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Loss of miR-122 expression in liver cancer correlates with suppression of the hepatic phenotype and gain of metastatic properties

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Abstract

Growing evidence indicates that microRNAs play a significant role in tumor development and may constitute robust biomarkers for cancer diagnosis and prognosis. In the present study, we evaluated the clinical and functional relevance of miR-122 expression in human hepatocellular carcinoma. We report that miR-122 is specifically repressed in a subset of primary tumors which are characterized by poor prognosis. We further demonstrate that the loss of miR-122 expression in tumor cells segregates with specific gene expression profiles linked to cancer progression, namely the suppression of hepatic phenotype and the acquisition of invasive properties. We identify liver-enriched transcription factors as central regulatory molecules in the gene networks associated with loss of miR-122, and provide evidence suggesting that miR-122 is under the transcriptional control of HNF1A, HNF3A, and HNF3B. We further demonstrate that loss of miR-122 results in an increase of cell migration and invasion and that restoration of miR-122 reverses this phenotype. In conclusion, miR-122 is a marker of hepatocyte-specific differentiation and an important determinant in the control of cell migration and invasion. From a clinical point of view, our study emphasizes miR-122 as a diagnostic and prognostic marker for HCC progression.

Keywords

hepatocellular carcinoma; microRNA; differentiation; metastasis; microarray

Introduction

HCC is the third most common cause of death from cancer world-wide, accounting for at least 600,000 deaths annually (Parkin *et al.*, 2005). The most prominent etiologies of HCC include chronic viral hepatitis B and C (HBV, HCV), alcoholic or nonalcoholic steatohepatitis, and aflatoxin intoxication (Thorgeirsson and Grisham, 2002; El-Serag, 2004). Although recent advances in functional genomics provide increasingly comprehensive portrayal of hepatocarcinogenesis (Thorgeirsson *et al.*, 2006; Villanueva *et al.*, 2007), the molecular pathogenesis of HCC remains poorly understood. Indeed, the

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clinical heterogeneity of HCC, the lack of good diagnostic markers and treatment strategies has rendered the disease a major challenge.

MicroRNAs (miRNAs) belong to an abundant family of endogenous, short, and non-coding RNAs which are believed to be key post-transcriptional regulators of gene activity. It has been widely demonstrated that miRNAs play important roles in the control of numerous biological processes, such as development, differentiation, proliferation, and apoptosis (Ambros, 2004). Therefore, it is not surprising that miRNAs have been implicated in cancer as well. Altered miRNA expression was observed in a large variety of neoplasms (Lu *et al.*, 2005), including HCC (Murakami *et al.*, 2006; Braconi and Patel, 2008; Budhu *et al.*, 2008; Ladeiro *et al.*, 2008; Li *et al.*, 2008; Wong *et al.*, 2008). Significance of miRNAs in cancer onset and progression has been supported by the experimental demonstrations of their oncogenic and tumor-suppressive properties (Esquela-Kerscher and Slack, 2006). In addition, miRNA genes have been found to be frequently located in cancer-associated genomic regions, such as fragile sites, minimal regions of loss of heterozygosity and minimal regions of amplification (Calin *et al.*, 2004).

MicroRNA-122 (miR-122) is the most abundant miRNA in the liver accounting for approximately 70% of the total miRNA population (Lagos-Quintana *et al.*, 2002; Chang *et al.*, 2004). Although miR-122 has been described as a liver specific miRNA, slight expression has been also reported in heart (Tang *et al.*, 2007). Several studies have emphasized the importance of miR-122 in liver homeostasis. In vivo, miR-122 has notably been implicated in fatty acid and cholesterol metabolism and HCV infection (Jopling *et al.*, 2005; Krutzfeldt *et al.*, 2005; Esau *et al.*, 2006; Chang *et al.*, 2008; Cheung *et al.*, 2008; Elmen *et al.*, 2008; Henke *et al.*, 2008). Expression of miR-122 has been found to be repressed in HCC (Kutay *et al.*, 2006; Murakami *et al.*, 2006; Gramantieri *et al.*, 2007; Braconi and Patel, 2008; Budhu *et al.*, 2008; Girard *et al.*, 2008; Ladeiro *et al.*, 2008; Lin *et al.*, 2008) as well as in nonalcoholic steatohepatitis (Cheung *et al.*, 2008). Recent work suggested that miR-122 deregulation is associated either with specific clinical risk factors (HBV, HCV) or HCC metastasis (Braconi and Patel, 2008; Budhu *et al.*, 2008; Girard *et al.*, 2008; Ladeiro *et al.*, 2008; Varnholt *et al.*, 2008).

Given the important role of miR-122 in liver pathology, we aimed i) to evaluate the diagnostic and prognostic significance of miR-122 expression in human HCC, and ii) to determine the functional implication of miR-122 deregulation in liver cancer development. By investigating the expression of miR-122 in 64 human HCC and 28 matched non-neoplastic surrounding liver tissues, we show that miR-122 repression in HCC correlates with clinically relevant parameters, such as etiology, survival, tumor size and differentiation status. Furthermore, we demonstrate that the loss of miR-122 expression correlates with distinct gene expression profiles characteristic of tumor progression (i.e. suppression of hepatic differentiation phenotype and gain of metastatic properties). Finally, we identify liver-enriched transcription factors as central functional regulators in the gene networks associated with the loss of miR-122 in human liver cancer and show that miR-122 is an important factor in the control of cellular migration and invasion.

Results

Heterogeneous expression of miR-122 in human HCC-derived cell lines

Natural properties of miRNAs, including their small size and the lack of polyadenylated tail, have made their quantification challenging. Here, the expression of miR-122 was first determined by real-time Q-RT-PCR in 21 HCC-derived cell lines (Lee and Thorgeirsson, 2002) using specific sets of primers (Figure 1 and SI Figure 1). Interestingly, the levels of miR-122 were extremely variable with more than 1000-fold expression differences among

the HCC cell lines. Some cell lines including PLC/PRF/5, Huh-1, and Hep40 exhibited very high levels of miR-122, whereas others, such as Hep3B and HepG2, expressed little or did not express miR-122 at all (e.g. SNU387, and SNU398). Specificity and reproducibility of PCR assays were confirmed by modulating the expression of miR-122 in vitro (SI Figure 2). We conclude that Q-RT-PCR is a reliable assay for quantification of the miR-122 expression and that HCC cell lines represent a suitable model for functional studies of miR-122 in liver cancer.

Loss of miR-122 expression in HCC is associated with poor prognosis

Next, the expression of miR-122 was analyzed in a panel of 64 primary liver tumors and 28 matched non-tumor surrounding liver tissues extensively characterized in our lab previously (Lee *et al.*, 2004a; Lee *et al.*, 2004b; Kaposi-Novak *et al.*, 2006; Lee *et al.*, 2006; Coulouarn *et al.*, 2008). HCC samples were representative of patients with various clinical outcomes and pathological features (Table 1). Interestingly, miR-122 levels were highly variable among the clinical samples (CV>2), suggesting that miR-122 expression in primary tumors was as heterogeneous as found in cell lines.

To further explore clinical relevance, the expression of miR-122 was analyzed as a function of various clinical parameters and tumor features (Figure 2). First, the expression levels of miR-122 were compared between HCC subtypes previously characterized by the experimentally well-defined gene expression signatures (Lee *et al.*, 2004a; Lee *et al.*, 2004b; Kaposi-Novak *et al.*, 2006; Lee *et al.*, 2006; Coulouarn *et al.*, 2008). As shown in Figure 2a, miR-122 was specifically repressed in the tumors which exhibited a bad prognosis signature. Accordingly, the overall survival time of the patients with low and high miR-122 expression in HCC was 30.3±8.0 and 83.7±10.3 months, respectively ($P<0.001$). MiR-122 was also repressed in a subset of HCC which harbored a hepatoblast signature, as well as a c-Met and a late TGF-β signature (Figure 2a). The hepatoblast signature, derived from hepatic progenitor cells, is specific for tumors with a poor differentiation status (Lee *et al.*, 2006) whereas c-Met and late TGF-β signatures are associated with subsets of tumors with poor prognosis and aggressive phenotype (Kaposi-Novak *et al.*, 2006; Coulouarn *et al.*, 2008). The loss of miR-122 expression was correlated with high proliferation and ubiquitination index along with low apoptotic index (Figure 2b). Supporting a recent observation (Varnholt *et al.*, 2008), the expression of miR-122 was clearly etiology-dependent since miR-122 was repressed only in HCC arising in HBV-infected livers (Figure 2c). No significant correlations ($P<0.05$) were observed between the status of HBV infection and the presence of hepatoblast, TGF-β or c-Met signatures. Finally, miR-122 repression was found to be associated with poor differentiation status and large tumor size (Figure 2c). Taken together, these data demonstrate that miR-122 expression in human HCC correlates with clinically relevant parameters and that loss of miR-122 is a marker of poor prognosis.

miR-122 levels define specific gene expression profiles in HCC

To get insights into the functional significance of miR-122 expression, tumors were divided into two groups. High and low miR-122 expressing tumors (15 and 17 cases, respectively) were defined by >2 fold changes in the median miR-122 expression across the samples. Gene expression profiling identified 509 non-redundant genes which were differentially expressed between the tumors with high vs. low miR-122 expression (SI Table 1). As expected, clustering analysis divided HCC into two distinct clusters (C1 and C2) defined by the level of miR-122 (Figure 3a). Differentially expressed genes were similarly organized in two clusters and included genes which displayed either positive (Cluster A) or negative (Cluster B) correlation with miR-122 expression (Figure 3a). Supporting our approach, cluster B included numerous predicted targets for miR-122 (e.g. ALDOA, CLIC1, and RHOA). The specific contribution of miR-122 to the HCC transcriptome was further

validated by testing the enrichment of the genes embedded in clusters A and B in the gene expression profiles derived from the experimental modulation of miR-122 levels (Krutzfeldt *et al.*, 2005). As shown in Figure 3b, genes highly expressed in the Clusters A and B (high and low miR-122 expressing HCC) were significantly enriched in the gene expression profiles corresponding to either control or mice treated with antagomir-122, respectively (Krutzfeldt *et al.*, 2005). In addition, the restoration of miR-122 expression in vitro to a large extend was able to shift the expression patterns of Cluster A and B genes from C2 (Low miR-122) toward C1 (High miR-122) (SI Figure 3). These results demonstrate that the expression of genes embedded in the clusters A and B is highly dependent on miR-122 expression.

Loss of miR-122 in HCC coincides with suppression of the hepatic phenotype

As shown in Figure 3a, the gene expression profiles were remarkably homogeneous within each subgroup of HCC, suggesting that miR-122 deregulation represents an important event during the course of HCC progression. To reveal the dominant signaling networks associated with the loss of miR-122 expression in HCC, systematic pathway and gene ontology analyses were performed using genes which either positively (Cluster A) or negatively (Cluster B) correlated with miR-122 expression. Ingenuity Pathway Analysis revealed a clear relationship between the miR-122 associated gene expression signature and the functional categories linked to liver cancer and liver metabolism (Table 2). Genes embedded in Cluster A were implicated in molecular and cellular functions characteristic of well differentiated hepatocytes (Table 2 and SI Table 1). These genes were involved in lipid metabolism (APO-A1, -C4, -F), acute phase response (ITIH3, SERPIN-C1, -F2), detoxification (CYP-2C9, -3A4, 4A11), and coagulation (F12, F13B). Moreover, high expression of miR-122 significantly correlated with the global activation of an extensive regulatory network centered on HNF4A (Figure 4a). Enrichment of HNF4A targets among miR-122-positive HCC suggests the involvement miR-122 in maintaining hepatocyte differentiation thereby implying that the loss of miR-122 contributes to suppression of the hepatic phenotype in HCC with poor prognosis (Figure 2).

Relationship between miR-122 and liver-enriched transcription factors

As a paradigm, HCC-derived cell lines were divided into two groups based on the miR-122 expression levels. Gene expression profiling identified 300 genes which were differentially expressed as a function of miR-122 expression (SI Figure 4). Consistent with our findings in primary tumors, high levels of miR-122 in HCC-derived cell lines were correlated with high expression of genes involved in the pathways characteristic of fully differentiated hepatocytes (SI Table 2). These included genes encoding apolipoproteins (APO-A1), coagulation factors (F2), and acute phase proteins (AHSG). More importantly, miR-122 exhibited a strong positive correlation with the expression level of numerous liver-enriched transcription factors essential for hepatocytic differentiation, such as HNF1A, HNF3A, HNF3B, HNF4A, HNF4G, and HNF6 (SI Figure 5). Gene network analysis not only confirmed activation of the HNF4A signature in HCC-derived cell lines expressing miR-122 but also revealed strong interactions with other liver-enriched transcription factors (Figure 4b).

In this context, HNF4A was directly evaluated as a function of miR-122 expression. As shown in Figure 4c–e, HNF4A expression was directly correlated with the expression of miR-122, both on mRNA and protein levels. Furthermore, in the high miR-122 expressing cell lines, HNF4A was particularly enriched in the nuclear fraction as compared to the cytoplasmic fraction reflecting its greater transcriptional activity (Figure 4e). The latter is consistent with a central role of HNF4 revealed by the network analysis in the miR-122 overexpressing HCC cells lines (Figure 4b). Despite a strong positive correlation, the exact

relationship between miR-122 and HNF4A appears to be highly complex. The lack of miR-122 seed region in the 3'UTR of HNF4A transcript makes HNF4A an unlikely direct target of miR-122. Accordingly, the experimental modulation of miR-122 expression did not affect HNF4A mRNA and protein levels (data not show). It is also dubious that HNF4A directly drives the expression of miR-122 since the silencing of HNF4A by siRNA did not significantly change the expression of miR-122 (Figure 5). Conversely, the knock-down of HNF1A, HNF3A or HNF3B was able to reduce miR-122 expression implying that mir-122 may be under transcriptional control of these transcription factors (Figure 5). These results provide new insight into the transcriptional regulation of miR-122 gene and implicate miR-122 as a marker of hepatocyte differentiation.

Loss of miR-122 coincides with acquisition of invasive phenotype

Lastly, we analyzed the genes which were negatively correlated with miR-122 (Cluster B in Figure 3a). Expression profiling indicated that loss of miR-122 expression was associated with a higher expression of numerous genes related to metastasis, including genes involved in cell motility (RAC1, RHOA), angiogenesis (VEGF), hypoxia (HIF1A) and epithelial-mesenchymal transition (vimentin) (SI Table 1, SI Figure 6). Notably, RHOA and RAC1 were two central molecules in a network associated with cell motility (Figure 6a). Therefore, we investigated the impact of miR-122 loss on cell motility. As shown in Figure 6b, the experimental inhibition of miR-122 in Huh-1 cells resulted in an increase of migration and invasion. Further validating the importance of miR-122 in the control of cell motility, the ectopic expression of miR-122 was able to reduce the migration and invasion of SK-Hep1 cells (Figure 6c). These data emphasize the critical role of the loss of miR-122 expression during tumor progression and the potential relevance of miR-122 restoration for HCC therapy.

Discussion

Accumulating evidence suggests a causal role for miRNAs in human cancer (Esquela-Kerscher and Slack, 2006). In this study, we have evaluated the clinical and functional relevance of liver specific miR-122 for human HCC. We show that the repression of miR-122 correlates with clinically relevant parameters, such as etiology, tumor size and differentiation grade. More importantly, miR-122 repression is specific for liver tumors which are characterized by poor prognosis and coincides with suppression of hepatic phenotype as well as gain of metastatic properties.

Our findings demonstrate a link between the expression levels of miR-122 and HBV or HCV infection. By analyzing the expression of miR-122 in a cohort of HCC with different etiology, we have established that down-regulation of miR-122 occurs mainly in the HCC arising in the HBV-infected livers. In the HCC developed with HCV infection, miR-122 expression was either maintained or increased, in agreement with recent observations (Varnholt *et al.*, 2008). Interestingly, the efficiency of HCV replication has been found to be closely correlated with the miR-122 expression levels (Jopling *et al.*, 2005; Chang *et al.*, 2008), and further studies have implicated miR-122 in HCV translation, suggesting involvement of miR-122 in HCV tropism to the liver (Henke *et al.*, 2008). Therefore, we speculate that HCV not only requires the host-expressed miR-122 for its replication, but it may also either directly induce miR-122 expression or indirectly inhibit miR-122 down-regulation.

Similar to the transcription factors, tissue specificity of miRNA expression may imply a role in differentiation. Due to the high abundance in the liver, it has been suggested that miR-122 may maintain the hepatic differentiation status by repressing the genes which are normally not expressed by hepatocytes (Girard *et al.*, 2008). The temporal expression pattern of

miR-122 during liver development is consistent with its involvement in liver differentiation (Chang *et al.*, 2004). In support of this notion, recent work showed that promotion of endodermal differentiation of human embryonic stem cells (hESC) by NaButyrate was associated with an increase of miR-122 expression and a parallel activation of several hepatocyte-specific genes (Tzur *et al.*, 2008). According to our findings in human HCC and HCC-derived cell lines, the expression of miR-122 was tightly correlated with the expression of numerous genes characteristic of the differentiated hepatocytes. In agreement with the current view of miR-122 as a regulator of lipid and cholesterol metabolism, the expression of abundant apolipoprotein genes was found to be correlated with miR-122 levels (Krutzfeldt *et al.*, 2005; Esau *et al.*, 2006; Elmen *et al.*, 2008). The specific down-regulation of miR-122 in poorly differentiated tumors as well as in tumors characterized by the hepatoblast gene signature supported miR-122 as an important determinant of hepatic differentiation. Moreover, in two independent datasets, we identified HNF4A as the central regulatory transcription factor in the network of genes which were differentially expressed as a function of miR-122 expression. HNF4A is a liver-enriched transcription factor controlling the differentiation-dependent gene expression in developing and adult livers (Odom *et al.*, 2004). Although we initially speculated that HNF4A was the missing link between the differentiation status and the transcription of miR-122 gene, the additional gene silencing experiments suggested a more important role for other liver-enriched transcription factors, such as HNF1A, HNF3A, and HNF3B. The promoter region of miR-122 gene has not been yet identified but a binding site for HNF3B has been located 5kb upstream of miR-122 (SI Figure 7). Although further experiments are needed to address the functionality of this binding site, our data provide the first evidence for the tissue specificity of miR-122 expression.

Using a comparative genomic approach based on the experimentally well defined gene expression signatures, we have previously identified several clinically relevant subtypes of HCC with significant differences in the biological properties and clinical outcome (Lee *et al.*, 2004a; Lee *et al.*, 2004b; Kaposi-Novak *et al.*, 2006; Lee *et al.*, 2006; Coulouarn *et al.*, 2008). Importantly, we showed here that the repression of miR-122 was characteristic of HCC which displayed a hepatoblast, c-Met or late TGF-β signature. These HCC subtypes were characterized by increased invasiveness, tumor recurrence and decreased patient survival whereby raising a possibility than the loss of miR-122 may predispose to the acquisition of invasive phenotype. Consistent with this, miR-122 was recently described as a metastasis-related miRNA in HCC (Budhu *et al.*, 2008), and repression of miR-122 was reported in highly metastatic human breast cancer cell lines (Tavazoie *et al.*, 2008). Supporting these data, our results demonstrate that HCC cell lines known to exhibit a more invasive phenotype also displayed a decreased miR-122 expression (Coulouarn *et al.*, 2008). Additionally, a significant enrichment of genes related to metastasis (RHOA, VEGF, HIF1A, Vimentin) was observed both in HCC and cell lines lacking miR-122 expression (SI Table 1). Accordingly, we provide direct evidence that the modulation of miR-122 greatly influenced the propensity of cancer cells to migrate and invade. Indeed, the loss of miR-122 resulted in the acquisition of migrating and invasive properties and the restoration of miR-122 was able to reverse this phenotype. In an attempt to identify the underlying molecular mechanisms responsible for the impact of miR-122 on cell motility, we used microRNA target prediction algorithms on genes negatively correlated with miR-122. Interestingly, an evolutionary conserved binding site for miR-122 was detected in the 3'UTR of RHOA mRNA (SI Figure 8a), and the analysis of the secondary structure of RHOA 3'UTR indicated that the predicted binding site coincided with a hairpin-loop structure, making this sequence likely accessible for an interaction with miR-122 (SI Figure 8b). RHOA is frequently overexpressed in HCC and several groups correlated the increase of RHOA expression with tumor progression, cell differentiation, and metastasis (reviewed in Grise *et al.*, 2009). Moreover, RHOA overexpression in liver has been directly correlated

with the acquisition of an aggressive phenotype, both in vitro and in vivo (Yoshioka *et al.*, 1995; Xue *et al.*, 2008). In addition, gene reporter experiments suggested a direct functional interaction between miR-122 and RHOA 3'UTR (SI Figure 8c). Nevertheless, the experimental inhibition or induction of miR-122 failed to demonstrate a consistent modulation of RHOA on a protein level. Also, the acquisition of invasive properties observed upon miR-122 inhibition (Figure 6b) appeared to be independent of RHOA since the silencing of RHOA did not significantly abrogated this response. The absence of RHOA modulation on a protein level could be due to an enhanced stability of the protein. Alternatively, RHOA transcript may be tightly regulated by positive and negative signals. For example, the increase in the oncogenic miR-155, which was recently reported to target and reduce the level of RHOA (Kong *et al.*, 2008), may counteract the miR-122 effect. These observations raise important questions regarding the regulation of transcript stability by microRNAs and suggest that the integration of activating and inhibitory signals driven by different microRNAs may define the level of a particular transcript. Interestingly, while writing this manuscript, Tsai *et al.*, published that miR-122 regulates intrahepatic metastasis by controlling the disintegrin and metalloprotease ADAM17 (Tsai *et al.*, 2009). Together, these data not only emphasize the critical role of miR-122 in migration and invasion but suggest that miR-122 contribution to tissue remodeling is broader than a direct effect on actin cytoskeleton organization through a RHOA-dependant mechanism.

In conclusion, our work sheds new light on the clinical and functional implications of miR-122 expression in liver cancer. We identified miR-122 as a new diagnostic and prognostic microRNA marker for HCC progression. Given that the modulation of miR-122 expression became a reality in animal models (Czech, 2006; Elmen *et al.*, 2008) our study provides a rationale for classification and development of novel therapy for human HCC.

Methods

Human tissue samples

A total of 64 HCC tissues and 28 matched non-tumor surrounding liver tissues were obtained from patients undergoing partial hepatectomy as treatment for HCC. Tissue banking was approved by the Institutional Review Board of US National Cancer Institute. Determination of proliferation, apoptosis and ubiquitination index was described previously (Lee *et al.*, 2004b). Total RNAs were isolated using the CsCl density gradient centrifugation method (Coulouarn *et al.*, 2006).

Cell lines

HCC-derived cell lines were obtained from American Type Culture Collection (ATCC, Rockville, MD) and RINKEN Cell Bank (Japan) and maintained in Dulbecco's modified Eagle medium/F12 medium supplemented with 2mM glutamine and 10% fetal bovine serum, as described (Lee and Thorgeirsson, 2002). Total RNAs were extracted from cells at 80% confluence using RNeasy mini kit (Qiagen, Valencia, CA).

Cell transfection

Transfections were carried out using siPORT™ NeoFX™ according to the manufacturer's instruction (Ambion, Austin, TX). RNA and protein analysis were conducted after 48 hours incubation in normal culture condition. miRIDIAN microRNA Hairpin Inhibitors and Mimics from Dharmacon (Waltham, MA) were used for the specific inhibition and overexpression of miR-122. Amount of small RNA was adjusted to a final concentration of 20 nM. Efficiency and specificity of the transfections were evaluated by confocal microscopy and Q-RT-PCR (SI Figure 9). Silencer select siRNAs along with the Negative Control #1 (Ambion, Austin, TX) were used for the specific knock-down of HNF1A

(s13870), HNF3A (s6689), HNF3B (s6692), HNF4A (s6698), FOXO1 (s5259), and RHOA (s760).

Real time RT-PCR of miR-122

Expression of miR-122 was measured using *mir*Vana Q-RT-PCR miRNA Detection Kit and SYBR® Green I (Ambion, Austin, TX). Total RNA were reverse transcribed at 37°C for 30 min with an ArrayScript Enzyme mix including miR-122 specific RT primers according to the manufacturer's instructions. Quantitative PCR analysis was performed with an IQ-5 real-time PCR detection system (Bio-Rad Laboratories, Hercules, CA) in a 96-well reaction plate. Melting analysis of the PCR products was conducted to validate the amplification of the specific product. Quantitative values were determined using the $2^{-\Delta\Delta Ct}$ method, where the ΔCt value of a given RNA sample was calculated by subtracting the Ct value of miR-122 from the Ct value of 5S small RNA used for normalization.

DNA microarray analysis

Gene expression data used in this study (GEO accession numbers: Human microarray platform, GPL1528; human HCC microarray data, GSE1898) were generated using a genome-wide set of long-mer human oligonucleotides obtained from Illumina and printed at the Advanced Technology Center (National Cancer Institute). Target preparation and hybridization on microarrays were previously described (Lee and Thorgeirsson, 2002). Differentially expressed genes were identified by a univariate two-sample t-test with a random variance model. Permutation P values for significant genes ($P < 0.01$) were computed based on 10.000 random permutations as previously described (Coulouarn *et al.*, 2006). Expression profiles were clustered by the Cluster 3.0 program using an unsupervised hierarchical procedure and visualized with the TreeView program (Coulouarn *et al.*, 2006).

Gene Set Enrichment Analysis (GSEA) and pathway analysis

GSEA was performed using the web-based tool developed by Broad Institute (www.broad.mit.edu/gsea/). Enrichment scores were determined after 1,000 permutations. Ingenuity Pathway Analysis tool was used to examine the functional associations between differentially expressed genes and to generated the highest significant gene networks (www.ingenuity.com). Biologically relevant networks were identified using the scoring system provided by Ingenuity.

miRNA target prediction

Potential miR-122 target among genes negatively correlated with miR-122 expression were determined using the publicly available miRanda (www.microrna.org) and TargetScan (www.targetscan.org) algorithms. Secondary structure of potential target transcripts was explored using the web-based RNA analyzer tool (www.wb2x01.biozentrum.uni-wuerzburg.de).

RHOA 3' UTR reporter analysis

The human sequence of the RHOA 3' UTR was amplified by PCR using Phusion high-fidelity DNA polymerase (New England Biolabs, Ipswich, MA) and cloned into a SacI- and HindIII-cut pMIR-Report luciferase vector (Ambion, Austin, TX). A mutant construct was generated by introducing mutations in the putative miR-122 binding site. Mutations were introduced by stand overlap PCR using mutagenic primers. All constructs were sequenced to confirm that the desired mutations had been obtained. Firefly luciferase constructs containing the wild-type or the mutant variant of RHOA 3' UTR were co-transfected with a β-galactosidase control vector in the presence of 20 nM miR-122 inhibitor or negative control. Transfections were performed with 0.1 μg firefly luciferase constructs and 0.01 μg

β-galactosidase normalization vector using Lipofectamine 2000. Luciferase and β-galactosidase activities were assessed 48 hours after transfection using a dual-light chemiluminescent reporter gene assay system (Applied biosystems, Foster City, CA).

Invasion and migration assays

The effect of miR-122 on invasion and migration was evaluated by using the BD BioCoat Matrigel Invasion Chambers as described (Coulouarn *et al.*, 2008).

Immunoblotting

Protein expression analysis was as previously described (Coulouarn *et al.*, 2006). Protein extraction was performed using M-PER and NE-PER reagents (Pierce, Rockford, IL). Twenty micrograms of protein extract were subjected to SDS/PAGE and transferred to PVDF membranes. Membranes were probed with a goat polyclonal HNF4A antibody and a rabbit polyclonal RHOA antibody (Santa Cruz Biotechnology, Santa Cruz, CA). Detection was performed by ECL (Amersham Biosciences, Piscataway, NJ) after incubation of a HRP-conjugated secondary antibody (Santa Cruz Biotechnology, Santa Cruz, CA).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Abbreviations

HCC	hepatocellular carcinoma
miRNA	microRNA

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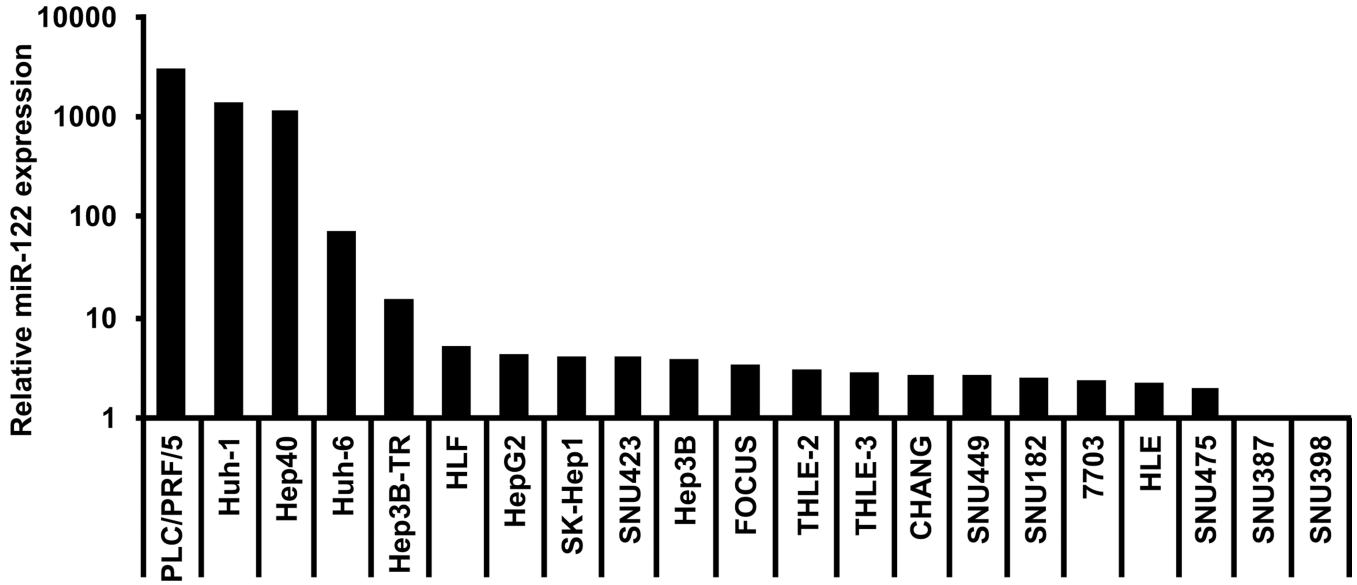

Figure 1.

Relative expression of miR-122 in human HCC-derived cell lines. Expression of miR-122 was evaluated in 21 human HCC-derived cell lines by Q-RT-PCR and normalized to the level of 5s RNA. Data are represented as fold induction relative to the lowest miR-122 expressing SNU-398 cell line using the $2^{-\Delta\Delta Ct}$ method. The great heterogeneity of miR-122 expression indicated that HCC-derived cell lines represent excellent natural models for functional studies of miR-122 in liver cancer.

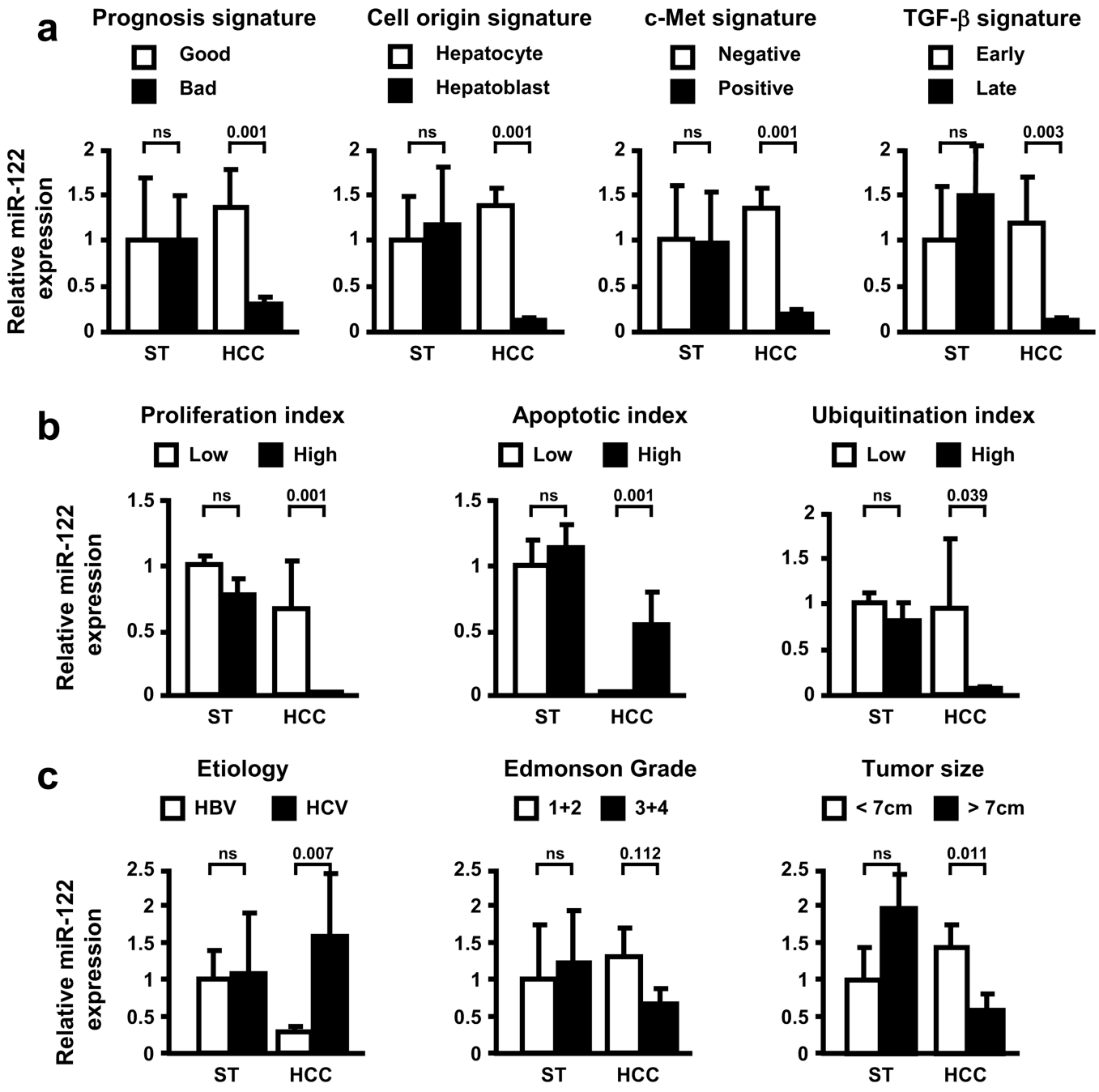

Figure 2.

Repression of miR-122 correlates with clinically relevant parameters in human HCC. Expression of miR-122 was evaluated by Q-RT-PCR in 64 human HCC and 28 non-tumor surrounding liver tissues (ST). (**a**) Expression of miR-122 was evaluated in ST and HCC tissues as a function of experimentally well-defined gene expression signatures (described in Lee *et al.*, 2004a; Kaposi-Novak *et al.*, 2006; Lee *et al.*, 2006; Coulouarn *et al.*, 2008). Data indicated that miR-122 was specifically repressed in HCC with bad prognosis which harbor hepatoblast, c-Met and late TGF-β gene expression signatures. (**b**) The assessment of miR-122 expression as a function of biochemical parameters indicated that miR-122 was particularly repressed in HCC with high proliferation and ubiquitination index, and low

apoptotic index (Lee *et al.*, 2004b). (**c**) The evaluation of miR-122 expression as a function of etiology, Edmonson grade and tumor size indicated that miR-122 was repressed in HCC arising from HBV-infected livers and was associated with poorly differentiated and large size tumors.

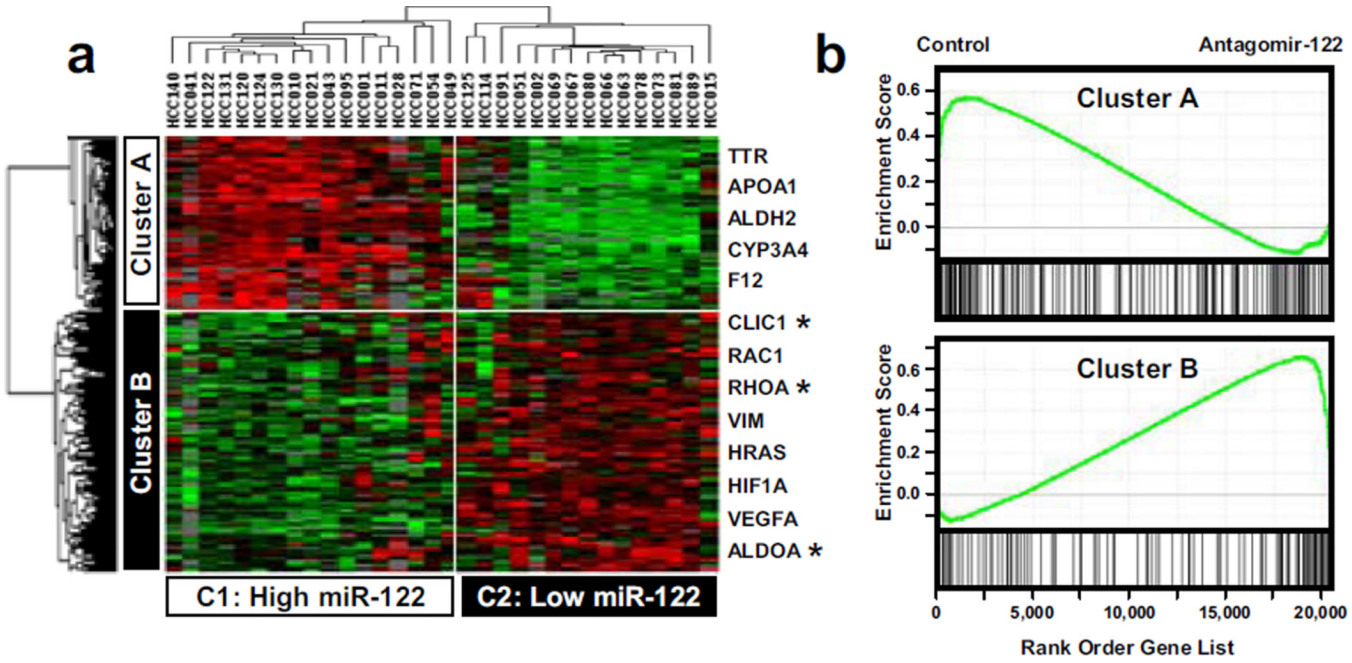

Figure 3.

miR-122 expression defines specific gene expression profiles in human HCC. (**a**) Hierarchical cluster analysis of 509 genes differentially expressed in liver primary tumors as a function of miR-122 expression. Data are presented in a matrix format in which rows and columns represent genes and HCC samples, respectively. Differentially expressed genes are grouped in two clusters, A and B. HCC samples cluster in two groups, C1 and C2, which greatly differ in term of miR-122 level (C1: 2.7±0.4; C2: 0.6±0.4; mean±s.e.m; $P<0.001$). Genes included in cluster A and cluster B are up- and down-regulated in cluster C1 HCC, respectively. As expected cluster B includes several predicted miR-122 target genes (*). (**b**) Gene Set Enrichment Analysis using the genes embedded in the clusters A and B as signatures and the gene expression profiles derived from the experimental inhibition of miR-122 in mice (Krutzfeldt *et al.*, 2005). Cluster A and B genes are significantly enriched in the profiles corresponding to control mice (Enrichment Score ES=0.578, $P<0.001$), and mice treated with antagomir-122 (ES=0.658, $P<0.001$), respectively.

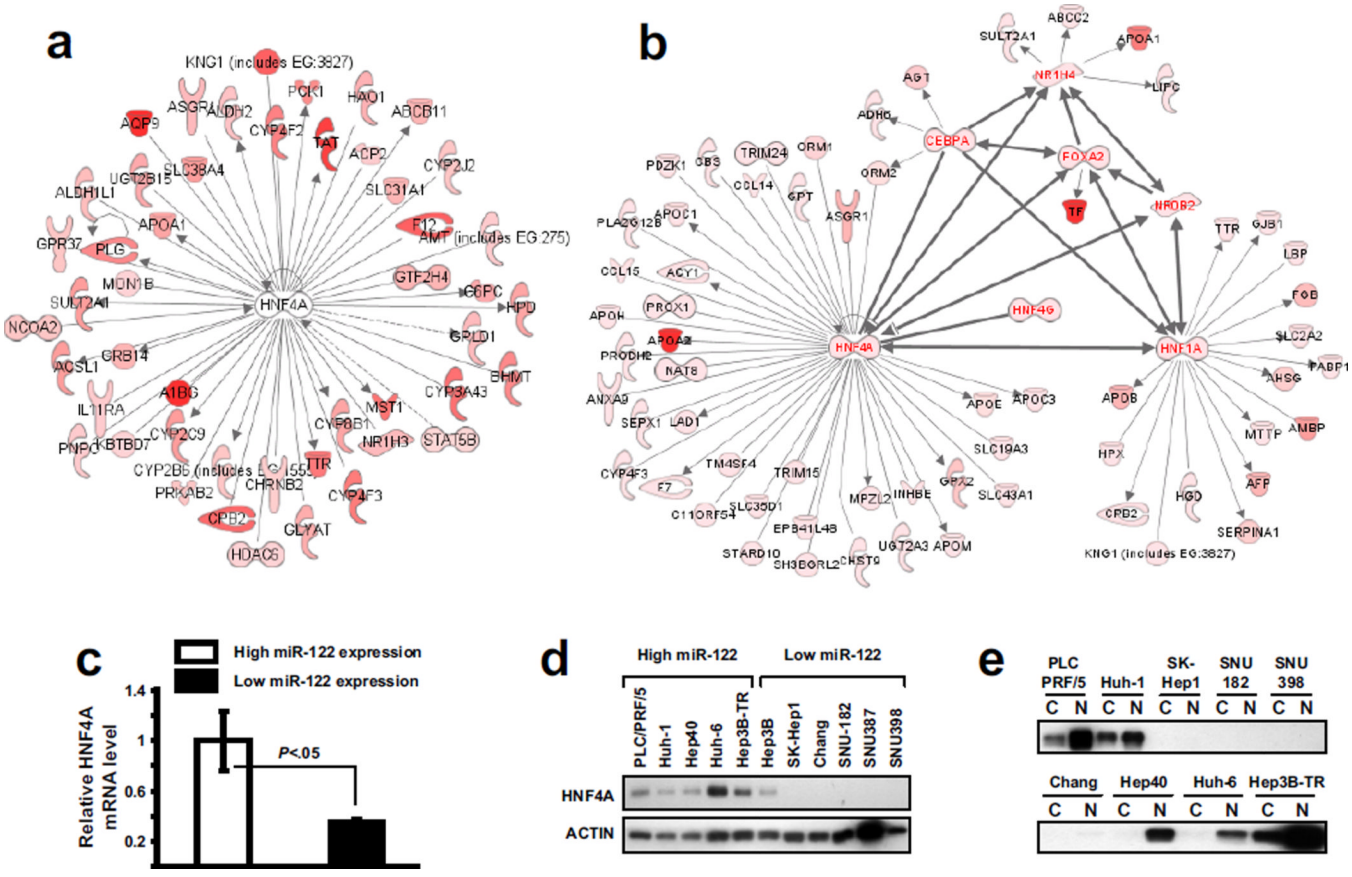

Figure 4.

High level of miR-122 in HCC defines a specific gene expression profile linked to HNF4A. (**a–b**) Ingenuity Pathway Analysis applied to the genes which expression was positively correlated with the expression of miR-122 in human primary HCC (**a**) and HCC-derived cell lines (**b**). Liver-enriched transcription factors, HNF4A in particular, were identified as the central regulators in the gene signature associated with a high miR-122 expression. (**c–e**) In HCC-derived cell lines the loss of miR-122 is associated with a loss of HNF4A. (**c**) The evaluation of HNF4A mRNA level in HCC-derived cell lines indicated a positive correlation between HNF4A and miR-122. (**d**) The assessment of HNF4A expression in protein extracts isolated from the cell lines which exhibit high vs. low miR-122 further indicates a positive correlation between miR-122 and HNF4A expression. High miR-122 expressing cell lines, such as PLC/PRF/5, Huh-1, Hep40 or Huh-6, exhibit a considerably higher HNF4A protein levels as compared to SNU-182, SNU-387, and SNU-398 lines which exhibit low miR-122levels. (**e**) Analysis of HNF4A expression in cytoplasmic (C) and nuclear (N) compartments indicates a nuclear enrichment of HNF4A in the high miR-122 expressing cell lines.

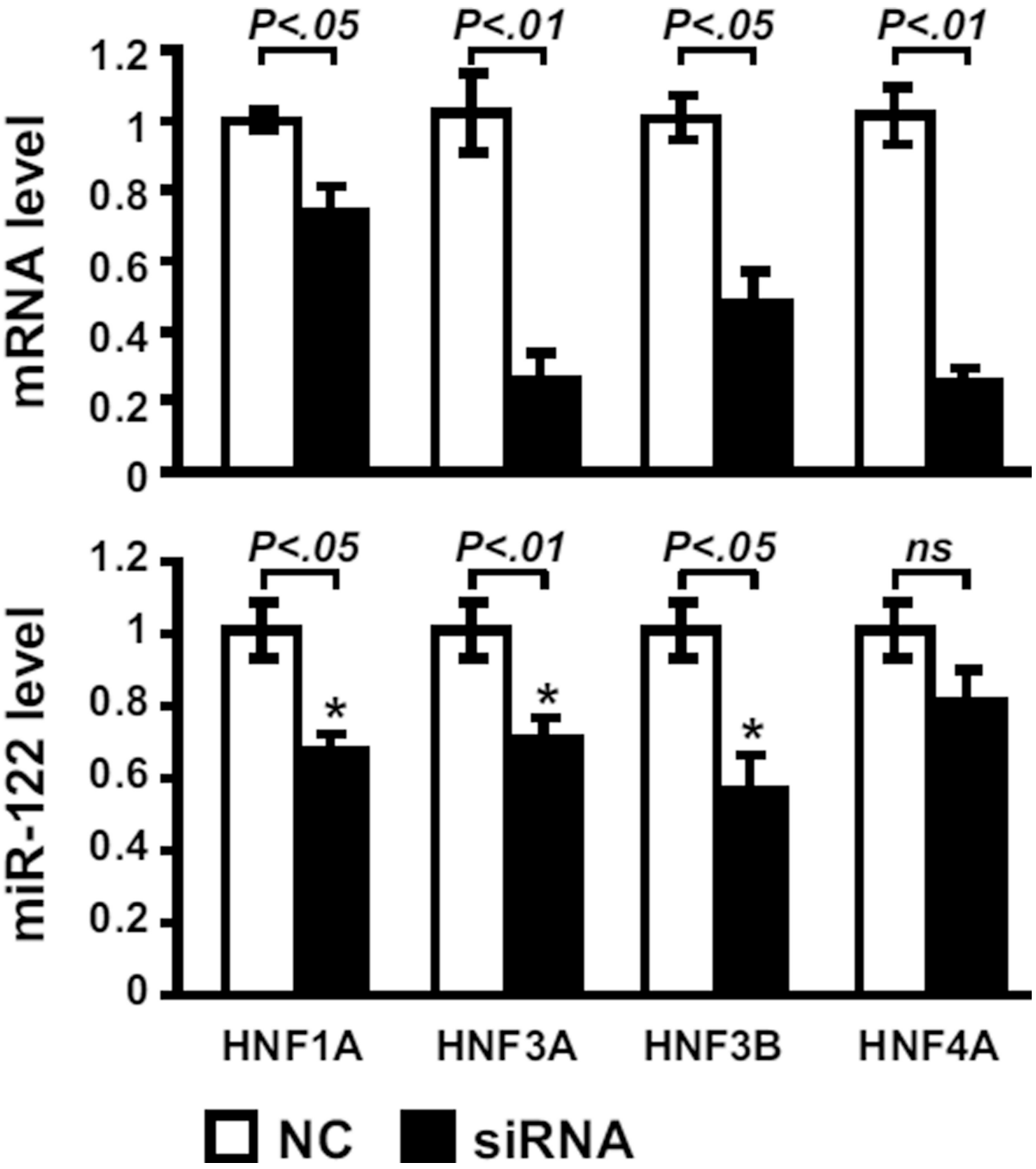

Figure 5.

The silencing of HNF1A, HNF3A, and HNF3B results in a decrease of miR-122 level. The high miR-122 expressing PLC/PRF/5 cell line was transfected with 40 nM of specific siRNA directed against HNF-1A, -3A, -3B, and -4A (siRNA, black column) vs. negative control (NC, white column). Forty eight hours after the transfection, the efficiency of the silencing was confirmed by the decreased level of respective mRNA (upper panel). As shown in the lower panel, the silencing of HNF-1A, -3A, and -3B significantly reduces the level of miR-122.

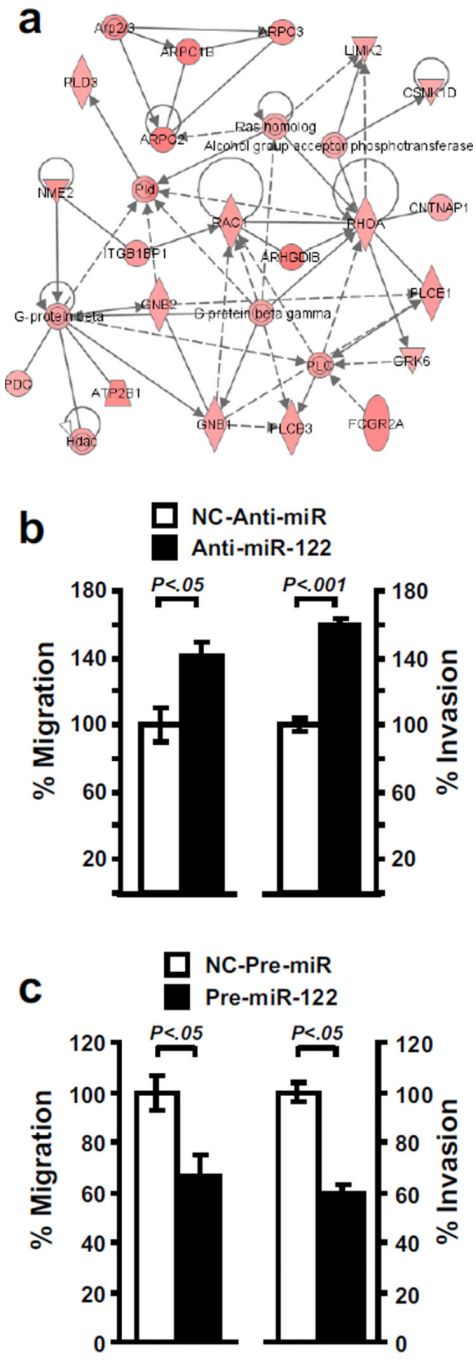

Figure 6.

miR-122 impacts the propensity of cancer cells to migrate and invade. (**a**) Ingenuity Pathway Analysis was performed on the genes up-regulated in HCC samples which exhibit a low miR-122 expression (Cluster B in Figure 3). This procedure revealed a network associated with cell motility and identified RAC1 and RHOA as two central regulators. (**b**) Inhibition of miR-122 results in the acquisition of migrating and invasive properties. The effect of miR-122 inhibition on cellular migration and invasion was assessed in Huh-1 by transfecting 20 nM of either anti-miR-122 (black column) or negative control (NC-Anti-miR, white column). Invasive and migrating activity was measured by using the BD

BioCoat Matrigel Invasion Chambers. Data indicate that the loss of miR-122 increases the migrating and invasive properties of Huh-1 cells. (**c**) Conversely, in the miR-122 negative SK-Hep1 cell line the restoration of miR-122 by transfecting miR-122 precursor molecules results in a decreased migration and invasion (Pre-miR-122, black column) as compared to cells transfected with negative control (NC-Pre-miR, white column).

Table 1

Clinical and pathological features of HCC cases

Gender	**# cases**
Female	16
Male	48
Age	
Median (range)	62 (34–85)
< 60	27
> 60	37
Survival (months)	
Mean ± s.e.m.	34.8 ± 4.9
Cirrhosis	
No	33
Yes	31
Plasma α-fetoprotein	
< 300 ng/mL	41
> 300 ng/mL	23
Etiology	
HBV	18
HCV	13
HBV + HCV	3
ALD	13
ALD + Viral infection [a]	4
other	2
unknown	11
Tumor size	
< 5 cm	34
> 5 cm	30
Edmondson-Steiner grade	
Grade 1	1
Grade 2	26
Grade 3	33
Grade 4	4
Vascular invasion	
No	14
Yes	29
Gene signatures [b]	
Good prognosis	40
Bad prognosis	24
Hepatocyte	49
Hepatoblast	15
c-Met Negative	45

Gender	# cases
c-Met positive	19
TGF-β Early	12
TGF-β Late	14

[a] Either HVB or HCV infection

[b] From our previously published HCC stratification (Lee *et al.*, 2004a; Kaposi-Novak *et al.*, 2006; Lee *et al.*, 2006; Coulouarn *et al.*, 2008)

Table 2

Ingenuity Pathway Analysis of genes correlated with miR-122 expression in HCC

Positive correlation	Negative correlation
Disease and disorders	
Hepatic system disease ($P<10^{-6}$)	Cancer ($P<10^{-9}$)
Molecular and cellular functions	
Lipid metabolism ($P<10^{-9}$)	Cell cycle ($P<10^{-5}$)
Amino acid metabolism ($P<10^{-3}$)	Cell. assembly & org. ($P<10^{-5}$)
Physiological system development and functions	
Tumor morphology ($P<10^{-3}$)	Immune response ($P<10^{-3}$)
Embryonic development ($P<10^{-3}$)	Connective tissue dev. ($P<10^{-3}$)
	Tumor morphology ($P<10^{-3}$)
Top canonical pathways	
Fatty acid metabolism ($P<10^{-10}$)	Actin cytosk. Signaling ($P<10^{-4}$)
Xenobiotic metabolism ($P<10^{-5}$)	Hypoxia signaling ($P<10^{-3}$)
Coagulation system ($P<10^{-5}$)	
FXR/ RXR Activation ($P<10^{-4}$)	
Top Tox functions: Hepatotoxicity	
Liver cholestasis ($P<10^{-6}$)	Hepatocellular carcinoma ($P<10^{-3}$)