldwide. MicroRNAs, inhibiting gene expression by targeting various transcripts, are involved in genomic dysregulation during hepatocellular tumorigenesis. In previous studies, microRNA-198 (miR-198) was shown to be significantly downregulated in HCV-positive hepatocellular carcinoma (HCC). Herein, the function of miR-198 in hepatocellular carcinoma cell growth and gene expression was studied.

In hepatoma cell-types with low levels of liver-specific transcription factor HNF1α indicating a low differentiation grade, miR-198 expression was most downregulated. However, miR-198 treatment did not restore the expression of the liver-specific transcription factors HNF1α or HNF4α. Importantly, overexpression of miR-198 in Pop10 hepatoma cells markedly reduced cell growth. In agreement, comprehensive gene expression profiling by microarray hybridisation and real-time quantification revealed that central signal transducers of proliferation pathways were downregulated by miR-198. In contrast, genes mediating cellular adherence were highly upregulated by miR-198. Thus, the low expression of E-cadherin and claudin-1, involved in cell adhesion and cell-cell contacts, was abolished in hepatoma cells after miR-198 overexpression. This definite induction of both proteins by miR-198 was shown to be accompanied by a significantly impaired migration activity of hepatoma Pop10 cells.

In conclusion, miR-198 acts as a tumor suppressor by repression of mitogenic and motogenic pathways diminishing cell growth and migration.



## 1. Introduction

Hepatocellular carcinoma (HCC) is a primary liver cancer that is the fifth most common cancer, leading to nearly 600,000 deaths a year, worldwide. Hepatitis B and C infections are frequent causes for HCC development. Other risk factors like aflatoxin B1, excessive alcohol abuse, drugs, hemochromatosis, and obesity play also an important role in development and progression of HCC [1–3].

Development of HCC is a multistep process, in which nearly 80% of HCC cases evolve from liver cirrhosis [2–5]. During progression of HCC, chromosomal and genetic alterations as well as epigenetic abnormalities can result in cellular dysfunction [3,5,6]. Chromosomal and epigenetic disarray leads to HCC progression by affecting the main proliferative cellular pathways [3,6,7]. Hepatocyte growth factor/hepatocyte growth factor receptor (HGF/MET) is affected by point mutations leading to a constitutive expression or overexpression. The insulin like growth factor IGF-2 mediates its high mitogenic potential by activation of the phosphatidylinositol 3-kinase (PI3K) and protein kinase B (AKT/PKB), in addition to Raf/mitogen activated protein kinase (MAPK) pathways [3,4,7,8]. In response to IGF-2 signaling, transcriptional upregulation of vascular epidermal growth factor (VEGF), and

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cyclin B as well as the protooncogene c-myc are initiated, enhancing cell proliferation and angiogenesis of the tumor. In addition, the HGF/MET pathway, a key mitogenic and motogenic pathway responsible for hepatic cell proliferation, is dysregulated during hepatocarcinogenesis [9–11].

In addition to the chromosomal and epigenetic alterations, genetic dysregulation of essential signaling pathways is orchestrated by tumor suppressor and oncogenic microRNAs. MicroRNAs (miRNAs) are small, non-coding RNA molecules that regulate gene expression on the post-transcriptional level by binding to and inhibiting target transcripts. During hepatocarcinogenesis, a wide variety of miRNA are dysregulated [12–15]. Thus, recent findings have shown that miR-21, miR-221 and miR-222 are increased in HCC [16,17]. miR-21 is a highly potent inhibitor of expression of many tumor suppressor proteins such as the phosphatase and tensin homolog (PTEN) protein and is thereby an enhancer of cell proliferation and migration [16]. Similarly, both miR-221 and miR-222 are strongly increased in HCC. Their overexpression results in reduced apoptosis and altered cell cycle control by targeting expression of many cell cycle associated proteins [18,19]. However, most miRNA species are downregulated during hepatocarcinogenesis [13,20]. miR-122 is the most abundant miRNA in liver and composes about 70% of all miRNA in hepatocytes [21]. Most reports have shown that miR-122 is strongly downregulated in HCC [22,23]. Loss of miR-122 affects hepatic metabolism, in particular the synthesis of cholesterol [24] and also cell cycle regulation [23]. In addition, miR-101, miR-145, and miR-199b are miRNAs which are downregulated after hepatocarcinogenesis [12,25].

In previous studies, we have demonstrated that miR-198 is one of the most prominently downregulated miRNA in HCC. Interestingly, the decrease of miR-198 was linked to the degree of liver injury and hepatocarcinogenesis [20]. miR-198 is moderately diminished in livers with cirrhosis, but even more so after development of dysplastic foci and after progression of HCC [20]. Furthermore, Cheung et al. have observed that miR-198 is decreased in liver tissues of patients with non-alcoholic fatty liver disease (NAFLD) [26]. Sung et al. identified miR-198 as a regulator of cyclin T [27] and, importantly, Tan et al. described that miR-198 targets the expression of the HGF receptor, Met, in Huh-7 hepatoma cells [28].

In the present study, we analyzed the impact of miR-198 on signaling pathways in hepatoma cells. Here, we demonstrate that overexpression of miR-198 in hepatoma cells leads to a marked inhibition of cell growth and cell migration. Comprehensive expression profiling in response to miR-198 underlines its crucial role in hepatocellular carcinogenesis, pointing to a dysregulation of immune responsive signaling, but most notably to central mitogenic and motogenic pathways.

## 2. Material and methods

### 2.1. Cell culture and transfection of miRNA

Human hepatoma cells Huh-7 [29], Hep3B [30], and Pop10 [31] were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum (FCS) in 5% $CO_2$ at 37 °C in humidified atmosphere. For transfection with miRNA, $3\times10^5$ cells were seeded on each well of a 6-well plate and transfected with either scrambled RNA [50 nM], miR-198 mimic [50 nM], or siRNA against HNF1α [10 nM] (caggacaagcatggtcccaca), HNF4α [10 nM] (aacctagagattgttacagaa), E-cadherin [30 nM] (tttgactgtaatcacacca tctgtg), or claudin-1 [10 nM] (gcatcctcctgggagtgatagcaat) from Thermo Scientific Dharmacon (Bonn, Germany) using lipofectamine 2000 (Invitrogen, Karlsruhe, Germany) according to the instructions of the manufacturer. Four hours after transfection, the medium was changed and cells were maintained in DMEM supplemented with 10% FCS for 24 h. Finally, cells were harvested for protein or RNA isolation.

### 2.2. Determination of cell growth, proliferation, and apoptosis

For analyses of cell viability, 24 h after transfection of Pop-10 hepatoma cells were treated with reagents of the Cell Titer 96 Aqueous One Solution Cell Proliferation Assay (Promega, Mannheim, Germany) according to the manufacturer. Cell viability was the measured at a wavelength $\lambda = 450$ nM as recommended by the contractor.

Apoptosis of transfected hepatoma cells was analyzed by the TUNEL assay using the In Situ Cell Death Detection Kit from Roche Applied Science (Mannheim, Germany) following the manufacturer's instructions. Apoptotic cells were quantified by counting fluorescent nuclei.

Cell proliferation was measured 24 h after miR-198 transfection of Pop-10 hepatoma cells by application of the ClickiT-Edu-Proliferation Kit (Invitrogen, Grand Island, NY, USA) due to the manufacturer's instructions. Detection of cell proliferation was performed by visual counting of fluorescence positive cells using the cell^P software (Olympus Europe Holding GmbH, Hamburg, Germany).

### 2.3. Determination of cell migration

For examination of the influence of miR-198 on cell migration, hepatoma cells were transfected with 50 nM scrambled RNA or miR-198 mimic. After 24 h, cell monolayers were scratched and monitored within 24 h. In addition, the Boyden chamber system was used for migration detection. $7\times10^4$ cells were seeded on the transwells (6.5 mm) with 8 μm pores (Corning B.V. Life Sciences, Amsterdam, The Netherlands). During seeding, cells were transfected either with 50 nM scrambled RNA or miR-198 in DMEM containing 2% FCS. Analyses of cell migration were performed after hematoxylin staining by light microscopy using the cell^P software for statistical evaluation (Olympus Europe Holding GmbH).

### 2.4. Total RNA isolation

Total RNA was isolated using Qiazol reagent following the instructions of the manufacturer (Qiagen, Hilden, Germany). RNA quantity was determined by the ND-1000 NanoDrop spectrophotometer (NanoDrop, Wilmington, DE, USA) and RNA quality was analyzed by microcapillary electrophoresis using the nanoRNA chip (2100 BioAnalyser, Agilent Technologies, Waldbronn, Germany).

### 2.5. Transcript and miRNA quantification by real-time PCR

Total RNA (1 μg) was reverse transcribed and polyadenylated using the miScript reverse transcription kit (Qiagen) as instructed. 10 ng cDNA was then applied for real-time PCR using Power SYBR Green PCR MasterMix (Applied Biosystems Life Technologies, Darmstadt, Germany) and transcript specific primer sets (Supplemental Table 1). For quantification of miRNA levels, 2 ng cDNA was applied for real-time PCR, using specific miScript Primer Assays from Qiagen. All reactions were performed in triplicates.

mRNA transcripts were normalized to hypoxanthin-phosphoribosyltransferase (HPRT) transcript levels. For normalization of the miRNA levels RNU6B was used as reference. Transcript and miRNA levels were calculated by the ΔΔCt-method. Primer used for quantification and validation analyses are listed in Supplemental Table 1.

### 2.6. Expression profiling by microarray hybridization

For gene expression profiling, hepatoma Pop10 cells were transfected with either 50 nM scrambled RNA or miR-198 for 24 h. Total RNA quantity and quality was monitored by microcapillary electrophoresis (2100 BioAnalyser, Agilent Technologies, Waldbronn, Germany). RNA samples of 300 ng and a RIN≥9 were reverse transcribed, followed by an in vitro transcription. Samples were chemically linked

with biotin and hybridized to Affymetrix Human Gene 1.0 ST Arrays (Affymetrix, Santa Clara, CA, USA). All cell experiments for gene expression profiling were performed in triplicates.

Hybridization arrays were scanned with the GeneChip Scanner 3000 7 G, controlled by GeneChip Operating Software (GCOS, Affymetrix). Raw data was processed with the Robust Multiarray

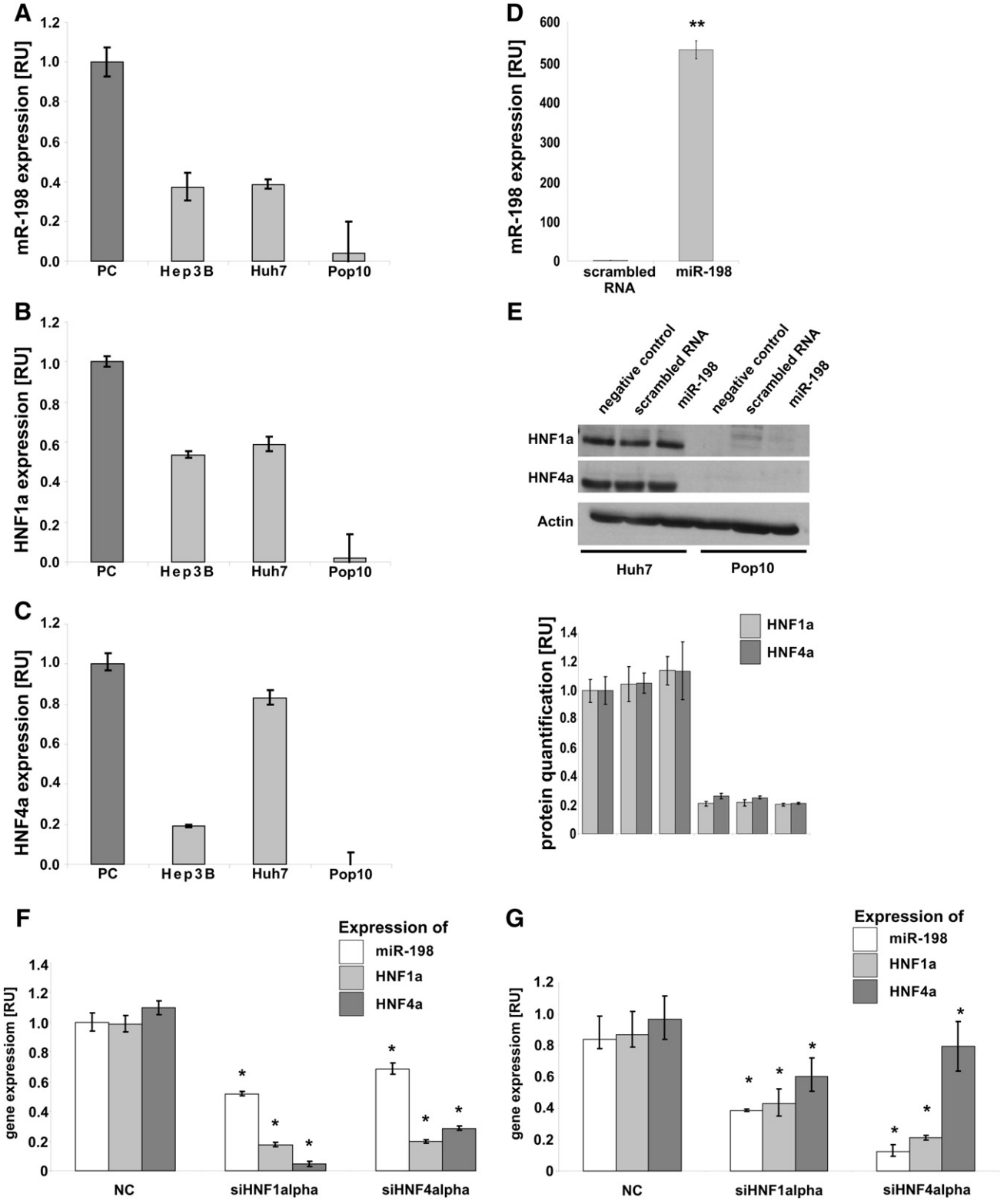


**Fig. 1.** miR-198 impact on HNF1α and HNF4α expression in different hepatoma cell types. Expression of miR-198, HNF1α, and HNF4α in normal liver parenchyma (PC) and in different hepatoma cells of Hep3B, Huh-7, and Pop10 cell lines was quantified by real-time PCR (A, B, C). miR-198 transfection in Pop10 cells resulted in increased miR-198 levels (D). After treatment with miR-198, protein levels of HNF1α and HNF4α were analyzed by Western blotting in Huh-7 and Pop10 cells, either untreated (negative control) or treated with scrambled RNA or miR-198. Actin protein levels were used for normalization (E). HNF1α and HNF4α were knocked down in Huh7 (F) and in Pop10 cells (G) by specific siRNA (siHNF1alpha, siHNF4alpha) [10 nM] for 24 h. Quantification of HNF1α, HNF4α, and miR-198 expression levels were performed by real-time PCR (F, G). miRNA and transcript levels were indicated by relative units [RU] (A–G).

Average (RMA) algorithm, then variance stabilized and transformed to a logarithmic scale.

Differences in gene expression were statistically analyzed by application of the unpaired Mann–Whitney test. Gene ontology (GO) was performed with GO-terms with p-value less or equal to 0.05 (enrichment score). In addition, array raw data were analyzed using the GeneSpring GX (Agilent Technologies) and Partec (Partec, Saint Luois, MO, USA) software. Expression profiles involved in certain pathways were indicated by BioCarta, KEGG, and GenMapp.

### 2.7. Western blot analysis

Cells were lysed with RIPA buffer containing protease inhibitors (10 μg/ml) (Roche Applied Biosystems, Mannheim, Germany). 10 μg of protein lysates were separated and incubated with anti-claudin (1:1000) (Cell Signaling, Danvers, MA, USA), anti E-cadherin (1:1000)(Dako, Hamburg, Germany), or anti-actin (1:2500) (Sigma-Aldrich Chemie GmbH, Munich, Germany) antibodies. After incubation with either anti-mouse or anti-rabbit secondary antibodies, both (1:5000) (Dianova, Hamburg, Germany) signals were detected by enhanced chemiluminescence and quantified using a FLUORCHEM FC2 Alpha Ease (Biozym, Hess. Oldendorf, Germany).

### 2.8. mRNA quantification in human HCC

Total RNA was extracted from 52 formalin fixed and paraffin embedded (FFPE) HCV positive HCC tissues as described previously [20]. For real-time PCR claudin-1 specific primer sets were designed (Supplemental Table 1) which produce very short amplicons, enabling quantitative PCR on RNA of FFPE material. Real-time PCR was carried out as described in Section 2.5, but for absolute quantification a standard curve was applied.

### 2.9. Statistical analyses

Statistical analyses were performed with SPSS software 17 (Chicago, IL, USA). Data are reported as means ± SEM. Variances were calculated using ANOVA and, statistical analyses were performed by Student's *t*-test. A value of $p<0.05$ was considered statistically significant.

## 3. Results

### 3.1. miR-198 expression in hepatoma cells of certain differentiation stages

Since previous data have shown that miR-198 is predominantly decreased during hepatocarcinogenis [20], we first studied the expression levels of miR-198 in different hepatoma cell lines. In Hep3B and Huh-7 cells and especially in Pop10 cells, the miR-198 concentration was very low in comparison to human parenchymal cells of normal liver (Fig. 1A). Quasdorff et al. have characterized different hepatoma cell types by their expression of hepatic metabolizing proteins and the liver-specific transcription factors, HNF1α and HNF4α [31]. Low levels of HNF1α and HNF4α indicate the advanced dedifferentiation of hepatocellular carcinoma cells, mimicking HCC progression [31]. Interestingly, both miR-198 and HNF1α transcripts were lowest in Pop10 cells. Therefore, the miR-198 expression profiles highly corresponded to HNF1α transcript levels in the different hepatoma cell types (Fig. 1A-C). In order to show the link of miR-198 and expression of liver-specific transcription factors, we compensated the low miR-198 expression in hepatoma cells by transfection of mature miR-198. miR-198 treatment resulted in a more than 500-fold increase of miR-198 in Pop10 cells (Fig. 1D). Subsequently, the protein levels of HNF1α and HNF4α were determined in Huh-7 and Pop10 cells in response to miR-198 overexpression. Western blot analysis confirmed the lower expression of both transcription factors in highly dedifferentiated Pop10 than in Huh-7 cells. Neither in Pop10 nor in Huh-7 cells miR-198 treatment did change the protein levels of HNF1α and HNF4α (Fig. 1E). Interestingly, efficient knockdown of the liver-specific transcription factors HNF1α and HNF4α by siRNA resulted in a definitive decrease of miR-198 expression level (Fig. 1F and G). In comparison to the regulation of miR-198 in Huh7 cells (Fig. 1F), in Pop10 cells miR-198 expression was moderately downregulated after efficient HNF1α and HNF4α silencing (Fig. 1G). This can be explained by the fact that Pop10 cells already hardly express miR-198.

### 3.2. High impact of miR-198 on hepatoma cell growth

Next, we addressed whether miR-198 might act as a tumor suppressor miRNA by affecting hepatoma cell growth. Therefore, we transfected hepatoma cells with miR-198 and measured cell viability (Fig. 2A), apoptosis (Fig. 2B) and cell proliferation (Fig. 2C). miR-198 treatment resulted in a marginal reduction of cell viability in Huh-7. In Pop10 cells, however, whose endogenous miR-198 expression was nearly completely abolished (Fig. 1A), a pronounced decrease of cell viability of more than 60% was detected after miR-198 overexpression (Fig. 2A). Analysis of apoptosis by TUNEL assays revealed only a small decrease in the number of apoptotic hepatoma cells, but a significant inhibition of cell proliferation was shown after miR-198 treatment (Fig. 2C).

### 3.3. Overexpression of miR-198 results in alteration of central cellular signaling pathways

After providing evidence that miR-198 acts as a potent tumor suppressor miRNA by inhibition of hepatoma cell growth, we aimed

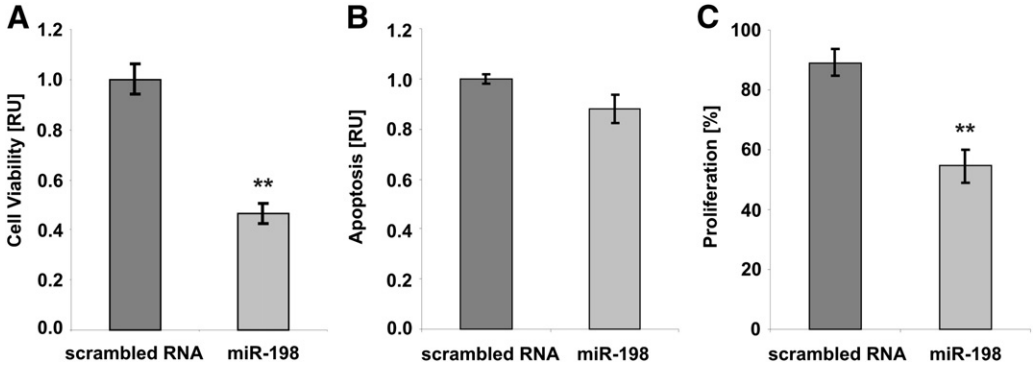


**Fig. 2.** miR-198 overexpression leads to inhibited cell growth of hepatoma cells. Hepatoma Pop10 cells transfected with miR-198 revealed an inhibitive influence of miR-198 on cell viability (A), apoptosis (B), and cell proliferation (C). Cell viability and apoptosis were indicated in relative units [RU] and the values of both, cell viability and apoptosis after scrambled RNA transfection were set to RU = 1 (A, B). For all assays, four independent experiments were performed.

to shed light on signaling pathways that are affected by miR-198. Therefore, a comprehensive gene expression analysis in Pop10 hepatoma cells was carried out by microarray hybridization after miR-198 treatment. Cluster analysis revealed that miR-198 overexpression influenced a wide range of gene expression profiles (Fig. 3A). In total, expression of 85 genes were significantly altered with a fold change higher than 1.5-fold. 49 genes were upregulated and 36 genes downregulated (Supplemental Tables 2 and 3). Those genes, that were most differentially expressed after miR-198 treatment are summarized in Fig. 3B and findings were validated by real-time PCR quantification shown in the Fig. 4.

Follistatin-like1 (FSTL1), whose exon sequence harbors the miR-198 gene, was the most reduced gene. In contrast to the interferon-responsive genes that were induced by miR-198 overexpression, genes

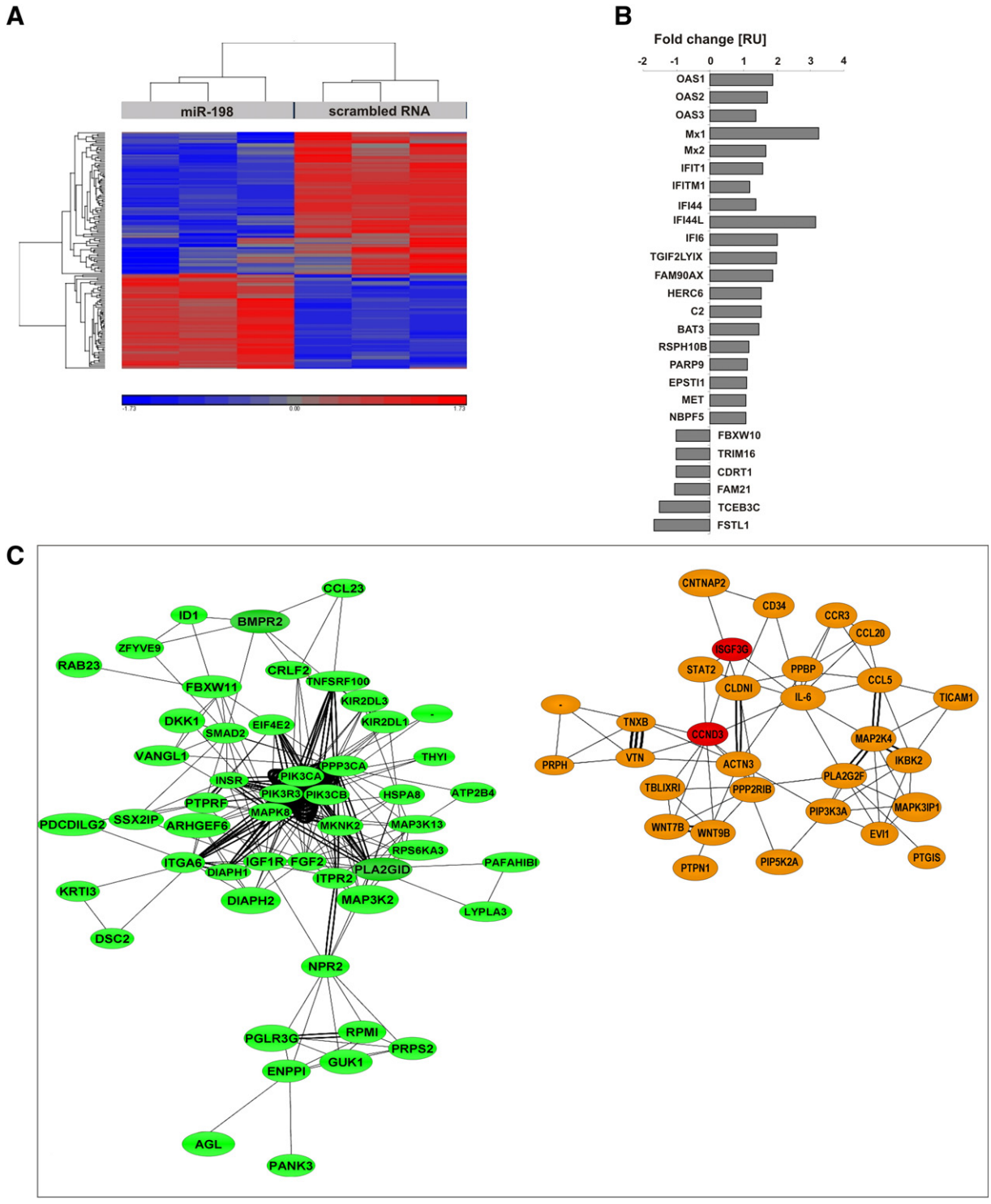


**Fig. 3.** Altered gene expression by miR-198 overexpression. Influence of miR-198 treatment on gene expression of Pop10 cells was proven by hybridization microarray analysis (A). The fold change of the expression of most significantly dysregulated genes after miR-198 transfection was demonstrated (B). Downregulated (C left; shown in green) and upregulated genes (C right; shown in red and orange) were analyzed for their linkage in interacting pathways by the Biorag database (http://www.biorag.org/ provided by the Arizona Cancer Center; SW Environmental Health Sci Center) and presented as signaling pathway trees. Highly upregulated genes cyclin *3D* (*CCND3*) and interferon-stimulated transcription factor-γ (*ISGF3G*) are implicated in deep red (C).

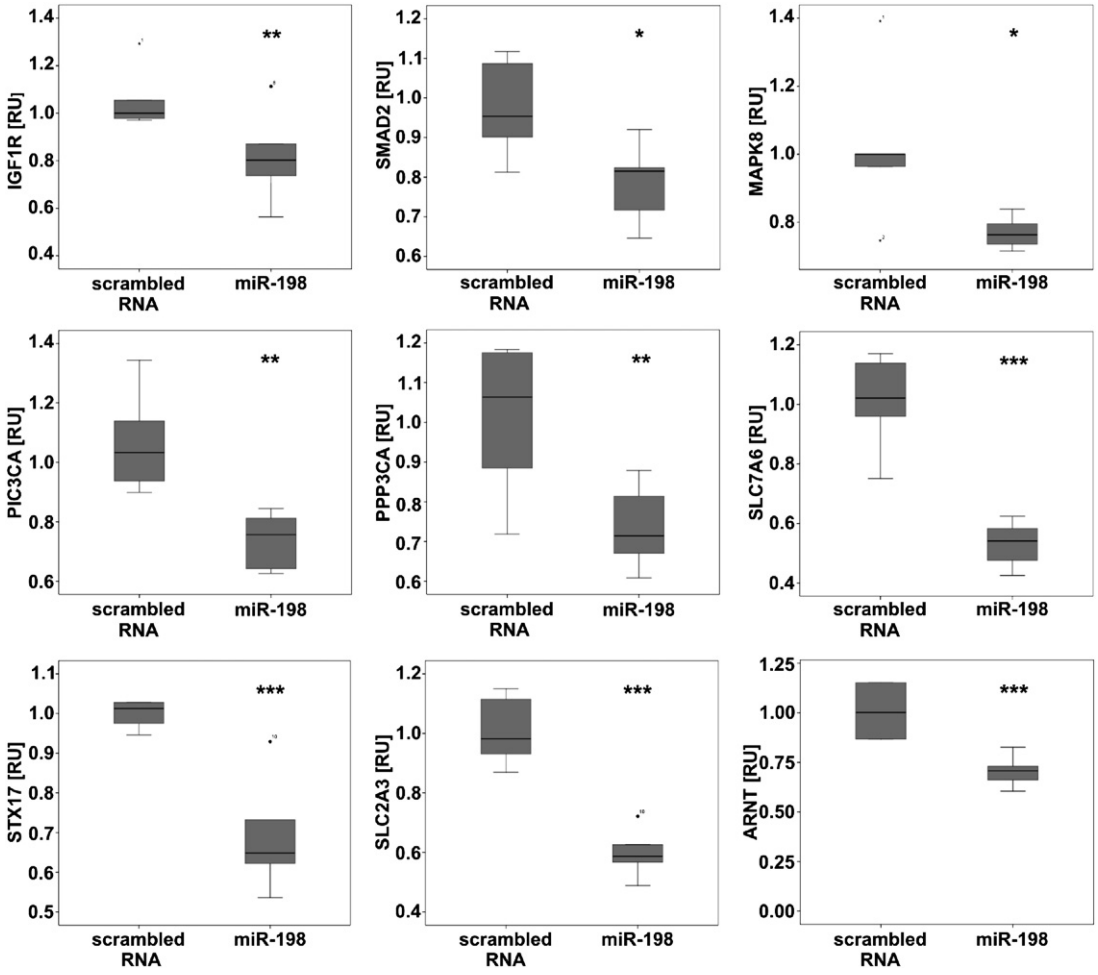


**Fig. 4.** Validation of selected significantly dysregulated genes after miR-198 induction. Expression profiles of genes, identified by microarray analysis as significantly dysregulated after miR-198 overexpression in Pop10 cells, were validated by real-time PCR. For validation further six independent experiments of miR-198 overexpression were performed and total RNA, isolated from scrambled and miR-198 treated Pop-10 cells, was analyzed. Downregulation of nine genes after miR-198 treatment was proven. The transcript levels were normalized using HPRT as a reference and indicated in Relative Units [RU].

associated to proliferation and migration pathways were repressed (Fig. 3C). Importantly, the differential pathway analysis by BioCarta, KEGG, and GenMapp showed a highly interactive functional link of significantly dysregulated genes after miR-198 overexpression (Fig. 3C).

### *3.4. Dysregulation of miR-198 mediates proliferative and morphogenic pathways*

The function of differentially expressed genes was further investigated by *in silico* inquest, classifying their role into five functional processes by GO analyses: cell proliferation and apoptosis, cell migration, metabolism, and gene regulation (Table 1). Thus, some key signal transducers of cellular activation and proliferation including phosphoinositol 3-kinase (PI3KCA), the receptor of the highly potent mitogens IGF-1 and IGF-2, and the antiproliferative growth factors TGF-β as well as its receptor TGF-β receptor type 1 and 2, confirm the important role of miR-198 in HCC growth and migration (Table 1). The significant dysregulation of these identified genes after miR-198 treatment was verified by quantitative analysis of transcriptional expression levels, shown in Fig. 4.

### *3.5. Synthesis of E-cadherin and claudin-1 in hepatocellular carcinoma depends on miR-198 expression*

Since after miR-198 overexpression the tight-junction protein claudin-1 (CLDN1) encoding gene was one of the most prominently upregulated genes identified by microarray analyses (Fig. 3C, Table 1), we studied expression of claudin-1 and also of E-cadherin more thoroughly. In addition to claudin-1, the cell adherence protein E-cadherin was selected, because its loss, which is observed during hepatocarcinogenesis, is suggested to be an important prerequisite of cancer progression [32–34].

Compensation of reduced miR-198 levels in hepatoma Pop10 cells by miR-198 treatment resulted into a more than 4-fold increase of claudin-1 expression. Furthermore, E-cadherin was also highly upregulated in response to miR-198 treatment (Fig. 5B). The marked upregulation of both claudin-1 and E-cadherin synthesis could also be shown at the protein level (Fig. 5C).

Next, we studied if the influence of E-cadherin and claudin-1 could also be observed on a collection of liver samples with different grades of HCC. Both expression levels were reduced in HCC with low miR-198 levels (Supplemental Fig. S1). In agreement with our in vitro-studies, real-time quantification of miR-198 and claudin-1 on RNA extracts from HCV positive HCC biopsies [20] revealed a highly significant correlation of miR-198 with claudin-1 expression levels ($r^2_{Spearman} = 0.691$, $p = 0.0009$) (Supplemental Fig. S1).

The loss of claudin-1 and E-cadherin during HCC development is suggested to be involved in enhanced invasive activity of hepatocellular carcinoma cells [32,34,35]. Therefore, we addressed the question, whether miR-198 induced expression of these proteins leads to impaired cell migration. For this purpose, we measured migration activity after scratching monolayers of hepatoma cells, overexpressing

**Table 1**
Signaling pathways affected by miR-198 overexpression.

| Signaling pathway | Genes[a] |
|---|---|
| Immune response | OAS1/2/3; IFI6/6L/T1/T1M/27; IRF9; ISG15; Mx1/2; EPSTI1; |
| Cell proliferation | MET; PIK3CA; IGF1R; STX17; MAP2K4; MAPK8; SMAD2; CCND3; PPP2R1B; E2F1; RB1; ARHGAP19; |
| Apoptosis | CASP9; PIK3CA/CB/R3; IGF1R; TGFBR2; BMPR3; FAS; PPP3CA/R1; |
| Cell metabolism | HMGCL; SLC7A6; SLC2A3; EDEM1; TOMM20; PTPMT1; |
| Cell migration | PIK3CA; TGFBR1/2; BMPR3; ACTN3; CLDN1; ITGB2; CCND3; ZEB2; RB1; ARHGAP19 |
| Gene regulation | RB1; TBP; SP100; FAS; PML; TAF1/9B; ARNT; DIAPH1; CARM1; GTF2H1/3; |

[a] Genes, significantly dysregulated ($p \leq 0.05$) after miR-198 overexpression in hepatoma cells, were classified into cell signaling pathways by Biocarta, KEGG, and GenMAPP.

miR-198. The slot distances, that were determined between the edges of the scratched wounds, demonstrated that migration activity was efficiently inhibited by approximately 75% in hepatoma cells treated with miR-198 (Fig. 5D, E). The miR-198 influence on cell migration could also be proven by the Boyden chamber system (Supplemental Fig. S2). Treatment of Pop10 hepatoma cells with siRNA against E-cadherin and claudin-1, resulted in a strong activation of cell motility. The enhanced migration, induced by E-cadherin and claudin-1 RNA silencing, was partly abolished by miR-198 overexpression (Supplemental Fig. S2).

## 4. Discussion

In the present study, novel evidence is shown that in the liver miR-198 may act as a potent tumor suppressor miRNA by inhibition of cell growth and migration. miR-198 is markedly decreased in non-alcoholic steatohepatitis and especially during HCV related hepatocarcinogenesis [20,26]. In agreement with our previous findings, which demonstrated the loss of miR-198 during progression of hepatocarcinogenesis [20], miR-198 repression is more pronounced in hepatocellular carcinoma cell types with an advanced dedifferentiation grade, indicated by low levels of liver-specific transcription factors. Although miR-198 seems to be closely associated with the expression of the liver-specific transcription factor HNF1α, it does not affect the expression of HNF1α and HNF4α (Fig. 1).

Compensation of miR-198 expression, which is nearly completely abolished in Pop10 cells (Fig. 1), leads to a highly efficient retardation of cell growth. However, concordantly to previous data of Tan et al., who describe no effect of miR-198 on HepG2 and Hep3B cell proliferation [28], inhibition of cell growth by miR-198 was only moderate when using Huh-7 cells. Since Huh-7 cells are characterized by lower division rates [29] and higher endogenous miR-198 levels than Pop10 cells (Fig. 1), the influence of additional miR-198, transfected into cells, is not as distinctive as in a cell type with low miR-198 levels. These findings support the data of Tan et al., who described only a moderate miR-198 effect on proliferation of HGF stimulated HepG2 and Hep3B expressing miR-198 [28]. Thus, cell division and viability may directly depend on the endogenous miR-198 concentration and

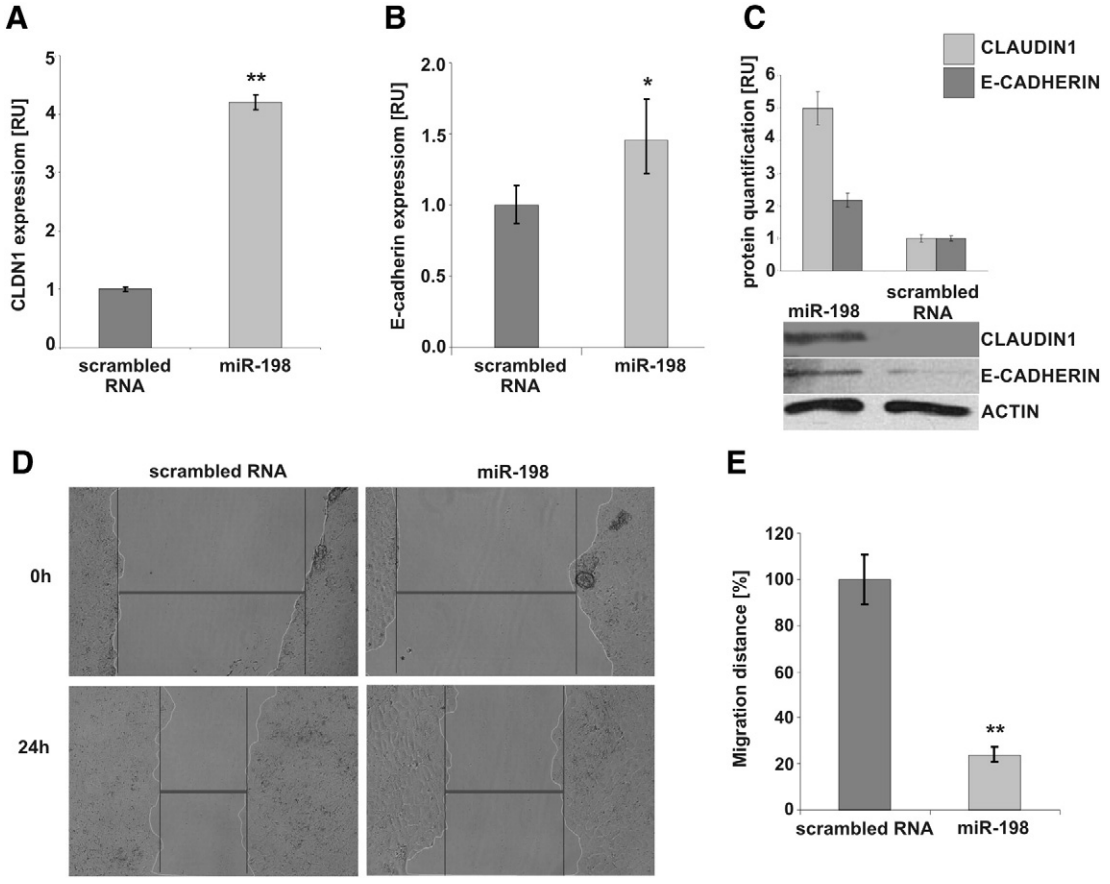


**Fig. 5.** Claudin-1 and E-cadherin synthesis were highly affected by miR-198 overexpression. Real-time PCR analyses of miR-198 transfected hepatoma cells indicated a high effect of miR-198 on claudin-1 (CLDN1) (A) and E-cadherin (B) expression 24 h after treatment. Upregulation of claudin-1 and E-cadherin by miR-198 overexpression was proven at the protein level by Western blotting (C). Migration analyses of hepatoma cells that were scratched 24 h posttransfection (D) presented a reduction of cell migration by miR-198. The distance between the scratching borders [%] indicated the migration within 24 h (E). Values of hepatoma cells transfected with scrambled RNA were set to 100%. For all assays, four independent experiments were performed.

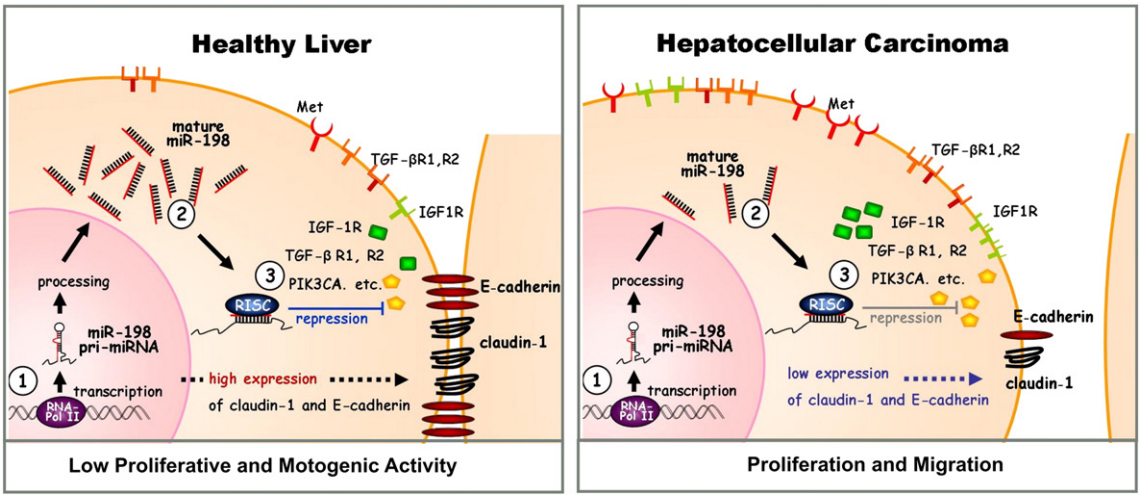


**Fig. 6.** Tumor suppressor activities of miR-198 in healthy liver and hepatocellular carcinoma. In healthy liver, miR-198 is more present than in HCC (1) and inhibits the mitogenic IGF-1R and Met induced signaling pathways by repression of receptor and PIK3CA signal transducer synthesis (2). Abundant miR-198 expression leads to high claudin-1 and E-cadherin expression (3). After hepatocarcinogenesis miR-198 is suppressed (1), leading to high expression of mitogenic signal transducers and of the TGF-β receptors R1 and R2 (2), but to low expression of E-cadherin and claudin-1 (3). Altered expression profiles due to low miR-198 expression in hepatocellular carcinoma cells contribute to increased proliferation and migration.

miR-198 joins the group of antiproliferative miRNAs, recently described to impede cell growth of hepatoma cells such as miR-29c, miR-145, miR-125b, and miR-199b [25,36,37].

miR-198 regulates hepatoma cell migration in addition to the inhibition of cell growth (Figs. 2 and 5D, E), confirming recent data of Tan et al., who reported the blockade of HGF-induced motogenic effects on hepatoma cells by miR-198 [28]. In our study we demonstrate the impact of miR-198 on hepatocellular migration activity by miR-198 regulation of E-cadherin and claudin-1 (Fig. 5A, B, C). Since miR-198 positively affects the synthesis of both proteins, the miR-198 influence might be either indirect or due to posttranscriptional positive regulation by stabilization of the E-cadherin and claudin-1 mRNA by putative miRNA interaction to the 5′-untranslated region [38].

The decrease of the cell adhesion protein E-cadherin in cancer cells is mediated by β-catenin after TGF-β exposure. Loss of E-cadherin from the epithelial cell membrane then results in less cell adherence and promotion of migration [32–34,39]. Therefore, the disengaged E-cadherin/miR-198 axis during hepatocarcinogenesis is suggested to contribute to enhanced hepatoma cell migration that in turn leads to increased metastatic activities of HCC.

In addition to E-cadherin, the expression of the tight-junction associated protein claudin-1 is highly controlled by miR-198 (Fig. 5). The family of claudins consists of 25 identified members, all involved in maintenance of cellular polarity, cell growth, and cell differentiation, but with different roles in cell migration [40,41]. Claudin-7 decreases cell migration and invasion in lung cancer [42], but claudin-2 is found to trigger lung cancer cell migration by induced matrix-metalloproteinase (MMP)-9 release [43]. Furthermore, claudin-6, 7, and 9 enhance gastric adenocarcinoma cell migration and invasion [44]. Yoon et al. have demonstrated that claudin-1 contributes to migration [45], but these findings were not confirmed by Higashi et al. [35]. Claudins are often dysregulated in cancer [41] and claudin-1 was shown to be significantly reduced in HCC. Furthermore, the loss of claudin-1 is associated with a poor prognosis of HCC [35]. Therefore, the newly identified regulation of claudin-1 by miR-198 in this study is of particular importance, although its function is controversially discussed.

In addition to claudin-1, we found miR-198 mediated dysregulation of a wide range of signal transducing proteins that take part in cell motility and cell growth reactions by gene expression profiling. Genes involved in cell growth and migration were significantly affected by miR-198 (Table 1; Fig. 3). Thus, we recognized by bioinformatic analyses, highly potent mitogenic mediators such as IGF-1 receptor and the catalytic subunit of phosphoinositol-3-kinase (PIK3CA), but also both receptors of TGF-β. Interestingly, by increasing the miR-200 expression and downregulating the Zeb-1 and Zeb-2 proteins (data not shown), miR-198 seems also to be involved in the TGF-β/ZEB/miR-200 signaling network that is known to regulate tumor cell invasion by E-cadherin expression [46]. Whereas stimulated IGF-2 signaling is mainly described as a malignant event occurring in HCC [4], the activation of the PIK3CA pathways is a widespread occurrence observed in many cancer types [47,48]. The remarkable inhibition of these proliferative and motogenic pathways, shown in this study (summarized in Fig. 6), emphasizes the extensive function of miR-198 as tumor suppressor, that might be not only taking part in HCC, but also in other cancer types.

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