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miR-148a Plays a Pivotal Role in the Liver by Promoting the Hepatospecific Phenotype and Suppressing the Invasiveness of Transformed Cells

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MicroRNAs (miRNAs) are evolutionary conserved small RNAs that post-transcriptionally regulate the expression of target genes. To date, the role of miRNAs in liver development is not fully understood. By using an experimental model that allows the induced and controlled differentiation of mouse fetal hepatoblasts (MFHs) into mature hepatocytes, we identified miR-148a as a hepatospecific miRNA highly expressed in adult liver. The main finding of this study revealed that miR-148a was critical for hepatic differentiation through the direct targeting of DNA methyltransferase (DNMT) 1, a major enzyme responsible for epigenetic silencing, thereby allowing the promotion of the "adult liver" phenotype. It was also confirmed that the reduction of DNMT1 by RNA interference significantly promoted the expression of the major hepatic biomarkers. In addition to the essential role of miR-148a in hepatocyte maturation, we identified its beneficial effect through the repression of hepatocellular carcinoma (HCC) cell malignancy. miR-148a expression was frequently down-regulated in biopsies of HCC patients as well as in mouse and human HCC cell lines. Overexpressing miR-148a led to an enhancement of albumin production and a drastic inhibition of the invasive properties of HCC cells, whereas miR-148a silencing had the opposite consequences. Finally, we showed that miR-148a exerted its tumor-suppressive effect by regulating the c-Met oncogene, regardless of the DNMT1 expression level. *Conclusion*: miR-148a is essential for the physiology of the liver because it promotes the hepatospecific phenotype and acts as a tumor suppressor. Most important, this report is the first to demonstrate a functional role for a specific miRNA in liver development through regulation of the DNMT1 enzyme. (HEPATOLOGY 2013;58:1153-1165)

MicroRNAs (miRNAs) constitute a group of evolutionary conserved small noncoding RNA molecules that finely regulate gene expression by complementary base pairing with the 3'-untranslated regions (3'-UTRs) of target messenger RNAs (mRNAs). The past decades have seen an increasing recognition of the overall significance of miRNAs in regulating a wide variety of fundamental biological phenomena and diseases,[1,2] including cancer.[3-5] The functional significance of miRNAs in cell specification and vertebrate development has been recently tackled.[6] For instance, miR-124 and miR-9,[7,8]

Abbreviations: 5-Aza, 5-Aza-2'-deoxycytidine; Afp, alpha-fetoprotein; Alb, albumin; Ab, antibody; Ck19, cytokeratin 19; CLD, chronic liver disease; c-Met, hepatocyte growth factor receptor; COBRA, combined bisulfite restriction analysis; Cyp, cytochrome P450; Dnmt, DNA methyltransferase; E-cadherin, epithelial cadherin; FBS, fetal bovine serum; G6pc, glucose-6-phosphatase; HBV, hepatitis B virus; HCC, hepatocellular carcinoma; HCV, hepatitis C virus; HGF, hepatic growth factor; MFH, mouse fetal hepatoblast; mRNAs, messenger RNAs; miRNA, microRNA; PAS, periodic acid-Schiff; RT-qPCR, reverse-transcription quantitative polymerase chain reaction; siRNA, small interfering RNA; Tat, tyrosine aminotransferase; 3'-UTR, 3'-untranslated region.

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two brain-enriched miRNAs, appear to be essential in neurogenesis, whereas miR-27b is relevant for myogenesis.[9] To date, little is known regarding the role and function of miRNAs in liver development. Hand et al. provided the first link between miRNAs and hepatobiliary development by emphasizing the functional role of miR-30a during biliary morphogenesis in zebrafish.[10] In humans, miR-122 might be of prime interest because it represents more than 70% of the total amount of miRNAs expressed in the adult liver, where it acts in metabolism regulation and hepatic homeostasis.[11]

During development, epigenetic modifications are essential for the modulations of tissue-specific gene expression that promote cell differentiation.[12] Epigenetic silencing includes reversible DNA methylation, which is primarily orchestrated by DNA methyltransferases (DNMTs). DNMT1 represents the major enzyme responsible for the maintenance of DNA methylation patterns during replication.[13] In contrast, DNMT3a and DNMT3b have been identified as *de novo* methyltransferases, which methylate DNA during early development and gametogenesis,[14] although DNMT1 also possesses *de novo* methylation activity. Inactivation of the DNMT1 enzyme in mice results in loss of genomic imprinting and leads to early embryonic lethality.[15] In addition, studies using methylation-deficient mouse embryos (*Dnmt1*$^{-/-}$, *Dnmt3a*$^{-/-}$, and *Dnmt3b*$^{-/-}$) have demonstrated that restoring DNA methylation is essential for development.[14,16] More recently, Sen et al. observed the enrichment of DNMT1 protein in epidermal progenitors, where it is required to maintain proliferative strength and suppress differentiation.[17] Their study also showed that DNMT1 depletion was associated with the altered proliferation and transition from progenitors to premature epidermal cells. In the liver, DNMT1 expression is frequently increased in tissues affected by chronic hepatitis and cirrhosis and, more dramatically, in hepatocellular carcinoma (HCC), in which DNMT1 augmentation correlates with poor prognosis.[18,19]

This study aimed to investigate the potential role of miRNAs in hepatic development. By taking advantage of an experimental primary cell-culture model that can trigger hepatic differentiation, we performed mouse miRNA microarray analyses and identified 10 miRNAs, which were selected for their predicted aptitude to target DNMT1. Among those miRNAs, miR-148a showed a strong induction in differentiating liver progenitors. Conversely, DNMT1 expression presented a rapid decline after stem cell entry into the differentiation process. We reported a correlation between the elevation of miR-148a and the promotion of the hepatospecific phenotype through the silencing of DNMT1. Because a significant down-regulation of miR-148a was observed in HCC, the role of miR-148a in liver cancer was also considered. We demonstrated the ability of miR-148a to suppress the invasive properties of transformed hepatic cells by inhibiting c-Met expression. In line with these findings, miR-148a was shown to play an essential role in the fate of the liver by inducing hepatospecific gene expression and suppressing tumor cell invasion.

Materials and Methods

Mouse Fetal Hepatoblast Model. Mouse fetal hepatoblasts (MFHs) were isolated and triggered to differentiate into mature hepatocytes as previously described.[20] Briefly, the method was based on the selective harvesting of hepatic parenchymal stem cells from mouse fetuses (E14.5). After their isolation, fetal liver tissues were dissociated physically and enzymatically in the presence of liberase (Liberase TM Research Grade; Roche Diagnostics, Mannheim, Germany). The sorting of epithelial cadherin (E-cadherin)-positive progenitors was performed using the biotin-anti-CD324 (E-cadherin) antibody (Ab) (eBioscience, Inc., San

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Diego, CA) and the EasySep Mouse Biotin Positive Selection Kit (STEMCELL Technologies Inc., Vancouver, British Columbia, Canada). From seeding, MFHs were maintained in a medium composed of the following mixture: William's E Medium, L-glutamine (2 mM), penicillin (50 IU/mL), and streptomycin (50 μg/mL), all from Gibco (Grand Island, NY), insulin (5 μg/mL; Sigma-Aldrich, St. Louis, MO), epidermal growth factor (25 ng/mL; Sigma-Aldrich), and 10% fetal bovine serum (FBS; HyClone; Thermo Fisher Scientific, Waltham, MA) supplemented with essential hepatocyte phenotype-promoting factors, including hepatic growth factor (HGF; 25 ng/mL; PeproTech Inc., Rocky Hill, NJ), oncostatin M (12.5 ng/mL; Sigma-Aldrich), hydrocortisone 21-hemisuccinate (5×10^{-7} M; Sigma-Aldrich), and dexamethasone (10^{-7} M; Sigma-Aldrich). The medium was replaced daily.

HCC Cell Lines and Human Samples. Mouse Hepa 1-6 and human HepG2 and Hep3B cells were purchased from the American Type Culture Collection (Manassas, VA). Huh-7 cells were from Riken BioResource Center (RIKEN BRC, Ibaraki, Japan). Cells were maintained in Dulbecco's modified Eagle's medium (Gibco) supplemented with penicillin (100 IU/mL), streptomycin (100 μg/mL), and 10% FBS. Human samples included 39 pairs of primary HCCs and their corresponding nontumor tissues. All patients exhibited chronic liver disease (CLD) related to hepatitis B (HBV) or C virus (HCV) infection (n = 18 and 21, respectively). Normal liver samples were collected from patients who had surgical resection of metastasis in the liver. Human fetal livers were obtained from spontaneously aborted fetuses (see Supporting Table 2 for clinical data).

Additional Methods. miRNA and small interfering RNA (siRNA) transfection procedures for primary cultures and cell lines, DNA extraction, methylation assay, immunoblotting, total RNA extraction, miRNA microarray, miRNA, and mRNA expression analysis by reverse-transcription quantitative polymerase chain reaction (RT-qPCR), miRNA assessment in the serum of HCC patients, periodic acid-Schiff (PAS) staining, luciferase reporter assays, apoptotic activity, cell growth, wound healing, transwell invasion assays, and statistical tools are described in the Supporting Materials.

Results

MFH Is an Adequate Model for the Study of Hepatic Differentiation. To clarify the function of miRNAs in liver development, we used an *in vitro* model previously developed by our group based on the sorting of E-cadherin-positive fetal liver cells, called MFHs, and their induced differentiation into hepatocytes (Fig. 1A). MFHs underwent remarkable changes in morphology during the maturation-induced process that resulted in the formation of pronounced cell aggregates with cuboidal shape, polarity, and frequent binucleation (Fig. 1B). Importantly, mature-induced hepatocytes exhibited prominent glycogen storage ability. The molecular data were consistent with those observations and revealed a hepatospecific phenotype and progressive maturation of MFHs, as evidenced by the expression of the early (alpha-fetoprotein; Afp), mid- (albumin; Alb), and late (glucose-6-phosphatase [G6pc] and tyrosine aminotransferase [Tat]) hepatic markers (Fig. 1C). In addition, the major cytochrome P450s (CYPs) were similarly induced (Supporting Fig. 1). Conversely, the mRNA level of cytokeratin 19 (Ck19), which is commonly associated with liver stem cells and epithelial cells of the biliary tract, decreased rapidly after the initiation of the maturation process. Our data also indicate the rapid decline of Dnmt1 expression in association with MFH differentiation, whereas Dnmt3a and Dnmt3b increased progressively (Fig. 1D).

miR-148a Induction Is Observed in Hepatic Cells During Mouse Liver Development. To analyze the expression profile of miRNAs during hepatic differentiation, we performed an miRNA microarray by using the MFH model at different stages of maturation (Fig. 2A; all the miRNA microarray data are displayed in Supporting Table 1). Then, taking advantage of the combination of the publicly available search engines, miRNA (miRanda), TargetScan, and PicTar, we obtained a list of 12 miRNAs that could putatively target Dnmt1 (Table 1). Among those miRNAs, 10 were significantly expressed in differentiating MFHs. A family of three conserved miRNAs (miR-148a, miR-148b, and miR-152) was highlighted as a result of its remarkable expression pattern during the maturation process of MFHs (Fig. 2B). More explicitly, both microarray and RT-qPCR analyses revealed that miR-148a and miR-152 were gradually up-regulated from the MFH to the mature-induced hepatocyte stage (Fig. 2C). In contrast, miR-148b stayed unchanged. Obviously, miR-148a exhibited the most significant induction and highest expression level in mature hepatocytes. Similar profiles of expression for these miRNAs were obtained from fetal liver tissues during mouse development (Supporting Fig. 2). In addition, Dnmt1 expression was inversely correlated with the level of miR-148a

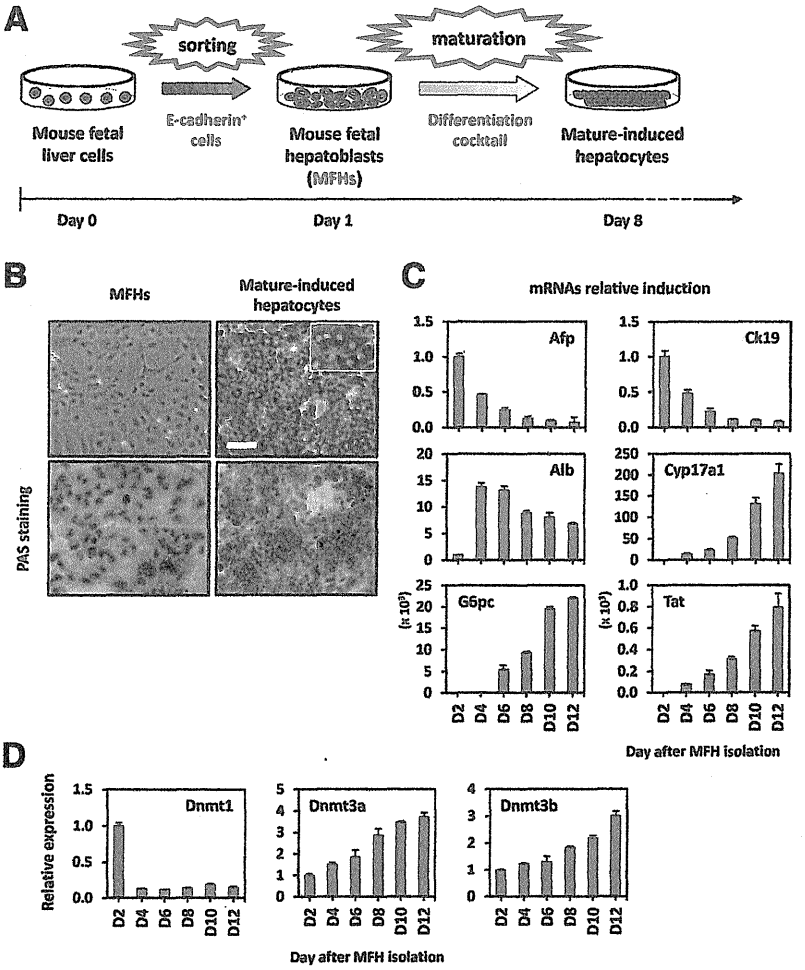

Fig. 1. Characterization of the MFH model. (A) Schematic representation of MFH purification and induced differentiation into mature hepatocytes after hepatotrophic factor stimulation. (B) Primary cultures of MFHs showing the radical changes undergone by undifferentiated hepatic progenitors to adopt the characteristic morphology of mature hepatocytes with polarity and frequent binucleation (white square). PAS staining revealed extensive glycogen storage in mature-induced hepatocytes, whereas MFHs were devoid of glycogen. Scale bar, 50 μm. Time course showing mRNA relative expression determined by RT-qPCR of (C) major hepatic markers and (D) Dnmt family members in the MFH model. The housekeeping gene, Gapdh, was used as an internal control to normalize the amount of complementary DNA.

in both *in vitro* and *in vivo* models, whereas Dnmt3a and Dnmt3b did not correlate. Consequently, it was hypothesized that miR-148a could play a critical role in liver development by regulating Dnmt1 expression.

miR-148a Is Down-Regulated in Human and Rodent HCC Cells. To explore the significance of miR-148a in the liver, we first compared expression profiles of miR-148a among mature-induced hepatocytes (MFH D8), undifferentiated hepatic stem cells (MFH D2), and the mouse HCC cell line, Hepa 1-6. Human Huh-7, HepG2, and Hep3B cells were also characterized in regard to normal adult and fetal hepatic tissues. As a result, a dramatic diminution of miR-148a was observed in both rodent (Fig. 3A) and human cell lines as well as in fetal livers (Fig. 3B). Moreover, the reduced expression of miR-148a was consistent with Dnmt1 augmentation in both species, arguing for a probable connection between miR-148a and Dnmt1. Thus, Spearman's rank correlation analysis showed that expression levels of DNMT1 and miR-148a in human samples were inversely correlated (rho: -0.609; $P = 0.0034$; Fig. 3C). To test the functional relevance of miR-148a down-regulation caused by DNA methylation, HCC cells were exposed to 5-Aza-2'-deoxycytidine (5-Aza). We found that demethylation treatment dramatically restored miR-148a expression in a dose-response manner in both Hepa 1-

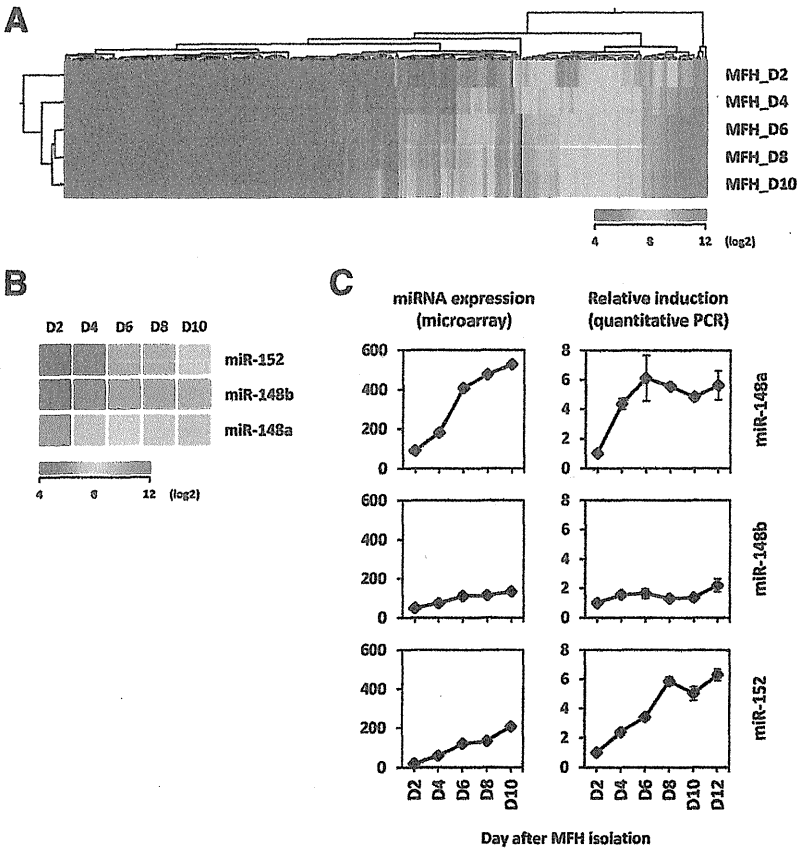

Fig. 2. Identification of miR-148a as a preponderant miRNA during hepatic differentiation. (A) miRNA global expression pattern during the process of MFH differentiation into mature hepatocytes. The scale bar encodes the logarithm of relative miRNA expression level. The 2-fold threshold was set to identify the miRNAs with significant differential expression. Microarray data are shown in Supporting Table 1. (B) Representative expression of miR-148a, miR-148b, and miR-152 selected for their significant induction during MFH differentiation and their predicted ability to target Dnmt1. (C) Differential expression of the miR-148a/148b/152 family evaluated by microarray and RT-qPCR. Relative expression levels determined by RT-qPCR were normalized against the endogenous control, RNU6B.

6 and HepG2 cell lines (Fig. 3D), indicating that a hypermethylation phenomenon is most likely responsible for the silencing of miR-148a in liver cancer cells. To verify this hypothesis, we first analyzed the genomic DNA sequence spanning of miR-148a and found that this gene had many CpG-rich regions (CpG islands) in its promoter. Subsequently, combined bisulfite restriction analysis (COBRA) was performed to examine the methylation status of the miR-148a promoter, which revealed hypermethylation of CpG islands in the miR-148a promoter in HepG2 cells, compared to normal human hepatocytes (Fig. 3E). We also observed that demethylation treatment by 5-Aza dramatically decreased the methylation status of the miR-148a promoter in both human and rodent HCC cell lines. Although the COBRA method did not reveal demethylation of the analyzed miR-148a CpG sites during the maturation process of MFHs (Supporting Fig. 3), bisulfite sequencing showed that the average methylation level of miR-148a was higher in undifferentiated MFHs (17.6% in MFH_D2), compared to differentiating cells (5.7% in MFH_D4), suggesting that a hypermethylation mechanism may participate in the regulation of miR-148a expression during development.

miR-148a Directly Modulates Dnmt1 Expression. We postulated that Dnmt1 inhibition during MFH maturation could be the result of its direct targeting by miR-148a. To explore this possibility, we first analyzed the consequences of miR-148a silencing or overexpression in HCC cell lines. The use of miR-148a mimics clearly affected Dnmt1 expression (Fig. 4A). Conversely, we observed a significant enhancement of Dnmt1 level after transfection with miR-148a antagonists up to 72 hours post-transfection. Dnmt1 contains a 3'-UTR element that is partially complementary to

Table 1. List of the miRNAs That Are Predicted to Target Mouse Dnmt1 and Their Respective Expression in the MFH Model (miRNA Microarray Data)

	1-MFH_D2			2-MFH_D4			3-MFH_D6			4-MFH_D8			5-MFH_D10		
	Raw Data	Log2 (Raw Data)	Log2 (sample/MFHD2)	Raw Data	Log2 (Raw Data)	Log2 (Sample/MFHD2)	Raw Data	Log2 (Raw Data)	Log2 (Sample/MFHD2)	Raw Data	Log2 (Raw Data)	Log2 (Sample/MFHD2)	Raw Data	Log2 (Raw Data)	Log2 (Sample/MFHD2)
mmu-miR-128-3p	20.24	4.34	1.00	20.55	4.36	1.01	27.63	4.79	1.10	29.01	4.86	1.12	36.18	5.18	1.19
mmu-miR-130a-3p	1,536.74	10.59	1.00	1,962.51	10.94	1.03	2,443.93	11.25	1.06	2,425.60	11.24	1.06	2,721.55	11.41	1.08
mmu-miR-130b-3p	103.95	6.70	1.00	106.84	6.74	1.01	111.44	6.80	1.01	100.15	6.65	0.99	111.61	6.80	1.02
mmu-miR-148a-3p	90.36	6.50	1.00	180.16	7.49	1.15	407.07	8.67	1.33	476.92	8.90	1.37	527.02	9.04	1.39
mmu-miR-148b-3p	49.51	5.63	1.00	74.49	6.22	1.10	110.86	6.79	1.21	114.13	6.83	1.21	133.33	7.06	1.25
mmu-miR-152-3p	18.54	4.21	1.00	58.77	5.88	1.40	120.96	6.92	1.64	134.04	7.07	1.68	207.02	7.69	1.83
mmu-miR-301a-3p	395.46	8.63	1.00	353.92	8.47	0.98	387.68	8.60	1.00	359.38	8.49	0.98	380.23	8.57	0.99
mmu-miR-301b-3p	13.26	3.73	1.00	10.48	3.39	0.91	10.85	3.44	0.92	10.78	3.43	0.92	10.89	3.45	0.92
mmu-miR-326-3p	1.00	0	0	17.29	4.11	0	23.84	4.58	0	25.06	4.65	0	35.23	5.14	0
mmu-miR-330-3p	1.00	0	0	1.00	0	0	1.00	0	0	1.00	0	0	1.00	0	0
mmu-miR-495-3p	281.06	8.13	1.00	521.99	9.03	1.11	554.68	9.12	1.12	384.00	8.58	1.06	368.08	8.52	1.05
mmu-miR-1192	1.00	0	0	1.00	0	0	1.00	0	0	1.00	0	0	1.00	0	0

miR-148a in both rodent and human species (Fig. 4B). The miRNA prediction databases that we interrogated identified Dnmt1 as a high-scoring predicted target of

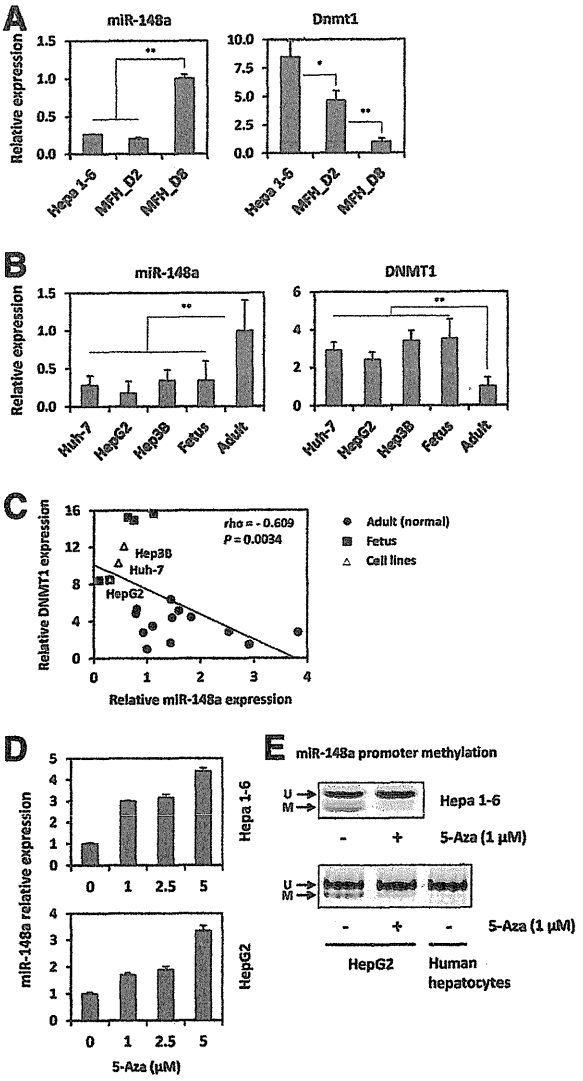

Fig. 3. miR-148a and Dnmt1 assessment in the liver. (A) The mouse hepatoma cell line, Hepa 1-6, was used to assess miR-148a and Dnmt1 mRNA levels, compared to undifferentiated hepatic stem cells (MFH D2) and mature-induced hepatocytes (MFH D8). (B) miR-148a and human DNMT1 expression were analyzed in the human HCC cell lines, Huh-7, HepG2, and Hep3B, and compared to a cohort of 13 normal livers as well as five lots of fetal livers. (C) Scatter plots of Spearman's correlation coefficient analysis between relative DNMT1 expression level and miR-148a. (D) Relative expression of miR-148a in Hepa 1-6 and HepG2 cells after 5 days of exposure to the hypomethylation agent, 5-Aza at 1, 2.5, and 5 μM. (E) COBRA of miR-148a promoter in Hepa 1-6 and HepG2 cells treated with or without 5-Aza for 5 days. Methylation status of the miR-148a promoter was also assessed in normal human hepatocytes. U, unmethylated; M, methylated. Statistical significance, compared to controls, was: $^{*}P < 0.05$ and $^{**}P < 0.01$ (*t* test) for RT-qPCR analysis.

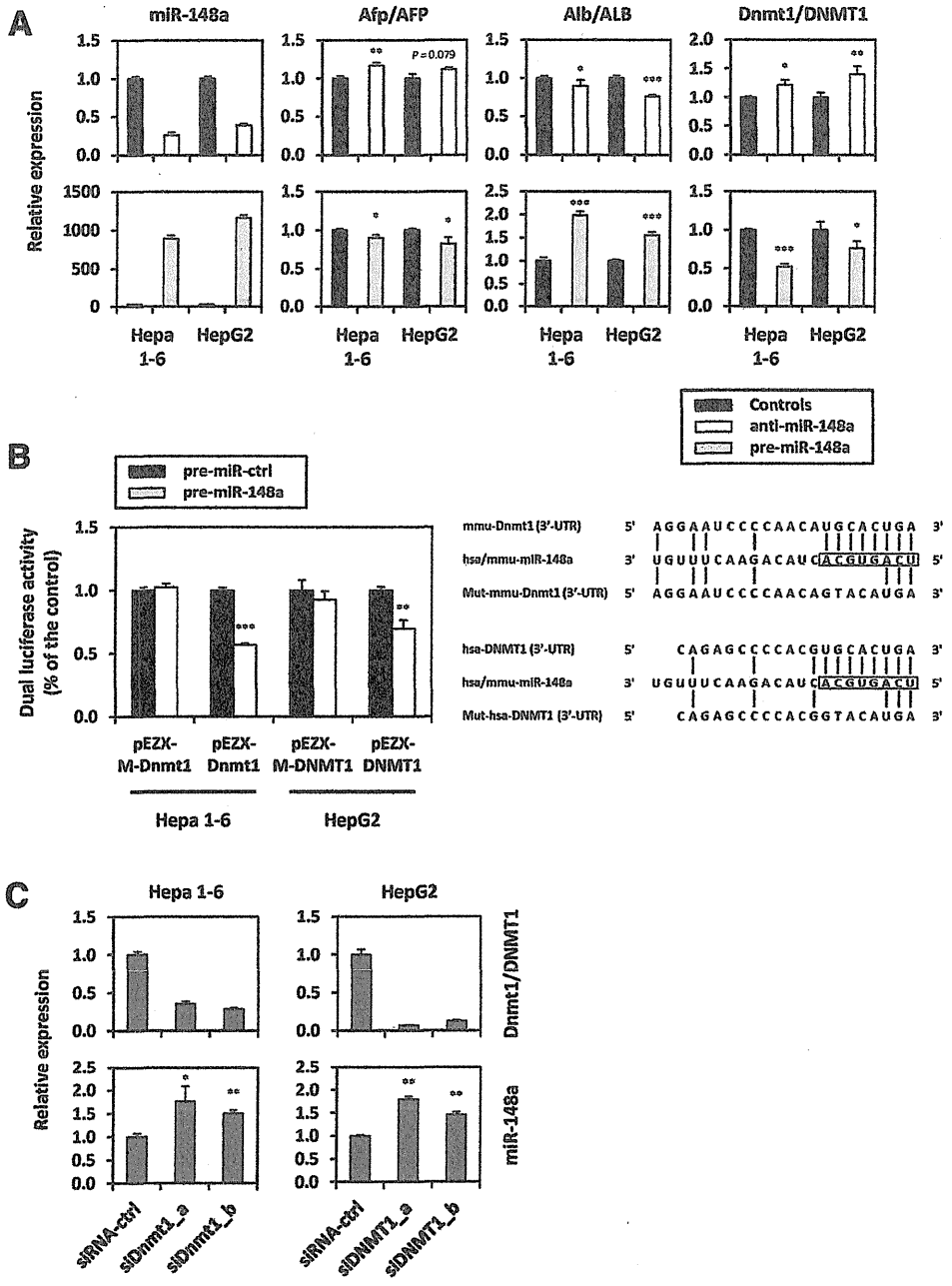

Fig. 4. Characterization of the relationship between miR-148a and DNMT1 in rodent and human models. (A) Relative expression of Dnmt1 and liver markers Afp and Alb, respectively, after experimental modulation of miR-148a in mouse Hepa 1-6 and human HepG2 cell lines. Cells were transfected using 100 ng of miR-148a mimics (pre-miR-148a) or antagonists (anti-miR-148a). Scramble miRNA mimics or antagonists were used as negative controls. Total RNAs were collected 72 hours post-transfection, and mRNA relative expression levels were determined by RT-qPCR. (B) Dual luciferase assay on Hepa 1-6 and HepG2 cells cotransfected with miR-148a mimics and the firefly/Renilla luciferase construct containing the mouse Dnmt1 or human DNMT1 3'-UTR. Mutated 3'-UTR sequences were used as negative controls, and ratios of firefly/Renilla luciferase activities were determined. Sequences indicate interaction sites between miR-148a and 3'-UTRs of mouse Dnmt1 and human DNMT1. (C) Transfection of Hepa 1-6 and HepG2 cells with siRNAs against mouse Dnmt1 and human DNMT1. Scramble siRNAs were used as negative controls (siRNA-ctrl). Total RNAs were used to analyze miR-148a expression by RT-qPCR 48 hours after transfection. Statistical significance: $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$ (*t* test).

miR-148a. Simultaneous transfection with miRNA mimics and a construct containing the mouse Dnmt1 3'-UTR inserted downstream of the luciferase coding sequence was performed in Hepa 1-6 cells. In this assay, miR-148a-forced expression decreased luciferase activity by 43.2% ± 1.1% ($P < 0.001$) from the control value,

whereas it failed to inhibit reporter activity in cells transfected with the vector containing a mutated sequence of the Dnmt1 3'-UTR (Fig. 4B). Comparable data were obtained using the 3'-UTR of human DNMT1 transfected into the human HCC cell line, HepG2. Then, to explore the role of DNMT1 in regulating expression of miR-148a, we silenced DNMT1 by using a siRNA approach in Hepa 1-6 and HepG2 cells. Both mouse Dnmt1 knockdown and human DNMT1 knockdown significantly induced miR-148a expression (Fig. 4C), reinforcing the idea of a regulatory circuit between DNMT1 and miR-148a as well as the existence of epigenetic regulation exerted by DNMT1 on miR-148a.

miR-148a Enhancement Promotes Hepatospecific Gene Expression Through Dnmt1 Inhibition During the Induced Differentiation of MFHs Into Mature Hepatocytes. The influence of miR-148a in hepatic differentiation was investigated by forcing its expression in the MFH primary culture model and evaluating the expression of major liver markers. Cells transfected with miR-148a mimics exhibited substantial overexpression of miR-148a, in contrast to its normal expression profile during MFH differentiation (Fig. 5A). Immunoblotting revealed that miR-148a overexpression dramatically increased the protein level of Alb in MFHs (Fig. 5B). The methylation status of the Alb promoter was also explored, which showed a progressive demethylation of CpG islands during hepatic differentiation (Supporting Fig. 3). Both 5-Aza treatment and miR-148a mimics contributed to the demethylation of Alb promoter, indicating the possible regulation of Alb expression by miR-148a through an epigenetic mechanism involving Dnmt1. RT-qPCR analysis demonstrated that miR-148a mimics enhanced the mRNA levels of Alb as well as the other major hepatic biomarkers, G6pc and Tat, whereas cells transfected by the control showed the standard differentiation process induced by the hepatotrophic factors (Fig. 5C). Moreover, miR-148a augmentation had no effect on Ck19 expression in MFHs, but it was associated with the increased expression of various CYPs (Supporting Fig. 4). Remarkably, we found evidence that miR-148a restoration in both mouse Hepa 1-6 and human HepG2 HCC cell lines was significantly related with the inhibition of the immature liver marker, Afp, whereas Alb expression was strongly enhanced, and *vice versa* (Fig. 4A). Last, the forced expression of miR-148a was correlated with a drastic repression of Dnmt1 in both the HCC (Fig. 4A) and MFH models (Fig. 5C). Western blotting analysis confirmed the negative correlation between miR-148a and DNMT1 expression levels (Fig. 5B). Indeed, the transfection of MFHs using miR-148a mimics promoted the decline of Dnmt1 that is normally observed during the differentiation process of these cells. To address the involvement of Dnmt1 in the establishment of the hepatic phenotype through its modulation by miR-148a, we finally analyzed the effect of Dnmt1 knockdown in the induced differentiation of MFHs. Consistent with miR-148a overexpression data, Dnmt1 inhibition led to the significant promotion of the major hepatic biomarkers that we assessed (Fig. 5D). Compared with MFHs transfected with negative control siRNAs, mRNA levels of Alb and advanced maturation biomarkers (G6pc, Tat, and Cyp17a1) appeared to be globally up-regulated 72 hours after Dnmt1 siRNA transfection.

In summary, these findings implicate Dnmt1 in the mechanisms controlling liver precursor maturation and indicate that miR-148a promotes the expression of adult hepatic genes by repressing Dnmt1 (Fig. 5E). In contrast, the occurrence of HCC malignancy may be associated with the deregulation of miR-148a, whereas maintenance of this miRNA seems to be essential for preserving the hepatospecific status of liver cells.

miR-148a Expression Is Frequently Decreased in the Liver of HCC Patients. We analyzed miR-148a expression in a cohort of 39 pairs of primary HCCs related to HBV or HCV infection and their adjacent nontumor regions. Tissues from normal liver (n = 13) were used as controls. miR-148a expression was reduced by more than 5-fold in HCC biopsies, relative to the normal liver group (median, 0.293 and 1.674, respectively; $P < 0.0001$, Mann-Whitney's U test; Fig. 6A). Interestingly, miR-148a was also inhibited in peritumoral non-neoplastic tissues, but to a lesser extent (median, 0.403; $P < 0.001$). We confirmed the possible correlation between miR-148a inhibition and advancement of the underlying liver disease by analyzing the expression level of miR-148a between early (chronic hepatitis) and advanced (precirrhotic/cirrhotic) fibrosis in nontumor tissues (Fig. 6B). Expression of miR-148a was significantly decreased in the cirrhotic samples, compared to the chronic hepatitis liver group (median, 0.247 and 0.473, respectively; $P < 0.0001$, Mann-Whitney's U test). Then, DNMT1 levels between tumors and their adjacent tissues were evaluated (Fig. 6C). Although DNMT1 expression was significantly down-regulated in tumors ($P = 0.0002$, Wilcoxon's signed-rank test), statistical analysis did not reveal significant correlation between DNMT1 and miR-148a expression in those clinical samples. Next, we compared the expression of miR-148a between

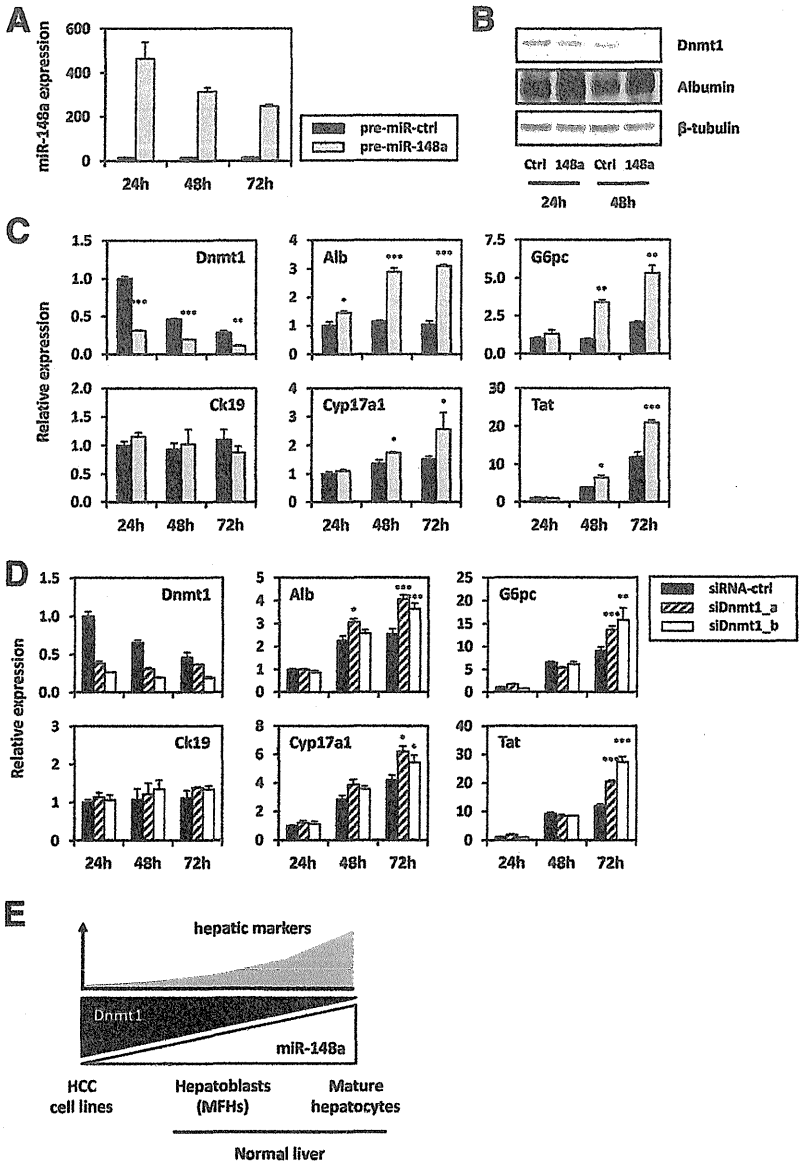

Fig. 5. Forcing expression of miR-148a promotes expression of hepatospecific genes during MFH-induced differentiation. MFH primary cultures were transfected using miR-148a mimics (100 nM) on the fourth day after cell isolation (MFH D4). Control miRNA mimics were used as negative controls. Total RNAs were extracted at the indicated times; then, miRNA and mRNA relative expression was determined by RT-qPCR. (A) MiR-148a overexpression in MFHs after transfection with miR-148a mimics (pre-miR-148a) or controls (pre-miR-ctrl). (B) Protein levels of Dnmt1 and Alb analyzed by immunoblotting 24 and 48 hours after MFH transfection by miR-148a mimics. β-tubulin was used as loading control. (C) Effect of miR-148a enforced induction on hepatic gene expression in the MFH model. mRNA levels of Alb and late hepatospecific makers (G6pc, Tat, and Cyp17a1) were analyzed. Dnmt1 and Ck19 were also evaluated in response to miR-148a overexpression. (D) Liver biomarker expression after Dnmt1 knockdown in MFHs. Transfections were performed the second day after cell sorting (MFH D2) using mouse Dnmt1 siRNAs, and RT-qPCR was performed at the indicated times. Statistical significance from control miRNAs and control siRNAs was reached at: $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$ (t test). (E) Schematic representation of the connection between miR-148a and Dnmt1 in the liver. During development, miR-148a expression is enhanced, inhibiting Dnmt1 and promoting induction of liver markers. In hepatic stem and HCC cells, miR-148a expression is repressed, leading to overexpression of Dnmt1 and silencing of hepatospecific genes.

tumors and their pair-matched normal tissues (Supporting Fig. 5). Of the 18 HBV-related HCC samples, miR-148a expression was decreased in 15 tumors, relative to their adjacent noncancerous hepatic regions ($P = 0.0268$, Wilcoxon's signed-rank test). In the 21 HCV-related HCCs, inhibition of miR-148a was observed in 12 HCC samples ($P = 0.9308$). The value of circulating miR-148a as a noninvasive HCC

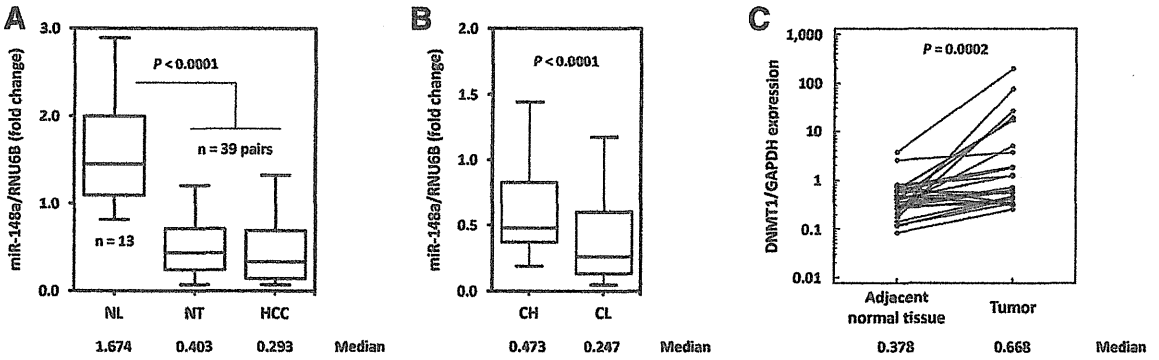

Fig. 6. Expression of miR-148a in liver clinical samples. (A) Plot boxes illustrating differential expression of miR-148a in 13 normal livers (NL), 39 primary HCCs (HCC), and their corresponding nontumor tissues (NT). Expression of miR-148a was normalized to RNU6B. Boxes show median and 25th and 75th percentiles, whereas vertical bars display the range of values. Within the box, 50% of values are shown, and 80% are included between the extremities of vertical bars. Mann-Whitney's U test indicated statistical underexpression of miR-148a in both HCC and adjacent NT tissues, compared to the normal liver group ($P < 0.0001$). (B) Expression of miR-148a in nontumor tissues between chronic hepatitis (CH) and precirrhotic/cirrhotic livers (CL). Statistical analyses showed significant inhibition in the CL group ($P < 0.0001$, Mann-Whitney's U test). (C) Comparison of DNMT1 expression levels between primary tumor and peritumoral non-neoplastic tissue from 24 randomly selected pairs. Statistical differences were analyzed with Wilcoxon's signed-rank test and indicated significant overexpression in tumor versus the normal group ($P = 0.0002$).

recurrence diagnostic marker in blood serum was also evaluated. Samples were collected in two steps from 11 HCC patients with HCV infection: (1) after surgical resection of the primary tumor and (2) subsequent to the diagnosis of HCC recurrence. We observed a diminution of circulating miR-148a in 8 patients after HCC recurrence ($P = 0.2783$, Wilcoxon's signed-rank test; Supporting Fig. 6).

The Rescue of miR-148a Suppresses HCC Cell Migration and Invasion by Indirectly Inhibiting the Hepatocyte Growth Factor Receptor Oncogene. As we highlighted the crucial role played by miR-148a in normal hepatic differentiation, it was of significant interest to consider the possible relationship between miR-148a deregulation and the promotion of hepatocyte transformation. First, the phenotype of Hepa 1-6 cells was characterized after the forced expression of miR-148a to investigate the effect of this miRNA on HCC cells. Notably, cell proliferation was not significantly altered by miR-148a mimics or antagonists (Fig. 7A), and induction of miR-148a had no effect on caspase activity (Supporting Fig. 7). However, the enforced expression of miR-148a substantially suppressed the motility of HCC cells in a wound-healing assay, whereas miR-148a agonists enhanced the recolonization of the wounds (Fig. 7B). In addition, overexpression of miR-148a remarkably altered the invasive abilities of Hepa 1-6 cells ($51.6\% \pm 10.15\%$ inhibition; $P < 0.001$), as revealed by the transwell migration assay (Fig. 7C). A similar observation was conducted using the human HCC cell line, Hep3B (data not shown). To evaluate whether the effect of miR-148a in the invasion of HCC cells is mediated by DNMT1 or another specific gene, functional analyses were performed using siRNA. We decided to focus on DNMT1 and hepatocyte growth factor receptor (c-Met), a frequently overexpressed oncogene in liver cancer and predicted target of miR-148a that was up-regulated in undifferentiated MFHs and Hepa 1-6 HCC cells (Supporting Fig. 8). In the presence of miR-148a mimics, c-Met mRNA levels appeared markedly decreased in Hepa 1-6, whereas miR-148a agonists promoted c-Met expression (Fig. 7D). However, c-Met 3'-UTR assays did not show a reduction of luciferase activity (Supporting Fig. 8), supporting an indirect effect of miR-148a on c-Met expression. Knockdown of c-Met using two distinct siRNAs attenuated cell proliferation (Fig. 7E) and dramatically abolished HCC cell invasion ($78.8\% \pm 7.7\%$ and $76.5\% \pm 7.5\%$ inhibition, respectively; $P < 0.001$; Fig. 7F). Remarkably, the use of siRNAs targeting Dnmt1 did not modify cell proliferation or invasion. These last results strongly suggest that miR-148a plays two distinct roles in the liver: (1) in the control of hepatic development by regulating DNMT1 and (2) in the modulation of HCC cell invasiveness by repressing the c-Met oncogene.

Discussion

DNA methylation plays an essential role in regulating stem cell differentiation and embryo development. Recently, Tsai et al. demonstrated that the pluripotency genes, *Oct4* and *Nanog*, which constitute a fundamental regulatory mechanism suppressing

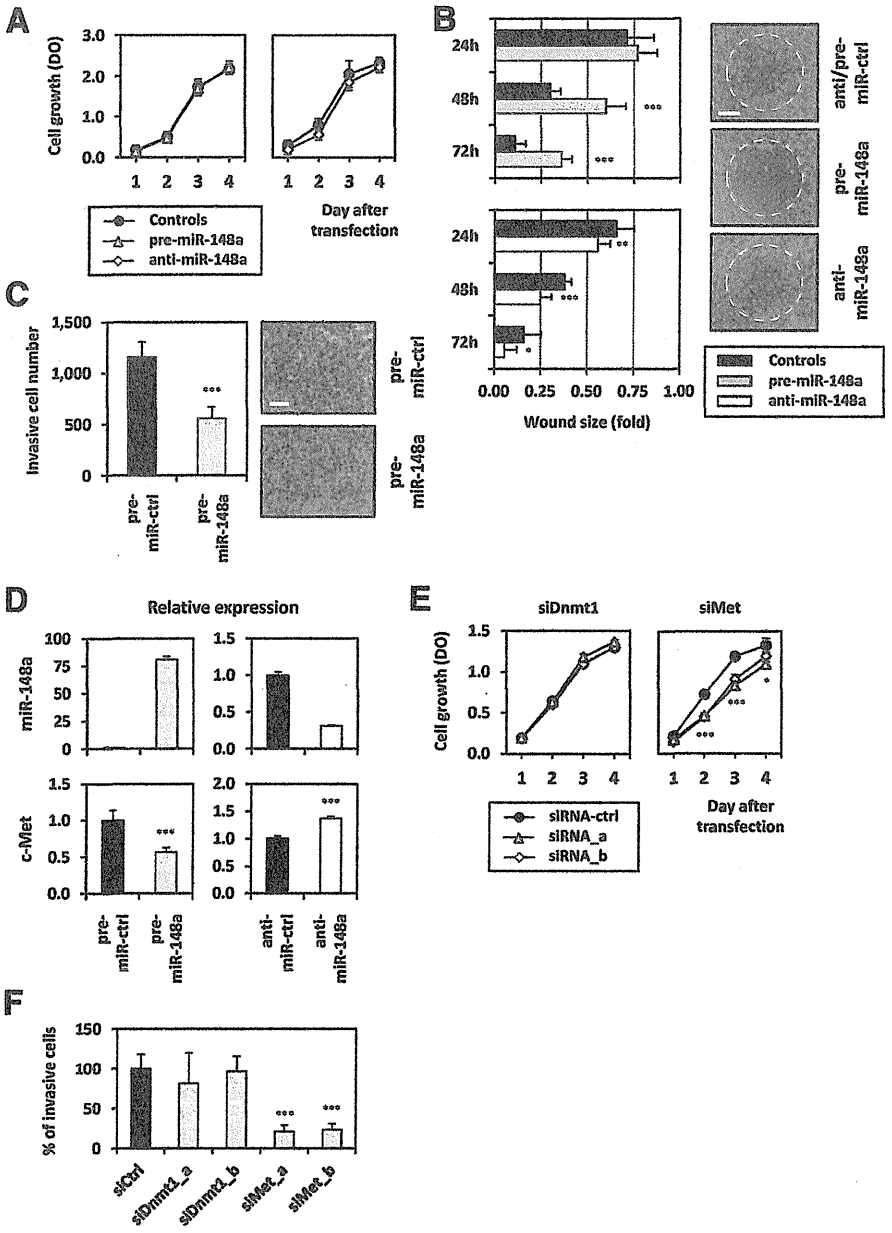

Fig. 7. Consequence of miR-148a rescue on HCC cell phenotype. (A) Hepa 1-6 cell growth determined at indicated times after miR-148a amplification or repression. No significant difference was found (*t* test). (B) Hepa 1-6 migratory abilities after miR-148a overexpression or inhibition in the presence of HGF (50 ng/mL). Cell monolayers were wounded 24 hours after transfection, and the sizes of the wounds were measured at indicated times. Bar: 500 μm. (C) Effect of miR-148a overexpression on cellular invasion ability. FBS (10%) and HGF (100 ng/mL) were used as chemoattractants for transwell invasion assays. Bar: 250 μm. (D) Expression of c-Met oncogene 48 hours after Hepa 1-6 transfection with miR-148a mimics or antagonists. (E) Assessment of Hepa 1-6 proliferation and (F) invasion ability after Dnmt1 and c-Met knockdown by using two distinct siRNAs for each. Statistical significance, compared to controls: $*P < 0.05$; $**P < 0.01$; $***P < 0.001$ (*t* test).

differentiation-associated genes, directly bind to the promoter of DNMT1 and enhance its expression.[21] In their report, mesenchymal stem cells exhibited a decreased proliferation rate when treated with an inhibitor of DNA methylation or transfected with DNMT1 short hairpin RNA, whereas the expression of genes associated with development regulators was increased. In agreement with this current work, our data clearly show the contribution of the DNMT1 enzyme in liver cell stemness as well as the existence of a micromanagement of DNMT1-related hepatic maturation controlled by miR-148a.

The deleterious consequences of DICER-silencing experiments in mouse embryonic stem cells demonstrated that miRNA processing plays a major role in development.[22] In the liver, Sekine et al. tested the consequence of DICER1 silencing by performing conditional knockout in hepatocytes.[23] Remarkably, hepatocytes exhibiting DICER1-specific depletion displayed a gene expression profile indicative of cell growth and dedifferentiation into liver progenitors. Although the role of miRNAs in cell specification has been addressed in a number of tissues,[6,24] little is known regarding the involvement of specific miRNAs in the control of hepatic development. miR-122 is probably an essential actor in liver ontogenesis, as suggested by its remarkable expression in the adult liver and its ability to induce CYPs in HCC cell lines.[25] The case of miR-148a also appears of prime interest in cell lineage determination, as previously described in hematopoietic stem cell specification[26] and myogenic differentiation.[27] In the last case, Zhang et al. showed the positive role of miR-148a in skeletal muscle development by the translational repression of ROCK1, an inhibitor of myogenesis.

Consistent with our results, other studies have demonstrated that miRNAs can control expression of DNMTs. In the liver, miR-140 can target the 3'-UTR of DNMT1 and control nuclear factor kappa B activity.[28] In addition, some splicing isoforms of DNMT3b have been found to be directly repressed by miR-148a.[29] Conversely, epigenetic mechanisms are considered essential for miRNA regulation.[30] The genomic sequence of miR-148a has been analyzed in a number of cancer cell lines with distinct tissue origins, as well as a large amount of CpG islands found in its promoter region. Thus, inactivation of miR-148a by DNA hypermethylation and DNMT1 overexpression has recently been demonstrated in pancreatic,[31] gastric,[32] and breast cancer.[33] Consequently, the network of feedback between miRNAs and epigenetic pathways appears to form a complex regulatory system that is essential to organize gene expression profile and maintain cell integrity. miR-148a and DNMT1 certainly constitute a regulatory circuit that is disrupted in HCC tissues. On the one hand, overexpression of DNMT1 leads to hypermethylation of the promoter region of miR-148a, causing its silencing. On the other hand, miR-148a alteration reduces its silencing action on DNMT1, resulting in augmentation of DNMT1 expression and maintaining hypermethylation of the miR-148a promoter.

Our data finally suggest that miR-148a restoration may provide a valuable strategy for therapeutic applications by inhibiting c-Met expression and repressing HCC cell invasion. Pertinent studies previously indicated that the use of miRNA precursors could contribute to the development of promising miRNA-based therapeutic methods. For instance, Kota et al. showed that systemic administration of miR-26a in rodents led to a remarkable slowdown of HCC progression without toxicity.[34] These observations suggest that the delivery of tumor-suppressor–type miRNAs, such as miR-148a and miR-122, which are highly expressed and therefore well tolerated in normal adult tissues, but lost in transformed cells, may provide a general strategy for miRNA replacement therapies. miR-148a also represents a valuable marker for the diagnosis and prognosis of HCC because its expression is frequently inhibited in liver cancer. Our observation that miR-148a alteration is not limited to the tumor site, but also affects the peritumoral nonneoplastic tissue, is noteworthy. This down-regulation is probably the consequence of the chronic inflammatory context inherent to hepatitis virus infection and liver fibrosis, which could represent an early event in CLDs, leading to augmentation of DNMT1 activity and aberrant DNA methylation. In this regard, Braconi et al. reported that the inflammation-associated cytokine, interleukin-6, regulates DNMT1 activity and methylation-dependent tumor-suppressor genes by modulating miR-148a/152 family expression in malignant cholangiocytes.[35] Furthermore, another study showed that miR-152 is frequently down-regulated in HBV-related HCC, inducing DNMT1 augmentation and aberrant DNA methylation.[36]

To conclude, our study demonstrates the existence of a dual role played by miR-148a in the liver. Importantly, we highlight a novel miRNA-mediated regulation mechanism in which miR-148a positively regulates hepatic differentiation by repressing DNMT1 expression. To our knowledge, this report is the first to demonstrate an effective promotion of the hepatospecific phenotype by modulating the expression of a single specific miRNA in a primary culture model using liver stem cells.

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CHAPTER 12

EXTRACELLULAR MicroRNAs AS POTENTIAL BIOMARKERS AND THERAPEUTIC TOOLS IN CANCER

Muriel Thirion[a] and Takahiro Ochiya[b,*]

ABSTRACT

Though circulating extracellular nucleic acids are not a recent discovery, the studies made over the past several years, especially on microRNAs (miRNAs), have demonstrated their importance in cancer. These nucleic acids, essential in cellular homeostasis and intercellular communication, have been detected in plasma, serum, urine, and other body fluids from healthy subjects as well as in diseased patients. These developments point to the role that miRNAs may play as novel diagnostic and prognostic markers. It has also opened the path to new therapeutic strategies such as immunotherapeutic nanovesicles and RNA interference (RNAi) delivery systems. In this review, we will summarize the present knowledges of the secretory mechanism, biological function and use of miRNAs as potential biomarkers and therapeutic tools in disease treatment.

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1. INTRODUCTION

Prior to the discovery of the double-helical structure of DNA by Watson and Crick, Mandel and Métais reported the presence of extracellular nucleic acids in the blood plasma (Mandel and Métais 1948). Due to lack of interest and proper molecular biological techniques to study circulating nucleic acids, this noteworthy work was unfortunately buried for almost 20 years until Tan and colleagues demonstrated the presence of circulating DNA in patients with systemic lupus erythematosus (Tan et al. 1966). The real breakthrough took place in 1994 when two groups detected the presence of mutated tumor-associated oncogenes in the plasma of patients with cancer (Sorenson et al. 1994, Vasioukhin et al. 1994). Since then, many studies demonstrated the presence of several extracellular nucleic acids (DNA, mRNA, miRNA) at high concentration in the blood of diseased patients (Shinozaki et al. 2007, Lawrie et al. 2008, Mitchell et al. 2008). These data were remarkable because they opened the path to new alternative approaches to the usual cancer screening tests that are invasive and inefficient in the early stages' detection.

Among the extracellular nucleic acids, miRNAs are of particular interest. Indeed, these small regulatory RNA molecules can modulate the expression of numerous specific mRNA targets and therefore play key roles in a variety of physiological and pathological processes (Bartel 2004). The expression pattern of miRNAs seems to be tissue-specific and altered expression has been reported in various cancers. In this review, we will thus focus on the potential usefulness of extracellular miRNAs as biomarkers and therapeutic tools in cancer.

2. ORIGIN OF EXTRACELLULAR miRNAs

In addition to being of small size, relatively abundant and tissue specific, circulating miRNAs are also very stable. However, RNases are present in large amounts in the blood. To assess the resistance mechanism of these extracellular miRNAs, Mitchell and colleagues designed synthetic miRNA with no homology to human sequences and added them to the plasma (Mitchell et al. 2008). These miRNAs were rapidly degraded unlike the endogenous plasma miRNAs, demonstrating that the latters should exit in a form resistant to RNase activity. At the same time, the circulating miRNAs were demonstrated to remain stable after being subjected to harsh conditions such as boiling, low/high pH or freeze-thaw cycles, conditions under which most RNA would be degraded (Chen et al. 2008). El-Hefnawy and coworkers showed that extracellular RNA was destroyed in presence of detergents, suggesting a protection by inclusion within lipid or lipoprotein vesicles or by attachment to proteins (El-Hefnawy et al. 2004).

2.1 Extracellular miRNAs Carried in Secreted Vesicles

Recent studies have revealed a novel genetic exchange between cells using miRNAs incorporated in and carried by extracellular vesicles (EV). These EV are called microvesicles, exosomes or others regarding to the size, density and secretion mechanisms. Their composition, origin and properties will be discussed later in this chapter. Presently the focus is on the actual knowledge about their origin (Fig. 1). The classical secretory pathway allows the cell to release soluble proteins and requires carrier and secretory vesicles that contain intraluminal components. By contrast, the secreted membranes vesicles contain cytoplasmic components. They can form at the plasma membrane by direct budding into the extracellular environment and produce large size (100–1000 nm), irregular shape microvesicles or smaller size round membrane particles. Alternatively, there is another type of particles called exosome-like vesicles. These are thought to be released from multivesicular bodies (MVB) but the mechanism is not clear yet. Finally, secreted vesicles can form inside internal compartments that subsequently fusion with the plasma membrane to release small size (30–100 nm) cup shape exosomes. A

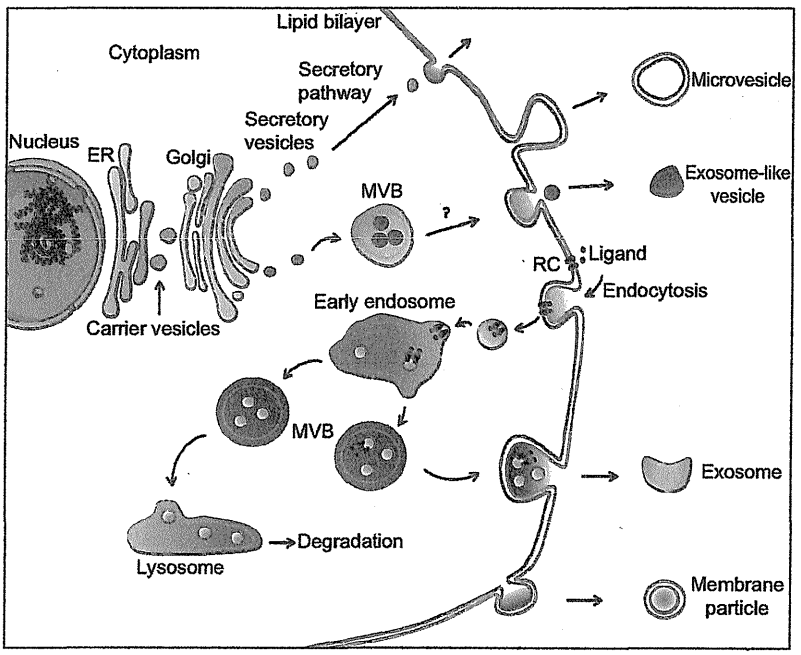

Figure 1. Biogenesis of various cellular secretory pathways. ER, endoplasmic reticulum; MVB, multivesicular bodies; Rc, receptor.

recent paper from our laboratory demonstrated that miRNAs are released through a ceramide-dependent secretory machinery and that the secretory miRNAs are transferable and functional in the recipient cells (Kosaka et al. 2010) (Fig. 2). In agreement with the finding by Kosaka and colleagues, some other reports showed the inhibition of neutral sphingomyelinase-2 (nSMase2), the enzyme that regulates the ceramide-dependent pathway, impairs the cellular export of miRNAs (Mittelbrunn et al. 2011, Kogure et al. 2011). Nevertheless, these data do not exclude other mechanisms for the secretion of miRNAs from the cells.

One of the first papers to report the existence of miRNA in small particles was from Valadi and coworkers (Valadi et al. 2007). The authors showed that vesicles released from human and murine mast cell lines contained mRNA and miRNA. Later, Hunter and colleagues demonstrated the presence of miRNAs in the blood's EV. These identified miRNAs had been previously shown to regulate the cell differentiation of blood cells, metabolic pathways and immune function (Hunter et al. 2008). To date, a large number of studies report extracellular particles as an important

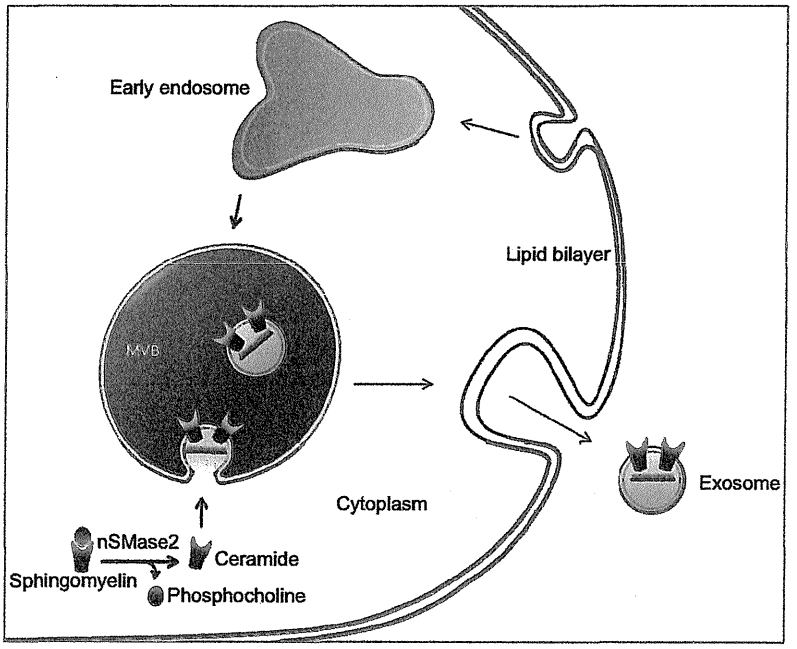

Figure 2. A model of secretory mechanism of microRNA involving exosomes. miRNA are brought to the multivesicular bodies (MVB), packaged into the exosomes and released from the cells, stimulated by the surge of cellular ceramide. nSMase2, neutral sphingomyelinase 2.

source of miRNA in the circulation and suggest a regulated miRNA-sorted mechanism. However, the exact mechanism of sorting and incorporation of miRNA to the vesicles and their secretion still needs to gain better understanding.

2.2 Extracellular miRNAs Associated with Protein Complexes

While the role of secreted vesicle as miRNA carrier is becoming increasingly recognized, some reports showed that an important part of the circulating miRNA is associated with RNA-binding proteins such as argonaute 2 protein (Ago2) and nucleophosmin 1 (NPM1).

Ago2 is the key effector protein of miRNA-mediated silencing. By using size-exclusion chromatography and immunoprecipitation techniques, Arroyo and coworkers demonstrated that circulating miRNA cofractionated with Ago2 complexes rather than with vesicles (Arroyo et al. 2011).

NPM1 is a nucleolar RNA-binding protein that is involved in ribosome biogenesis and transport. Wang and colleagues found that NPM1 was released outside the cell, independently from the vesicular fraction, and that this extracellular NPM1 bound miRNA and protected it from RNAse activity (Wang et al. 2010a).

It is still unclear whether these miRNA-protein complexes could target specifically and be functional in intercellular communication or rather be by-products of normal or dying cells. However, Wang and colleagues have shown that intracellular levels of ATP affected the exportation of miRNA, implying this process to be largely energy dependent (Wang et al. 2010a). This points to a possible role of the miRNA-protein complexes in cell–cell communication, as it has already been reported for miRNA-exosomes. This aspect is critical in determining the usefulness of circulating miRNAs as biomarkers and particularly in the context of therapeutic targets that will be discussed in the point 4 below.

2.3 Extracellular miRNAs Associated with Lipoprotein Complexes

Finally, extracellular miRNA was also found in association with lipoproteins. Vickers and coworkers demonstrated that high-density lipoprotein (HDL) could load plasma miRNA and deliver it to cells, leading to altered gene expression of these recipient cells (Vickers et al. 2011) (Fig. 3). To this end, this group first separated the exosome, low-density lipoprotein (LDL) and