apoptotic molecule involved in the TGF-beta1-induced apoptosis (Li et al., 2010a,b).

A number of studies have reported the dramatic role of aberrantly expressed miRNAs in HCC drug-resistance mechanisms. Importantly, Tonimaru and colleagues demonstrated that miR-21 over-expression increases the interferon (IFN)-alpha/5-fluorouracil (5-FU) drug resistance of HCC cells, whereas the use of miR-21 inhibitors renders the cells sensitive to the treatment (Tomimaru et al., 2010). Consequently, a moderated expression of miR-21 in HCC tissues was associated with a favorable response to the IFN-alpha/5-FU combination therapy and a better survival prognosis. Garofalo and colleagues further demonstrated that miR-221 and miR-222 are commonly over-expressed in HCC cells and, by targeting PTEN and TIMP3 tumor suppressors, induce TNF-related apoptosis-inducing ligand (TRAIL) resistance and enhance cellular migration through the activation the AKT pathway and metalloproteases (Garofalo et al., 2009). In the same study, the authors showed that the MET oncogene is implicated in miR-221/222 expression through its action on the c-Jun transcription factor.

HCC recurrence

Highly active drug-metabolizing pathways and multi-drug resistance transporter proteins are known to diminish the efficiency of current chemotherapeutic treatments. In addition, HCC recurrence after surgical resection of the primary tumor represents one of the characteristics leading to the low survival rate associated with liver cancer. Specific miRNA signatures have been linked to the increased risk of tumor recurrence and poor prognosis. The expression profiling of apoptosis-associated and metastasis-related miRNAs may provide clues for each patient to predict drug resistance and invasiveness of HCC that condition the recurrence of their disease. Fornari and colleagues demonstrated that miR-199a-3p repression observed in HCC leads to the over-expression of mTOR and MET, whereas the experimental restoration of miR-199a-3p reduces the growth and invasive properties of HCC cells and increases the apoptosis induced by doxorubicin (Fornari et al., 2010). Thus, an inverse correlation was revealed between miR-199a-3p and mTOR, as well as a shorter time to recurrence after tumor resection, in the patients with lower miR-199a-3p. Another study showed that low expression levels of miR-26 are well correlated with a better response to IFN-based treatment in patients with HCC but are associated with short survival (Ji et al., 2009).

The accurate assessment of cancer-related miRNA expression may predict the risk of relapse and represent an attractive prognostic tool. In particular, the high expression of miR-15b is associated with a low risk of tumor recurrence following surgical resection, as shown by Chung and colleagues who reported a negative correlation between miR-15b expression and the reappearance of HCC (Chung et al., 2010). Experimentally, targeting miR-15b with antagonists increased HCC cell proliferation and inhibited TRAIL-induced apoptosis *in vitro*, while the miR-15b precursor transfection decreased proliferation and enhanced apoptosis by repressing the anti-apoptotic Bcl-w. In addition to their prognostic significance, modulating the expression of specific drug resistance-related miRNAs may clearly represent a valuable method to improve apoptosis-sensitizing strategies for HCC treatment and avoid the recurrence of the tumor.

The "miRNA perspective" in liver cancer

The discovery of miRNAs has considerably modified and complexified conventional concepts regarding gene regulation. Concerning cancer biology, understanding the molecular mechanisms by which miRNAs promote carcinogenesis may lead to novel concepts in the diagnosis and treatment of a large number of malignancies. In addition to the deregulation of cancer-related miRNAs observed in HCC, an association has also been found between miRNA expression and the clinicopathological outcome of liver cancer (tumor growth, response to treatment, metastatic potential, and recurrence). Therefore, the use of a miRNA-based classification correlated with the etiology and the aggressiveness of the tumor could significantly enhance the molecular diagnosis accuracy of HCC and its classification, leading to the consideration of more appropriate therapeutic strategies. In this regard, several teams have reported particular miRNA expression profiles that could be considered as valuable HCC prognostic indicators (Villanueva et al., 2010). Budhu and collaborators defined a combination of 20 miRNAs as an HCC metastasis signature and showed that this 20 miRNA-based profile was capable of predicting the survival and recurrence of HCC in patients with multi-nodular or single tumors, including those at an early stage of the disease (Budhu et al., 2008). Remarkably, the highlighted expression profile showed a similar accuracy regarding patient prognosis when compared to the conventional clinical parameters, suggesting the clinical relevance of this miRNA signature. Consequently, the profiling of aberrantly expressed cancer-related miRNAs might establish the basis for the development of a rational system of classification in order to refine the diagnosis and the prediction of HCC evolution.

The potential implication of miRNAs as oncogenes or tumor suppressors supports the interest paid to cancer-related miRNAs in the past decade for the development of new curative approaches. MiRNAs represent relevant candidates as therapeutic targets, and several strategies have been reported to amend the altered expression of cancer-related miRNAs in the liver (Wang et al., 2012). First, miRNA replacement therapies use short RNA duplexes that mimic down-regulated miRNAs. On the other hand, miRNA inhibitors are chemically modified single-stranded oligonucleotides that antagonize the miRNAs over-expressed in cancer. In combination with

the latest developments, which render miRNA delivery safer and more efficient, the use of RNA interference (RNAi) therapeutic strategies will pave the way to innovative perspectives in the clinical management of HCC. Pertinent studies have already argued that miRNA-based therapy may represent an attractive approach to target hepatic primary tumors. For example, Kota and collaborators showed that a systemic administration of miR-26a in rodents led to a dramatic slow-down of HCC progression without notification of toxicity (Kota et al., 2009). Thus, the delivery of tumor suppressor miRNAs, which are typically highly expressed in the liver, but altered in HCC, may provide a valuable curative approach. However, miRNAs-based therapeutics are still in an early stage of development and more work will be required to identify relevant cancer-related miRNAs and understand the complex implication of these small non-coding RNAs in early or late HCC. In addition, as one miRNA can substantially affect the expression of several down-stream targets, precautions are necessary to avoid undesirable off-target effects. Finally, the safety of the reagents used to deliver miRNA mimics and antagomirs needs to be validated for future clinical applications.

Conclusion

Increasing evidence has highlighted the frequent alteration of miRNA expression in liver cancer, as well as the critical role of these small RNAs in tumorigenesis. Collectively, the investigative studies performed to date have resulted in a better understanding of cancer-related miRNA functions and their role as tumor suppressors and oncogenes. Given the implication of a large number of miRNAs in the control of key tumor suppressors and oncogenes, the deregulation of specific miRNAs has been shown to greatly influence HCC growth, invasiveness, treatment response, and liver tumor curability. From a diagnostic point of view, miRNA profiling (from hepatic tissues and sera) may be beneficial, as it offers additional information that could be used in combination with the conventional methods available for the clinical assessment of liver cancer. In addition, a better understanding of the processes leading to the deregulation of miRNA expression in HCC will yield further insight into the molecular mechanisms of tumorigenesis and provide a promising perspective regarding the development of new curative approaches.

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References

Aravalli R.N., Steer C.J. and Cressman E.N. (2008). Molecular mechanisms of hepatocellular carcinoma. Hepatology 48, 2047-2063.

Bai S., Nasser M.W., Wang B., Hsu S.H., Datta J., Kutay H., Yadav A., Nuovo G., Kumar P. and Ghoshal K. (2009). MicroRNA-122 inhibits tumorigenic properties of hepatocellular carcinoma cells and sensitizes these cells to sorafenib. J. Biol. Chem. 284, 32015-32027.

Banaudha K., Kaliszewski M., Korolnek T., Florea L., Yeung M.L., Jeang K.T. and Kumar A. (2011). MicroRNA silencing of tumor suppressor DLC-1 promotes efficient hepatitis C virus replication in primary human hepatocytes. Hepatology 53, 53-61.

Bartel D.P. (2004). MicroRNAs: genomics, biogenesis, mechanism, and function. Cell 116, 281-297.

Bartel D.P. (2009). MicroRNAs: target recognition and regulatory functions. Cell 136, 215-233.

Bhattacharya S.D., Garrison J., Guo H., Mi Z., Markovic J., Kim V.M. and Kuo P.C. (2010). Micro-RNA-181a regulates osteopontin-dependent metastatic function in hepatocellular cancer cell lines. Surgery 148, 291-297.

Borel F., Konstantinova P. and Jansen P.L. (2012). Diagnostic and therapeutic potential of miRNA signatures in patients with hepatocellular carcinoma. J. Hepatol. 56, 1371-1383.

Boutz D.R., Collins P.J., Suresh U., Lu M., Ramirez C.M., Fernandez-Hernando C., Huang Y., Abreu Rde S., Le S.Y., Shapiro B.A., Liu A.M., Luk J.M., Aldred S.F., Trinklein N.D., Marcotte E.M. and Penalva L.O. (2011). Two-tiered approach identifies a network of cancer and liver disease-related genes regulated by miR-122. J. Biol. Chem. 286, 18066-18078.

Braconi C., Huang N. and Patel T. (2010). MicroRNA-dependent regulation of DNA methyltransferase-1 and tumor suppressor gene expression by interleukin-6 in human malignant cholangiocytes. Hepatology 51, 881-890.

Budhu A., Jia H.L., Forgues M., Liu C.G., Goldstein D., Lam A., Zanetti K.A., Ye Q.H., Qin L.X., Croce C.M., Tang Z.Y. and Wang X.W. (2008). Identification of metastasis-related microRNAs in hepatocellular carcinoma. Hepatology 47, 897-907.

Buurman R., Gurlevik E., Schaffer V., Eilers M., Sandbothe M., Kreipe H., Wilkens L., Schlegelberger B., Kuhnel F. and Skawran B. (2012). Histone deacetylases activate hepatocyte growth factor signaling by fepressing microRNA-449 in hepatocellular carcinoma cells. Gastroenterology 143, 811-820.

Calin G.A. and Croce C.M. (2006). MicroRNA signatures in human cancers. Nat. Rev. Cancer. 6, 857-866.

Calin G.A. and Croce C.M. (2007). Chromosomal rearrangements and microRNAs: a new cancer link with clinical implications. J. Clin. Invest. 117, 2059-2066.

Calin G.A., Sevignani C., Dumitru C.D., Hyslop T., Noch E., Yendamuri S., Shimizu M., Rattan S., Bullrich F., Negrini M. and Croce C.M. (2004). Human microRNA genes are frequently located at fragile sites and genomic regions involved in cancers. Proc. Natl. Acad. Sci. USA 101, 2999-3004.

Chang T.C., Yu D., Lee Y.S., Wentzel E.A., Arking D.E., West K.M., Dang C.V., Thomas-Tikhonenko A. and Mendell J.T. (2008). Widespread microRNA repression by Myc contributes to tumorigenesis. Nat. Genet. 40, 43-50.

Chen X.M. (2009). MicroRNA signatures in liver diseases. World J. Gastroenterol. 15, 1665-1672.

Chen C., Ridzon D.A., Broomer A.J., Zhou Z., Lee D.H., Nguyen J.T., Barbisin M., Xu N.L., Mahuvakar V.R., Andersen M.R., Lao K.Q., Livak K.J. and Guegler K.J. (2005). Real-time quantification of microRNAs by stem-loop RT-PCR. Nucleic Acids Res. 33, e179.

Chen P.J., Yeh S.H., Liu W.H., Lin C.C., Huang H.C., Chen C.L., Chen D.S. and Chen P.J. (2012). Androgen pathway stimulates MicroRNA-216a transcription to suppress the tumor suppressor in lung cancer-1 gene in early hepatocarcinogenesis. Hepatology 56, 632-643.

Cheng J., Zhou L., Xie Q.F., Xie H.Y., Wei X.Y., Gao F., Xing C.Y., Xu X., Li L.J. and Zheng S.S. (2010). The impact of miR-34a on protein output in hepatocellular carcinoma HepG2 cells. Proteomics 10, 1557-1572.

Cheung O., Puri P., Eicken C., Contos M.J., Mirshahi F., Maher J.W., Kellum J.M., Min H., Luketic V.A. and Sanyal A.J. (2008). Nonalcoholic steatohepatitis is associated with altered hepatic MicroRNA expression. Hepatology 48, 1810-1820.

Chung G.E., Yoon J.H., Myung S.J., Lee J.H., Lee S.H., Lee S.M., Kim S.J., Hwang S.Y., Lee H.S. and Kim C.Y. (2010). High expression of microRNA-15b predicts a low risk of tumor recurrence following curative resection of hepatocellular carcinoma. Oncol. Rep. 23, 113-119.

Connolly E.C., Van Doorslaer K., Rogler L.E. and Rogler C.E. (2010). Overexpression of miR-21 promotes an in vitro metastatic phenotype by targeting the tumor suppressor RHOB. Mol. Cancer Res. 8, 691-700.

Datta J., Kutay H., Nasser M.W., Nuovo G.J., Wang B., Majumder S., Liu C.G., Volinia S., Croce C.M., Schmittgen T.D., Ghoshal K. and Jacob S.T. (2008). Methylation mediated silencing of MicroRNA-1 gene and its role in hepatocellular carcinogenesis. Cancer Res. 68, 5049-5058.

Ding J., Huang S., Wu S., Zhao Y., Liang L., Yan M., Ge C., Yao J., Chen T., Wan D., Wang H., Gu J., Yao M., Li J., Tu H. and He X. (2010). Gain of miR-151 on chromosome 8q24.3 facilitates tumour cell migration and spreading through downregulating RhoGDIA. Nat. Cell Biol. 12, 390-399.

El-Serag H.B. and Rudolph K.L. (2007). Hepatocellular carcinoma: epidemiology and molecular carcinogenesis. Gastroenterology 132, 2557-2576.

Elyakim E., Sitbon E., Faerman A., Tabak S., Montia E., Belanis L., Dov A., Marcusson E.G., Bennett C.F., Chajut A., Cohen D. and Yerushalmi N. (2010). hsa-miR-191 is a candidate oncogene target for hepatocellular carcinoma therapy. Cancer Res. 70, 8077-8087.

Esquela-Kerscher A. and Slack F.J. (2006). Oncomirs - microRNAs with a role in cancer. Nat. Rev. Cancer 6, 259-269.

Faggad A., Budczies J., Tchernitsa O., Darb-Esfahani S., Sehouli J., Muller B.M., Wirtz R., Chekerov R., Weichert W., Sinn B., Mucha C., Elwali N.E., Schafer R., Dietel M. and Denkert C. (2010). Prognostic significance of Dicer expression in ovarian cancer-link to global microRNA changes and oestrogen receptor expression. J. Pathol. 220, 382-391.

Fang Y., Xue J.L., Shen Q., Chen J. and Tian L. (2012). MicroRNA-7 inhibits tumor growth and metastasis by targeting the phosphoinositide 3-kinase/Akt pathway in hepatocellular carcinoma. Hepatology 55, 1852-1862.

Farazi P.A. and DePinho R.A. (2006). Hepatocellular carcinoma pathogenesis: from genes to environment. Nat. Rev. Cancer 6, 674-687.

Fornari F., Gramantieri L., Ferracin M., Veronese A., Sabbioni S., Calin G.A., Grazi G.L., Giovannini C., Croce C.M., Bolondi L. and Negrini M. (2008). MiR-221 controls CDKN1C/p57 and CDKN1B/p27 expression in human hepatocellular carcinoma. Oncogene 27, 5651-5661.

Fornari F., Gramantieri L., Giovannini C., Veronese A., Ferracin M., Sabbioni S., Calin G.A., Grazi G.L., Croce C.M., Tavolari S., Chieco P., Negrini M. and Bolondi L. (2009). MiR-122/cyclin G1 interaction modulates p53 activity and affects doxorubicin sensitivity of human hepatocarcinoma cells. Cancer Res. 69, 5761-5767.

Fornari F., Milazzo M., Chieco P., Negrini M., Calin G.A., Grazi G.L., Pollutri D., Croce C.M., Bolondi L. and Gramantieri L. (2010). MiR-199a-3p regulates mTOR and c-Met to influence the doxorubicin sensitivity of human hepatocarcinoma cells. Cancer Res. 70, 5184-5193.

Furuta M., Kozaki K.I., Tanaka S., Arii S., Imoto I. and Inazawa J. (2010). miR-124 and miR-203 are epigenetically silenced tumor-suppressive microRNAs in hepatocellular carcinoma. Carcinogenesis 31, 766-776.

Gailhouste L., Gómez-Santos L. and Ochiya T. (2013). Potential applications of miRNAs as diagnostic and prognostic markers in liver cancer. Front. Biosci. 18, 199-223.

Gao Y., Schug J., McKenna L.B., Le Lay J., Kaestner K.H. and Greenbaum L.E. (2011). Tissue-specific regulation of mouse microRNA genes in endoderm-derived tissues. Nucleic Acids Res. 39, 454-463.

Garofalo M., Di Leva G., Romano G., Nuovo G., Suh S.S., Ngankeu A., Taccioli C., Pichiorri F., Alder H., Secchiero P., Gasparini P., Gonelli A., Costinean S., Acunzo M., Condorelli G. and Croce C.M. (2009). miR-221&222 regulate TRAIL resistance and enhance tumorigenicity through PTEN and TIMP3 downregulation. Cancer Cell 16, 498-509.

Gatfield D., Le Martelot G., Vejnar C.E., Gerlach D., Schaad O., Fleury-Olela F., Ruskeepaa A.L., Oresic M., Esau C.C., Zdobnov E.M. and Schibler U. (2009). Integration of microRNA miR-122 in hepatic circadian gene expression. Genes Dev. 23, 1313-1326.

Gramantieri L., Ferracin M., Fornari F., Veronese A., Sabbioni S., Liu C.G., Calin G.A., Giovannini C., Ferrazzi E., Grazi G.L., Croce C.M., Bolondi L. and Negrini M. (2007). Cyclin G1 is a target of miR-122a, a microRNA frequently down-regulated in human hepatocellular carcinoma. Cancer Res. 67, 6092-6099.

Gramantieri L., Fornari F., Callegari E., Sabbioni S., Lanza G., Croce C.M., Bolondi L. and Negrini M. (2008). MicroRNA involvement in hepatocellular carcinoma. J. Cell. Mol. Med. 12, 2189-2204.

Gramantieri L., Fornari F., Ferracin M., Veronese A., Sabbioni S., Calin G.A., Grazi G.L., Croce C.M., Bolondi L. and Negrini M. (2009). MicroRNA-221 targets Bmf in hepatocellular carcinoma and correlates with tumor multifocality. Clin. Cancer Res. 15, 5073-5081.

Griffiths-Jones S. (2004). The microRNA Registry. Nucleic Acids Res. 32, D109-111.

Hanoun N., Delpu Y., Suriawinata A.A., Bournet B., Bureau C., Selves J., Tsongalis G.J., Dufresne M., Buscail L., Cordelier P. and Torrisani J. (2010). The silencing of microRNA 148a production by DNA hypermethylation is an early event in pancreatic carcinogenesis. Clin. Chem. 56, 1107-1118.

He X.X., Chang Y., Meng F.Y., Wang M.Y., Xie Q.H., Tang F., Li P.Y., Song Y.H. and Lin J.S. (2012). MicroRNA-375 targets AEG-1 in hepatocellular carcinoma and suppresses liver cancer cell growth in vitro and in vivo. Oncogene 31, 3357-3369.

Hou Y.Y., Cao W.W., Li L., Li S.P., Liu T., Wan H.Y., Liu M., Li X. and

Tang H. (2011). MicroRNA-519d targets MKi67 and suppresses cell growth in the hepatocellular carcinoma cell line QGY-7703. Cancer Lett. 307, 182-190.

Hsu S.H., Wang B., Kota J., Yu J., Costinean S., Kutay H., Yu L., Bai S., La Perle K., Chivukula R.R., Mao H., Wei M., Clark K.R., Mendell J.R., Caligiuri M.A., Jacob S.T., Mendell J.T. and Ghoshal K. (2012). Essential metabolic, anti-inflammatory, and anti-tumorigenic functions of miR-122 in liver. J. Clin. Invest. 122, 2871-2883.

Huang N., Lin J., Ruan J., Su N., Qing R., Liu F., He B., Lv C., Zheng D. and Luo R. (2012). MiR-219-5p inhibits hepatocellular carcinoma cell proliferation by targeting glypican-3. FEBS Lett. 586, 884-891.

Imam J.S., Buddavarapu K., Lee-Chang J.S., Ganapathy S., Camosy C., Chen Y. and Rao M.K. (2010). MicroRNA-185 suppresses tumor growth and progression by targeting the Six1 oncogene in human cancers. Oncogene 29, 4971-4979.

Ivanovska I., Ball A.S., Diaz R.L., Magnus J.F., Kibukawa M., Schelter J.M., Kobayashi S.V., Lim L., Burchard J., Jackson A.L., Linsley P.S. and Cleary M.A. (2008). MicroRNAs in the miR-106b family regulate p21/CDKN1A and promote cell cycle progression. Mol. Cell. Biol. 28, 2167-2174.

Ji J. and Wang X.W. (2009). New kids on the block: diagnostic and prognostic microRNAs in hepatocellular carcinoma. Cancer Biol. Ther. 8, 1686-1693.

Ji J., Shi J., Budhu A., Yu Z., Forgues M., Roessler S., Ambs S., Chen Y., Meltzer P.S., Croce C.M., Qin L.X., Man K., Lo C.M., Lee J., Ng I.O., Fan J., Tang Z.Y., Sun H.C. and Wang X.W. (2009). MicroRNA expression, survival, and response to interferon in liver cancer. N. Engl. J. Med. 361, 1437-1447.

Ji J., Zhao L., Budhu A., Forgues M., Jia H.L., Qin L.X., Ye Q.H., Yu J., Shi X., Tang Z.Y. and Wang X.W. (2010). Let-7g targets collagen type I alpha2 and inhibits cell migration in hepatocellular carcinoma. J. Hepatol. 52, 690-697.

Jopling C.L., Yi M., Lancaster A.M., Lemon S.M. and Sarnow P. (2005). Modulation of hepatitis C virus RNA abundance by a liver-specific MicroRNA. Science 309, 1577-1581.

Kent O.A. and Mendell J.T. (2006). A small piece in the cancer puzzle: microRNAs as tumor suppressors and oncogenes. Oncogene 25, 6188-6196.

Kondo Y., Kanai Y., Sakamoto M., Mizokami M., Ueda R. and Hirohashi S. (2000). Genetic instability and aberrant DNA methylation in chronic hepatitis and cirrhosis--A comprehensive study of loss of heterozygosity and microsatellite instability at 39 loci and DNA hypermethylation on 8 CpG islands in microdissected specimens from patients with hepatocellular carcinoma. Hepatology 32, 970-979.

Kosaka N., Iguchi H. and Ochiya T. (2010). Circulating microRNA in body fluid: a new potential biomarker for cancer diagnosis and prognosis. Cancer Sci. 101, 2087-2092.

Kosaka N., Iguchi H., Yoshioka Y., Hagiwara K., Takeshita F. and Ochiya T. (2012). Competitive interactions of cancer cells and normal cells via secretory microRNAs. J. Biol. Chem. 287, 1397-1405.

Kota J., Chivukula R.R., O'Donnell K.A., Wentzel E.A., Montgomery C.L., Hwang H.W., Chang T.C., Vivekanandan P., Torbenson M., Clark K.R., Mendell J.R. and Mendell J.T. (2009). Therapeutic microRNA delivery suppresses tumorigenesis in a murine liver cancer model. Cell 137, 1005-1017.

Krutzfeldt J., Rajewsky N., Braich R., Rajeev K.G., Tuschl T., Manoharan M. and Stoffel M. (2005). Silencing of microRNAs in vivo with 'antagomirs'. Nature 438, 685-689.

Ladeiro Y., Couchy G., Balabaud C., Bioulac-Sage P., Pelletier L., Rebouissou S. and Zucman-Rossi J. (2008). MicroRNA profiling in hepatocellular tumors is associated with clinical features and oncogene/tumor suppressor gene mutations. Hepatology 47, 1955-1963.

Lagos-Quintana M., Rauhut R., Yalcin A., Meyer J., Lendeckel W. and Tuschl T. (2002). Identification of tissue-specific microRNAs from mouse. Curr. Biol. 12, 735-739.

Lan F.F., Wang H., Chen Y.C., Chan C.Y., Ng S.S., Li K., Xie D., He M.L., Lin M.C. and Kung H.F. (2011). Hsa-let-7g inhibits proliferation of hepatocellular carcinoma cells by downregulation of c-Myc and upregulation of p16(INK4A). Int. J. Cancer 128, 319-331.

Le Sage C., Nagel R., Egan D.A., Schrier M., Mesman E., Mangiola A., Anile C., Maira G., Mercatelli N., Ciafre S.A., Farace M.G. and Agami R. (2007). Regulation of the p27(Kip1) tumor suppressor by miR-221 and miR-222 promotes cancer cell proliferation. Embo J. 26, 3699-3708.

Lee R.C., Feinbaum R.L. and Ambros V. (1993). The C. elegans heterochronic gene lin-4 encodes small RNAs with antisense complementarity to lin-14. Cell 75, 843-854.

Lewis B.P., Burge C.B. and Bartel D.P. (2005). Conserved seed pairing, often flanked by adenosines, indicates that thousands of human genes are microRNA targets. Cell 120, 15-20.

Li N., Fu H., Tie Y., Hu Z., Kong W., Wu Y. and Zheng X. (2009a). miR-34a inhibits migration and invasion by down-regulation of c-Met expression in human hepatocellular carcinoma cells. Cancer Lett. 275, 44-53.

Li Y., Tan W., Neo T.W., Aung M.O., Wasser S., Lim S.G. and Tan T.M. (2009b). Role of the miR-106b-25 microRNA cluster in hepatocellular carcinoma. Cancer Sci. 100, 1234-1242.

Li J., Fu H., Xu C., Tie Y., Xing R., Zhu J., Qin Y., Sun Z. and Zheng X. (2010a). miR-183 inhibits TGF-beta1-induced apoptosis by downregulation of PDCD4 expression in human hepatocellular carcinoma cells. BMC Cancer 10, 354.

Li L.M., Hu Z.B., Zhou Z.X., Chen X., Liu F.Y., Zhang J.F., Shen H.B., Zhang C.Y. and Zen K. (2010b). Serum microRNA profiles serve as novel biomarkers for HBV infection and diagnosis of HBV-positive hepatocarcinoma. Cancer Res. 70, 9798-9807.

Li D., Liu X., Lin L., Hou J., Li N., Wang C., Wang P., Zhang Q., Zhang P., Zhou W., Wang Z., Ding G., Zhuang S.M., Zheng L., Tao W. and Cao X. (2011a). MicroRNA-99a inhibits hepatocellular carcinoma growth and correlates with prognosis of patients with hepatocellular carcinoma. J. Biol. Chem. 286, 36677-36685.

Li J., Wang Y., Yu W., Chen J. and Luo J. (2011b). Expression of serum miR-221 in human hepatocellular carcinoma and its prognostic significance. Biochem. Biophys. Res. Commun. 406, 70-73.

Liang L., Wong C.M., Ying Q., Fan D.N., Huang S., Ding J., Yao J., Yan M., Li J., Yao M., Ng I.O. and He X. (2010). MicroRNA-125b suppressesed human liver cancer cell proliferation and metastasis by directly targeting oncogene LIN28B2. Hepatology 52, 1731-1740.

Lin C.J., Gong H.Y., Tseng H.C., Wang W.L. and Wu J.L. (2008). miR-122 targets an anti-apoptotic gene, Bcl-w, in human hepatocellular carcinoma cell lines. Biochem. Biophys. Res. Commun. 375, 315-320.

Lin J., Huang S., Wu S., Ding J., Zhao Y., Liang L., Tian Q., Zha R., Zhan R. and He X. (2011). MicroRNA-423 promotes cell growth and regulates G(1)/S transition by targeting p21Cip1/Waf1 in hepatocellular carcinoma. Carcinogenesis 32, 1641-1647.

Liu A.M., Poon R.T. and Luk J.M. (2010). MicroRNA-375 targets Hippo-signaling effector YAP in liver cancer and inhibits tumor properties. Biochem. Biophys. Res. Commun. 394, 623-627.

Liu J., Valencia-Sanchez M.A., Hannon G.J. and Parker R. (2005). MicroRNA-dependent localization of targeted mRNAs to mammalian P-bodies. Nat. Cell Biol. 7, 719-723.

Liu W.H., Yeh S.H., Lu C.C., Yu S.L., Chen H.Y., Lin C.Y., Chen D.S. and Chen P.J. (2009). MicroRNA-18a prevents estrogen receptor-alpha expression, promoting proliferation of hepatocellular carcinoma cells. Gastroenterology 136, 683-693.

Lu J., Getz G., Miska E.A., Alvarez-Saavedra E., Lamb J., Peck D., Sweet-Cordero A., Ebert B.L., Mak R.H., Ferrando A.A., Downing J.R., Jacks T., Horvitz H.R. and Golub T.R. (2005). MicroRNA expression profiles classify human cancers. Nature 435, 834-838.

Lujambio A. and Esteller M. (2007). CpG island hypermethylation of tumor suppressor microRNAs in human cancer. Cell Cycle 6, 1455-1459.

Lujambio A. and Lowe S.W. (2012). The microcosmos of cancer. Nature 482, 347-355.

Ma S., Tang K.H., Chan Y.P., Lee T.K., Kwan P.S., Castilho A., Ng I., Man K., Wong N., To K.F., Zheng B.J., Lai P.B., Lo C.M., Chan K.W. and Guan X.Y. (2010). miR-130b Promotes CD133(+) liver tumor-initiating cell growth and self-renewal via tumor protein 53-induced nuclear protein 1. Cell Stem Cell 7, 694-707.

Mayr C., Hemann M.T. and Bartel D.P. (2007). Disrupting the pairing between let-7 and Hmga2 enhances oncogenic transformation. Science 315, 1576-1579.

Melo S.A., Ropero S., Moutinho C., Aaltonen L.A., Yamamoto H., Calin G.A., Rossi S., Fernandez A.F., Carneiro F., Oliveira C., Ferreira B., Liu C.G., Villanueva A., Capella G., Schwartz S. Jr, Shiekhattar R. and Esteller M. (2009). A TARBP2 mutation in human cancer impairs microRNA processing and DICER1 function. Nat. Genet. 41, 365-370.

Meng F., Henson R., Wehbe-Janek H., Ghoshal K., Jacob S.T. and Patel T. (2007). MicroRNA-21 regulates expression of the PTEN tumor suppressor gene in human hepatocellular cancer. Gastroenterology 133, 647-658.

Meng X.Z., Zheng T.S., Chen X., Wang J.B., Zhang W.H., Pan S.H., Jiang H.C. and Liu L.X. (2011). microRNA expression alteration after arsenic trioxide treatment in HepG-2 cells. J. Gastroenterol. Hepatol. 26, 186-193.

Mott J.L. (2009). MicroRNAs involved in tumor suppressor and oncogene pathways: implications for hepatobiliary neoplasia. Hepatology 50, 630-637.

Murakami Y., Yasuda T., Saigo K., Urashima T., Toyoda H., Okanoue T. and Shimotohno K. (2006). Comprehensive analysis of microRNA expression patterns in hepatocellular carcinoma and non-tumorous tissues. Oncogene 25, 2537-2545.

Pineau P., Volinia S., McJunkin K., Marchio A., Battiston C., Terris B., Mazzaferro V., Lowe S.W., Croce C.M. and Dejean A. (2010). miR-221 overexpression contributes to liver tumorigenesis. Proc. Natl. Acad. Sci. USA 107, 264-269.

Salvi A., Sabelli C., Moncini S., Venturin M., Arici B., Riva P., Portolani N., Giulini S.M., De Petro G. and Barlati S. (2009). MicroRNA-23b mediates urokinase and c-met downmodulation and a decreased migration of human hepatocellular carcinoma cells. FEBS J. 276, 2966-2982.

Sato F., Tsuchiya S., Meltzer S.J. and Shimizu K. (2011). MicroRNAs and epigenetics. FEBS J. 278, 1598-1609.

Sekine S., Ogawa R., Ito R., Hiraoka N., McManus M.T., Kanai Y. and Hebrok M. (2009). Disruption of Dicer1 induces dysregulated fetal gene expression and promotes hepatocarcinogenesis. Gastroenterology 136, 2304-2315 e2301-2304.

Shen Q., Cicinnati V.R., Zhang X., Iacob S., Weber F., Sotiropoulos G.C., Radtke A., Lu M., Paul A., Gerken G. and Beckebaum S. (2010). Role of microRNA-199a-5p and discoidin domain receptor 1 in human hepatocellular carcinoma invasion. Mol. Cancer 9, 227.

Shigoka M., Tsuchida A., Matsudo T., Nagakawa Y., Saito H., Suzuki Y., Aoki T., Murakami Y., Toyoda H., Kumada T., Bartenschlager R., Kato N., Ikeda M., Takashina T., Tanaka M., Suzuki R., Oikawa K., Takanashi M. and Kuroda M. (2010). Deregulation of miR-92a expression is implicated in hepatocellular carcinoma development. Pathol. Int. 60, 351-357.

Shimizu S., Takehara T., Hikita H., Kodama T., Miyagi T., Hosui A., Tatsumi T., Ishida H., Noda T., Nagano H., Doki Y., Mori M. and Hayashi N. (2010). The let-7 family of microRNAs inhibits Bcl-xL expression and potentiates sorafenib-induced apoptosis in human hepatocellular carcinoma. J. Hepatol. 52, 698-704.

Su H., Yang J.R., Xu T., Huang J., Xu L., Yuan Y. and Zhuang S.M. (2009). MicroRNA-101, down-regulated in hepatocellular carcinoma, promotes apoptosis and suppresses tumorigenicity. Cancer Res. 69, 1135-1142.

Toffanin S., Hoshida Y., Lachenmayer A., Villanueva A., Cabellos L., Minguez B., Savic R., Ward S.C., Thung S., Chiang D.Y., Alsinet C., Tovar V., Roayaie S., Schwartz M., Bruix J., Waxman S., Friedman S.L., Golub T., Mazzaferro V. and Llovet J.M. (2011). MicroRNA-Based Classification of Hepatocellular Carcinoma and Oncogenic Role of miR-517a. Gastroenterology 140, 1618-1628 e1616.

Tomimaru Y., Eguchi H., Nagano H., Wada H., Tomokuni A., Kobayashi S., Marubashi S., Takeda Y., Tanemura M., Umeshita K., Doki Y. and Mori M. (2010). MicroRNA-21 induces resistance to the anti-tumour effect of interferon-alpha/5-fluorouracil in hepatocellular carcinoma cells. Br. J. Cancer 103, 1617-1626.

Tomimaru Y., Eguchi H., Nagano H., Wada H., Kobayashi S., Marubashi S., Tanemura M., Tomokuni A., Takemasa I., Umeshita K., Kanto T., Doki Y. and Mori M. (2012). Circulating microRNA-21 as a novel biomarker for hepatocellular carcinoma. J. Hepatol. 56, 167-175.

Tsai W.C., Hsu P.W., Lai T.C., Chau G.Y., Lin C.W., Chen C.M., Lin C.D., Liao Y.L., Wang J.L., Chau Y.P., Hsu M.T., Hsiao M., Huang H.D. and Tsou A.P. (2009). MicroRNA-122, a tumor suppressor microRNA that regulates intrahepatic metastasis of hepatocellular carcinoma. Hepatology 49, 1571-1582.

Villanueva A., Hoshida Y., Toffanin S., Lachenmayer A., Alsinet C., Savic R., Cornella H. and Llovet J.M. (2010). New strategies in hepatocellular carcinoma: genomic prognostic markers. Clin. Cancer Res. 16, 4688-4694.

Volinia S., Calin G.A., Liu C.G., Ambs S., Cimmino A., Petrocca F., Visone R., Iorio M., Roldo C., Ferracin M., Prueitt R.L., Yanaihara N., Lanza G., Scarpa A., Vecchione A., Negrini M., Harris C.C. and Croce C.M. (2006). A microRNA expression signature of human solid tumors defines cancer gene targets. Proc. Natl. Acad. Sci. USA 103, 2257-2261.

Wang Y., Lee A.T., Ma J.Z., Wang J., Ren J., Yang Y., Tantoso E., Li K.B., Ooi L.L., Tan P. and Lee C.G. (2008). Profiling microRNA expression in hepatocellular carcinoma reveals microRNA-224 up-regulation and apoptosis inhibitor-5 as a microRNA-224-specific target. J. Biol. Chem. 283, 13205-13215.

Wang B., Hsu S.H., Majumder S., Kutay H., Huang W., Jacob S.T. and Ghoshal K. (2010). TGFbeta-mediated upregulation of hepatic miR-181b promotes hepatocarcinogenesis by targeting TIMP3. Oncogene 29, 1787-1797.

Wang X.W., Heegaard N.H. and Orum H. (2012). MicroRNAs in Liver Disease. Gastroenterology 142, 1431-1443.

Wightman B., Ha I. and Ruvkun G. (1993). Posttranscriptional regulation of the heterochronic gene lin-14 by lin-4 mediates temporal pattern formation in C. elegans. Cell 75, 855-862.

Wong Q.W., Lung R.W., Law P.T., Lai P.B., Chan K.Y., To K.F. and Wong N. (2008). MicroRNA-223 is commonly repressed in hepatocellular carcinoma and potentiates expression of Stathmin1. Gastroenterology 135, 257-269.

Wong Q.W., Ching A.K., Chan A.W., Choy K.W., To K.F., Lai P.B. and Wong N. (2010). MiR-222 overexpression confers cell migratory advantages in hepatocellular carcinoma through enhancing AKT signaling. Clin. Cancer Res. 16, 867-875.

Wong C.C., Wong C.M., Tung E.K., Au S.L., Lee J.M., Poon R.T., Man K. and Ng I.O. (2011). The microRNA miR-139 suppresses metastasis and progression of hepatocellular carcinoma by down-regulating Rho-kinase 2. Gastroenterology 140, 322-331.

Wu J.F., Shen W., Liu N.Z., Zeng G.L., Yang M., Zuo G.Q., Gan X.N., Ren H. and Tang K.F. (2011a). Down-regulation of Dicer in hepatocellular carcinoma. Med. Oncol. 28, 804-809.

Wu L., Cai C., Wang X., Liu M., Li X. and Tang H. (2011b). MicroRNA-142-3p, a new regulator of RAC1, suppresses the migration and invasion of hepatocellular carcinoma cells. FEBS Lett. 585, 1322-1330.

Wu N., Liu X., Xu X., Fan X., Liu M., Li X., Zhong Q. and Tang H. (2011c). MicroRNA-373, a new regulator of protein phosphatase 6, functions as an oncogene in hepatocellular carcinoma. FEBS J. 278, 2044-2054.

Xie Q., Chen X., Lu F., Zhang T., Hao M., Wang Y., Zhao J., McCrae M.A. and Zhuang H. (2012). Aberrant expression of microRNA 155 may accelerate cell proliferation by targeting sex-determining region Y box 6 in hepatocellular carcinoma. Cancer 118, 2431-2442.

Xiong Y., Fang J.H., Yun J.P., Yang J., Zhang Y., Jia W.H. and Zhuang S.M. (2010). Effects of microRNA-29 on apoptosis, tumorigenicity, and prognosis of hepatocellular carcinoma. Hepatology 51, 836-845.

Xu T., Zhu Y., Xiong Y., Ge Y.Y., Yun J.P. and Zhuang S.M. (2009). MicroRNA-195 suppresses tumorigenicity and regulates G1/S transition of human hepatocellular carcinoma cells. Hepatology 50, 113-121.

Xu C., Liu S., Fu H., Li S., Tie Y., Zhu J., Xing R., Jin Y., Sun Z. and Zheng X. (2010a). MicroRNA-193b regulates proliferation, migration and invasion in human hepatocellular carcinoma cells. Eur. J. Cancer 46, 2828-2836.

Xu H., He J.H., Xiao Z.D., Zhang Q.Q., Chen Y.Q., Zhou H. and Qu L.H. (2010b). Liver-enriched transcription factors regulate microRNA-122 that targets CUTL1 during liver development. Hepatology 52, 1431-1442.

Xu J., Wu C., Che X., Wang L., Yu D., Zhang T., Huang L., Li H., Tan W., Wang C. and Lin D. (2011). Circulating microRNAs, miR-21, miR-122, and miR-223, in patients with hepatocellular carcinoma or chronic hepatitis. Mol. Carcinog. 50, 136-142.

Yamamoto Y., Kosaka N., Tanaka M., Koizumi F., Kanai Y., Mizutani T., Murakami Y., Kuroda M., Miyajima A., Kato T. and Ochiya T. (2009). MicroRNA-500 as a potential diagnostic marker for hepatocellular carcinoma. Biomarkers 14, 529-538.

Yang F., Yin Y., Wang F., Wang Y., Zhang L., Tang Y. and Sun S. (2010a). miR-17-5p Promotes migration of human hepatocellular carcinoma cells through the p38 mitogen-activated protein kinase-heat shock protein 27 pathway. Hepatology 51, 1614-1623.

Yang L., Ma Z., Wang D., Zhao W., Chen L. and Wang G. (2010b). MicroRNA-602 regulating tumor suppressive gene RASSF1A is overexpressed in hepatitis B virus-infected liver and hepatocellular carcinoma. Cancer Biol. Ther. 9, 803-808.

Yao J., Liang L., Huang S., Ding J., Tan N., Zhao Y., Yan M., Ge C., Zhang Z., Chen T., Wan D., Yao M., Li J., Gu J. and He X. (2010). MicroRNA-30d promotes tumor invasion and metastasis by targeting Galphai2 in hepatocellular carcinoma. Hepatology 51, 846-856.

Ying Q., Liang L., Guo W., Zha R., Tian Q., Huang S., Yao J., Ding J., Bao M., Ge C., Yao M., Li J. and He X. (2011). Hypoxia-inducible microRNA-210 augments the metastatic potential of tumor cells by targeting vacuole membrane protein 1 in hepatocellular carcinoma. Hepatology 54, 2064-2075.

Zeng C., Wang R., Li D., Lin X.J., Wei Q.K., Yuan Y., Wang Q., Chen W. and Zhuang S.M. (2010). A novel GSK-3 beta-C/EBP alpha-miR-122-insulin-like growth factor 1 receptor regulatory circuitry in human hepatocellular carcinoma. Hepatology 52, 1702-1712.

Zhang X., Liu S., Hu T., Liu S., He Y. and Sun S. (2009). Up-regulated microRNA-143 transcribed by nuclear factor kappa B enhances hepatocarcinoma metastasis by repressing fibronectin expression. Hepatology 50, 490-499.

Zhang J., Yang Y., Yang T., Liu Y., Li A., Fu S., Wu M., Pan Z. and Zhou W. (2010). microRNA-22, downregulated in hepatocellular carcinoma and correlated with prognosis, suppresses cell proliferation and tumourigenicity. Br. J. Cancer 103, 1215-1220.

Zhang J.F., He M.L., Fu W.M., Wang H., Chen L.Z., Zhu X., Chen Y., Xie D., Lai P., Chen G., Lu G., Lin M.C. and Kung H.F. (2011). Primate-specific microRNA-637 inhibits tumorigenesis in hepatocellular carcinoma by disrupting signal transducer and activator of transcription 3 signaling. Hepatology 54, 2137-2148.

Zheng F., Liao Y.J., Cai M.Y., Liu Y.H., Liu T.H., Chen S.P., Bian X.W., Guan X.Y., Lin M.C., Zeng Y.X., Kung H.F. and Xie D. (2012). The putative tumour suppressor microRNA-124 modulates hepatocellular carcinoma cell aggressiveness by repressing ROCK2 and EZH2. Gut 61, 278-289.

Zhu A., Xia J., Zuo J., Jin S., Zhou H., Yao L., Huang H. and Han Z. (2011). MicroRNA-148a is silenced by hypermethylation and interacts with DNA methyltransferase 1 in gastric cancer. Med. Oncol. 29, 2701-2709.

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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

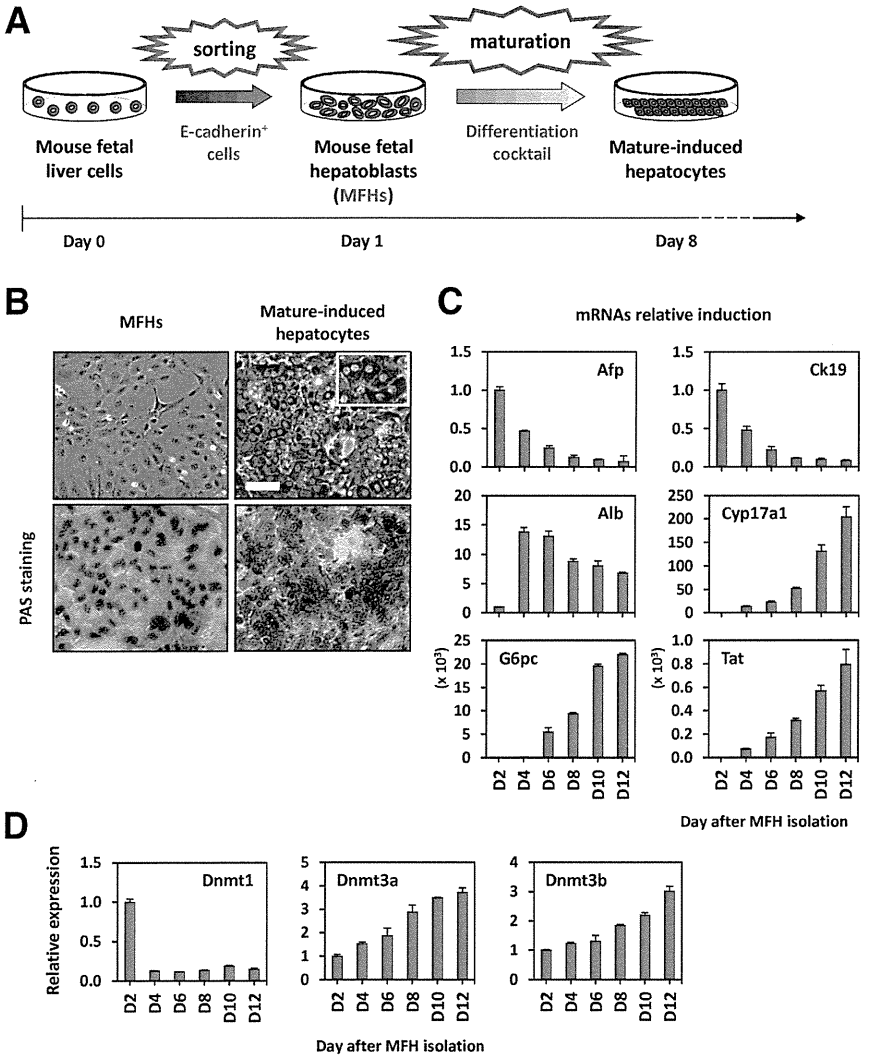

Fig. 1. Characterization of the MFH model. (A) Schematic representation of MFH purification and induced differentiation into mature hepatocytes after hepatotrophic factor stimulation. (B) Primary cultures of MFHs showing the radical changes undergone by undifferentiated hepatic progenitors to adopt the 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-

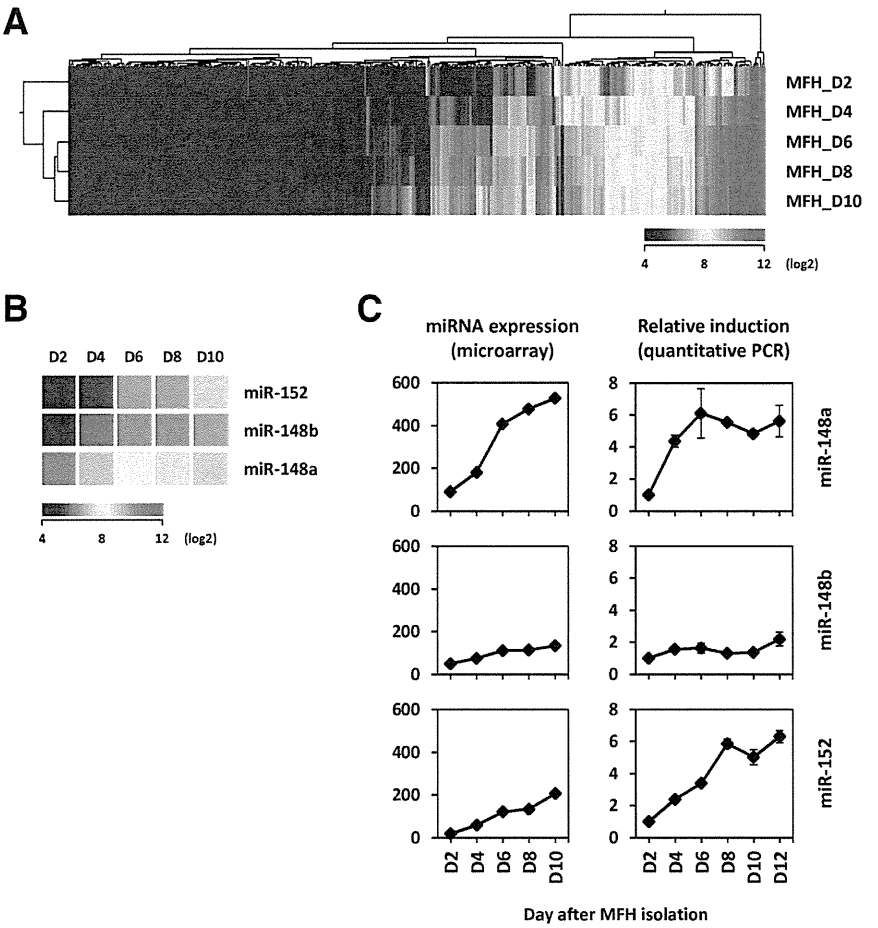

Fig. 2. Identification of miR-148a as a preponderant miRNA during hepatic differentiation. (A) miRNA global expression pattern during the process of MFH differentiation into mature hepatocytes. The scale bar encodes the logarithm of relative miRNA expression level. The 2-fold threshold was set to identify 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/MFDH2)
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

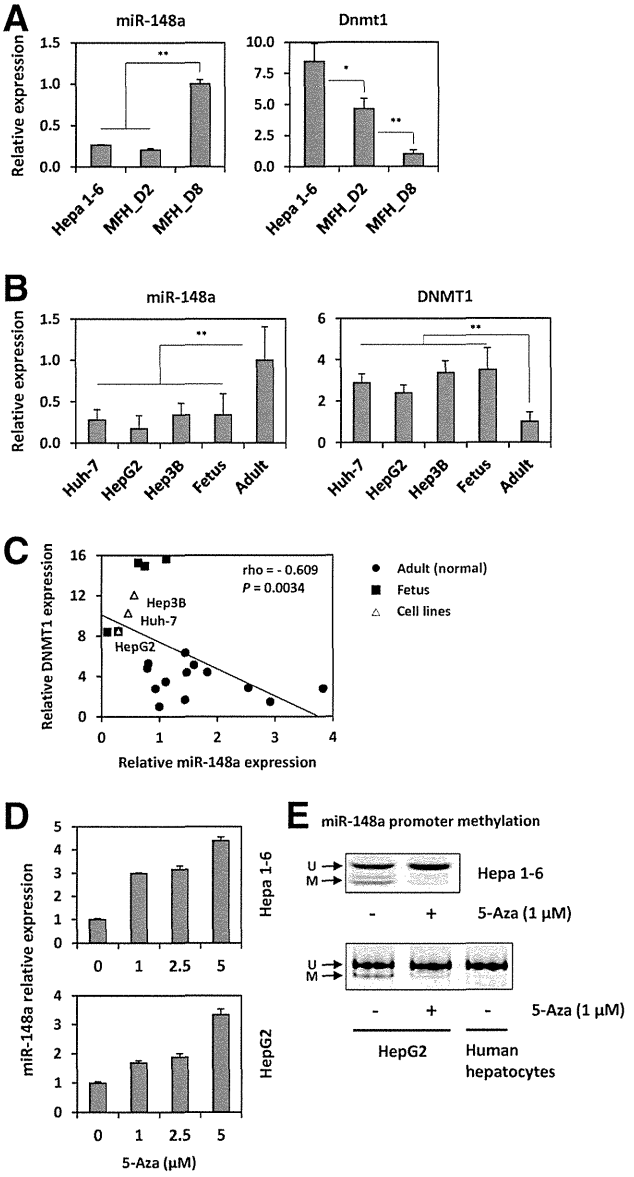

Fig. 3. miR-148a and Dnmt1 assessment in the liver. (A) The mouse hepatoma cell line, Hepa 1-6, was used to assess miR-148a and Dnmt1 mRNA levels, compared to undifferentiated hepatic stem cells (MFH D2) and mature-induced hepatocytes (MFH D8). (B) miR-148a and human DNMT1 expression were analyzed in 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.

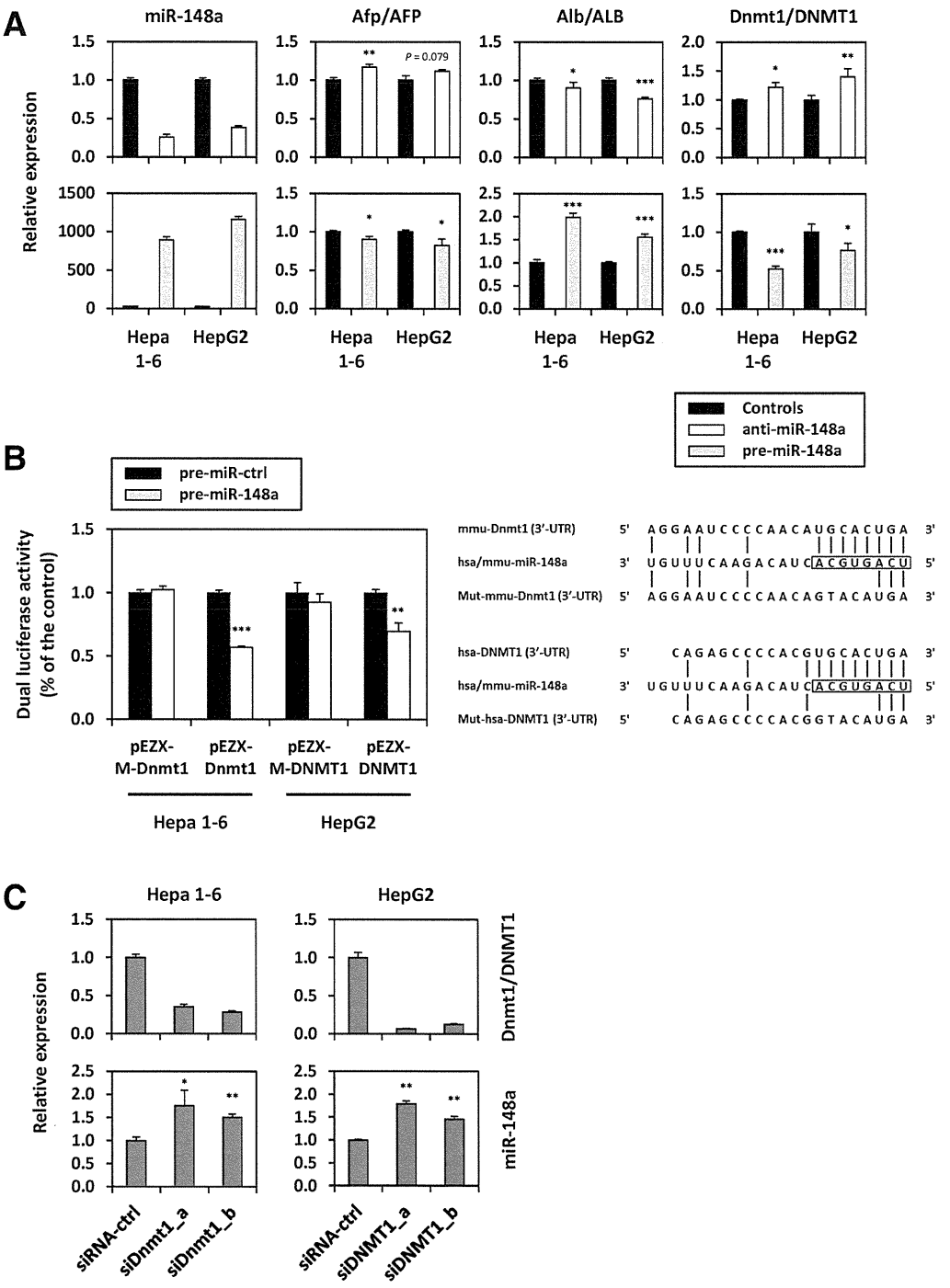

Fig. 4. Characterization of the relationship between miR-148a and DNMT1 in rodent and human models. (A) Relative expression of Dnmt1 and liver markers Afp and Alb, respectively, after experimental modulation of miR-148a in mouse Hepa 1-6 and human HepG2 cell lines. Cells were transfected using 100 ng 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

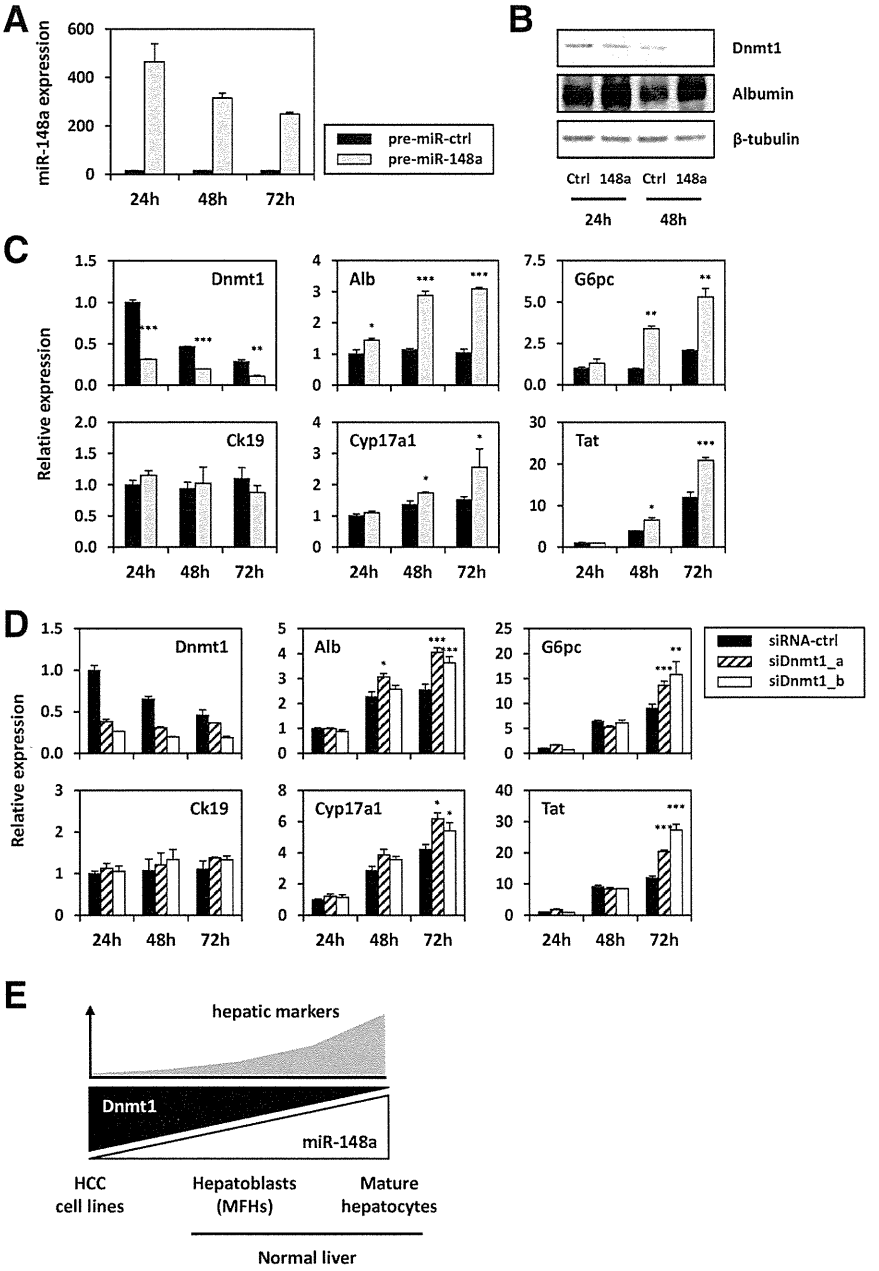

Fig. 5. Forcing expression of miR-148a promotes expression of hepatospecific genes during MFH-induced differentiation. MFH primary cultures were transfected using miR-148a mimics (100 nM) on the fourth day after cell isolation (MFH D4). Control miRNA mimics were used as negative controls. Total RNAs 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

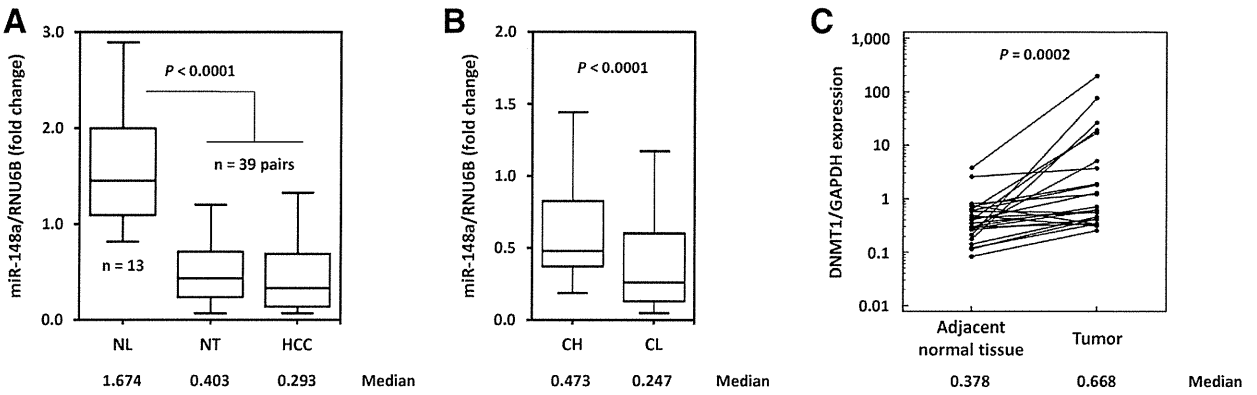

Fig. 6. Expression of miR-148a in liver clinical samples. (A) Plot boxes illustrating differential expression of miR-148a in 13 normal livers (NL), 39 primary HCCs (HCC), and their corresponding nontumor tissues (NT). Expression of miR-148a was normalized to RNU6B. Boxes show median and 25th and 75th 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

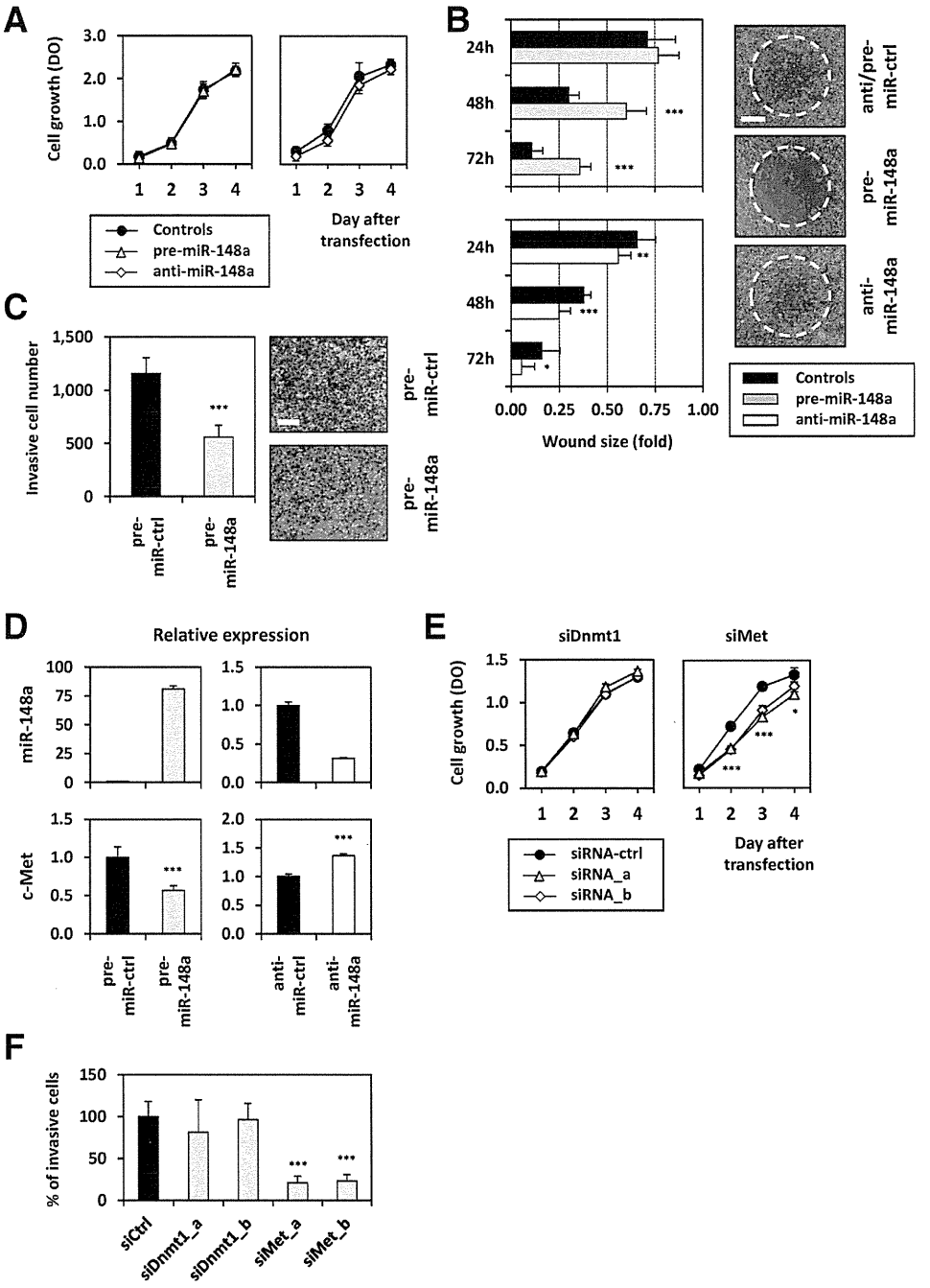

Fig. 7. Consequence of miR-148a rescue on HCC cell phenotype. (A) Hepa 1-6 cell growth determined at indicated times after miR-148a amplification or repression. No significant difference was found (*t* test). (B) Hepa 1-6 migratory abilities after miR-148a overexpression or inhibition in the presence 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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References

1. Ambros V. The functions of animal microRNAs. Nature 2004; 431:350-355.

2. Bartel DP. MicroRNAs: genomics, biogenesis, mechanism, and function. Cell 2004;116:281-297.
3. Calin GA, Croce CM. MicroRNA signatures in human cancers. Nat Rev Cancer 2006;6:857-866.
4. Gailhouste L, Ochiya T. Cancer-related microRNAs and their role as tumor suppressors and oncogenes in hepatocellular carcinoma. Histol Histopathol 2013;28:437-451.
5. Gailhouste L, Gómez-Santos L, Ochiya T. Potential applications of miRNAs as diagnostic and prognostic markers in liver cancer. Front Biosci 2013;18:199-223.
6. Gangaraju VK, Lin H. MicroRNAs: key regulators of stem cells. Nat Rev Mol Cell Biol 2009;10:116-125.
7. Cheng LC, Pastrana E, Tavazoie M, Doetsch F. miR-124 regulates adult neurogenesis in the subventricular zone stem cell niche. Nat Neurosci 2009;12:399-408.
8. Zhao C, Sun G, Li S, Shi Y. A feedback regulatory loop involving microRNA-9 and nuclear receptor TLX in neural stem cell fate determination. Nat Struct Mol Biol 2009;16:365-371.
9. Crist CG, Montarras D, Pallafacchina G, Rocancourt D, Cumano A, Conway SJ, et al. Muscle stem cell behavior is modified by microRNA-27 regulation of Pax3 expression. Proc Natl Acad Sci U S A 2009;106:13383-13387.
10. Hand NJ, Master ZR, Eauclaire SF, Weinblatt DE, Matthews RP, Friedman JR. The microRNA-30 family is required for vertebrate hepatobiliary development. Gastroenterology 2009;136:1081-1090.
11. Girard M, Jacquemin E, Munnich A, Lyonnet S, Henrion-Caude A. miR-122, a paradigm for the role of microRNAs in the liver. J Hepatol 2008;48:648-656.
12. Li E. Chromatin modification and epigenetic reprogramming in mammalian development. Nat Rev Genet 2002;3:662-673.
13. Hermann A, Gowher H, Jeltsch A. Biochemistry and biology of mammalian DNA methyltransferases. Cell Mol Life Sci 2004;61:2571-2587.
14. Okano M, Bell DW, Haber DA, Li E. DNA methyltransferases Dnmt3a and Dnmt3b are essential for de novo methylation and mammalian development. Cell 1999;99:247-257.
15. Chan MF, van Amerongen R, Nijjar T, Cuppen E, Jones PA, Laird PW. Reduced rates of gene loss, gene silencing, and gene mutation in Dnmt1-deficient embryonic stem cells. Mol Cell Biol 2001;21:7587-7600.
16. Lei H, Oh SP, Okano M, Juttermann R, Goss KA, Jaenisch R, et al. De novo DNA cytosine methyltransferase activities in mouse embryonic stem cells. Development 1996;122:3195-3205.
17. Sen GL, Reuter JA, Webster DE, Zhu L, Khavari PA. DNMT1 maintains progenitor function in self-renewing somatic tissue. Nature 2010;463:563-567.
18. Saito Y, Kanai Y, Sakamoto M, Saito H, Ishii H, Hirohashi S. Expression of mRNA for DNA methyltransferases and methyl-CpG-binding proteins and DNA methylation status on CpG islands and pericentromeric satellite regions during human hepatocarcinogenesis. HEPATOLOGY 2001;33:561-568.
19. Saito Y, Kanai Y, Nakagawa T, Sakamoto M, Saito H, Ishii H, et al. Increased protein expression of DNA methyltransferase (DNMT) 1 is significantly correlated with the malignant potential and poor prognosis of human hepatocellular carcinomas. Int J Cancer 2003;105:527-532.
20. Gailhouste L. Isolation and purification method of mouse fetal hepatoblasts. Methods Mol Biol 2012;826:33-47.
21. Tsai CC, Su PF, Huang YF, Yew TL, Hung SC. Oct4 and Nanog directly regulate Dnmt1 to maintain self-renewal and undifferentiated state in mesenchymal stem cells. Mol Cell 2012;47:169-182.
22. Bernstein E, Kim SY, Carmell MA, Murchison EP, Alcorn H, Li MZ, et al. Dicer is essential for mouse development. Nat Genet 2003;35:215-217.
23. Sekine S, Ogawa R, Ito R, Hiraoka N, McManus MT, Kanai Y, et al. Disruption of Dicer1 induces dysregulated fetal gene expression and promotes hepatocarcinogenesis. Gastroenterology 2009;136:2304-2315.e1-4.
24. Sayed D, Abdellatif M. MicroRNAs in development and disease. Physiol Rev 2011;91:827-887.
25. Xu H, He JH, Xiao ZD, Zhang QQ, Chen YQ, Zhou H, et al. Liver-enriched transcription factors regulate microRNA-122 that targets CUTL1 during liver development. HEPATOLOGY 2010;52:1431-1442.
26. Merkerova M, Vasikova A, Belickova M, Bruchova H. MicroRNA expression profiles in umbilical cord blood cell lineages. Stem Cells Dev 2010;19:17-26.
27. Zhang J, Ying ZZ, Tang ZL, Long LQ, Li K. MicroRNA-148a promotes myogenic differentiation by targeting the ROCK1 gene. J Biol Chem 2012;287:21093-21101.
28. Takata A, Otsuka M, Yoshikawa T, Kishikawa T, Hikiba Y, Obi S, et al. MiRNA-140 acts as a liver tumor suppressor by controlling NF-kappaB activity via directly targeting Dnmt1 expression. HEPATOLOGY 2013;57:162-170.
29. Duursma AM, Kedde M, Schrier M, le Sage C, Agami R. miR-148 targets human DNMT3b protein coding region. RNA 2008;14:872-877.
30. Sato F, Tsuchiya S, Meltzer SJ, Shimizu K. MicroRNAs and epigenetics. FEBS J 2011;278:1598-1609.
31. Hanoun N, Delpu Y, Suriawinata AA, Bournet B, Bureau C, Selves J, et al. The silencing of microRNA 148a production by DNA hypermethylation is an early event in pancreatic carcinogenesis. Clin Chem 2010;56:1107-1118.
32. Zhu A, Xia J, Zuo J, Jin S, Zhou H, Yao L, et al. MicroRNA-148a is silenced by hypermethylation and interacts with DNA methyltransferase 1 in gastric cancer. Med Oncol 2011.
33. Xu Q, Jiang Y, Yin Y, Li Q, He J, Jing Y, et al. A regulatory circuit of miR-148a/152 and DNMT1 in modulating cell transformation and tumor angiogenesis through IGF-IR and IRS1. J Mol Cell Biol 2013;5:3-13.
34. Kota J, Chivukula RR, O'Donnell KA, Wentzel EA, Montgomery CL, Hwang HW, et al. Therapeutic microRNA delivery suppresses tumorigenesis in a murine liver cancer model. Cell 2009;137:1005-1017.
35. Braconi C, Huang N, Patel T. MicroRNA-dependent regulation of DNA methyltransferase-1 and tumor suppressor gene expression by interleukin-6 in human malignant cholangiocytes. HEPATOLOGY 2010;51:881-890.
36. Huang J, Wang Y, Guo Y, Sun S. Down-regulated microRNA-152 induces aberrant DNA methylation in hepatitis B virus-related hepatocellular carcinoma by targeting DNA methyltransferase 1. HEPATOLOGY 2010;52:60-70.

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Review

Roles of microRNAs in the Hepatitis B Virus Infection and Related Diseases

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Abstract: The hepatitis B virus (HBV) is a small enveloped DNA virus that belongs to the *Hepadnaviridae* family. HBV can cause acute and persistent infection which can lead to hepatocellular carcinoma (HCC). MicroRNAs (miRNAs) play a crucial role in the main cellular events. The dysregulation of their expression has been linked to the development of the cancer as well as to viral interference. This chapter will describe the involvement of miRNAs in the case of HBV infection and their implication in the development of the HBV-related diseases.

Keywords: hepatitis B virus; microRNA; hepatocellular carcinoma

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1. Introduction

The microRNAs (miRNAs or miRs) are small non-coding RNAs of 19–23 nucleotides that play key roles in the regulation of almost every cellular process in all multicellular eukaryotes [1]. As intracellular pathogens, viruses are affected by these post-transcriptional modulators and have evolved to subvert them. Several viruses, especially the herpesviruses, encode for their own miRNAs that increase their replication potential and/or allow the evasion from the innate immune system [2]. Other viruses, such as the hepatitis B virus (HBV), modulate the cellular miRNAs in order to achieve the same effects.

HBV is a small enveloped DNA virus that belongs to the *Hepadnaviridae* family. It primarily infects hepatocytes and causes acute and chronic liver disease. Among the 2,000 million people