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Regulation of placenta growth factor by microRNA-125b in hepatocellular cancer

Gianfranco Alpini^{1,2}, Shannon S. Glaser¹, Jing-Ping Zhang³, Heather Francis^{1,4}, Yuyan Han¹, Jiao Gong³, Allison Stokes⁴, Taylor Francis⁴, Nathan Hughart⁴, Levi Hubble⁴, Shi-Mei Zhuang³, and Fanyin Meng^{1,4}

¹ Department of Medicine and Scott & White Digestive Disease Research Center, Texas A&M HSC COM and Scott & White Hospital, Temple, Texas.

² Research, Central Texas Veterans Health Care System, Temple, Texas.

³ Key Laboratory of Gene Engineering of the Ministry of Education, State Key Laboratory of Biocontrol, School of Life Sciences, Sun Yat-sen University, PR China

⁴ Research & Education, Scott & White Hospital, Temple, Texas.

Abstract

BACKGROUND & AIMS—MicroRNAs (miRNAs) are a class of small noncoding RNAs that can regulate gene expression by translation repression or mRNA degradation. Our aim was to evaluate the role of aberrantly expressed miRNAs in hepatocellular cancer (HCC).

METHODS—miRNA expression in HCC tissues and cells was evaluated by qPCR array and Taqman miRNA assay. Cell proliferation, motility, invasion and angiogenesis index were quantitated using commercial assays. DNA methylation status, matrix metalloproteinases (MMPs) mRNA expression was quantitated by real-time PCR analysis.

RESULTS—miRNA profiling identified a decrease in miR-125b expression in HCC tumor tissues and cell lines. The expression of miR-125b was significantly increased by the methylation inhibitor 5-aza-2'-deoxycytidine in HCC cells but not in normal controls, suggesting that the expression of miR-125b could be epigenetically modulated. Methylation-specific PCR revealed hypermethylation status of miR-125b in HCC cells instead of non-malignant controls. Cell proliferation, anchorage-independent growth, cell migration, invasion and angiogenesis were significantly decreased by the introduction of miR-125b precursor in HCC cell lines. Placenta growth factor was identified as a target of miR-125b by bioinformatics analysis and experimentally verified using luciferase reporter constructs. Overexpression of miR-125b in HCC cells decreased PIGF expression, and altered angiogenesis index. Furthermore, modulation of miR-125b also distorted expression of MMP-2 and -9, the mediators of enzymatic degradation of extracellular matrix.

CONCLUSIONS—Our studies showing epigenetic silencing of miR-125b contributes to an invasive phenotype provide novel mechanistic insights and identify a potential target mechanism that could be manipulated for therapeutic benefit in HCC.

Address for correspondence: Gianfranco Alpini, Ph.D or Fanyin Meng, Ph.D Scott & White Digestive Diseases Research Center Division Research, Central Texas Veterans Health Care System Scott & White and Texas A & M Health Science Center College of Medicine 702 SW H.K. Dodgen Loop, Temple, TX, 76704 Phone: 254-742-7044 or 254-724-3834 Fax: 254-724-9278 or 254-742-7181 galpini@medicine.tamhsc.edu or fmeng@tamu.edu.

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Keywords

microRNA; liver cancer; PIGF; invasion; angiogenesis; methylation

Introduction

Hepatocellular carcinoma (HCC) is the most common primary malignancy arising within the liver. Worldwide HCC is the fifth most common primary malignancy. HCC is the leading cause of death in patients with cirrhosis in Europe and the United States. Although the incidence of HCC in the United States has been low, both the incidence and the mortality from HCC have been increasing. Clinical outcomes for HCC reflect the potential for invasion, which results in intrahepatic spread and a high rate of recurrence following surgical resection. Thus, an understanding of the molecular mechanisms involved in tumor cell invasion and spread is important, and may lead to more effective therapeutic approaches for HCC.

MicroRNAs (miRNAs) are a group of non-coding RNA that are being increasingly recognized as important players in human cancers [3]. Aberrant expression of specific microRNAs, including miR-21, miR-122, miR-221, miR-222 and miR-125b, has been discovered in human HCC cells [22, 27]. Aberrant expression of miRNAs such as miR-21 and miR-122 alters cellular expression of PTEN and cyclin G1 [10, 22]. However, the contribution of the majority of aberrantly expressed miRNAs in tumor cell behavior in HCC is unknown.

The regulation of the tumor suppressor p53 by miR-125b suggests a potential role for miR-125b in tumor cell behavior [19]. E2F3, a transcription factor involved in cell cycle progression has been also identified as a target of miR-125b [14]. Although de-repression of E2F3 promotes neoplastic growth in tumors in which miR-125b is reduced, such as bladder and breast cancer, the silencing of miR-125b in human HCC indicates the presence of additional mechanisms by which miR-125b may contribute to cancer metastasis [2, 20-21]. Decreased expression of miR-125b has also been reported in other gastrointestinal and breast cancers [28]. In this study, we investigated the epigenetic regulation of miR-125b in hepatocarcinogenesis with respect to the regulation of the specific target gene which is involved in HCC tumor growth, metastasis and angiogenesis.

Materials and Methods

Human liver tissues and cell lines

HCC cell lines HepG2 and PLC/PRF-5 were obtained from ATCC (Manassas, VA) and cultured as recommended by the supplier. HCC tumor and normal control tissues were obtained from BioChain Institute, Inc. (Haywood, CA) and used for microRNA real-time PCR array analysis (Supplementary Table 1). Additional 19 Paired HCC patients and adjacent non-tumoral liver tissues were obtained from the Bank of Tumor Resources, Cancer Center, Sun Yat-sen University in Guangzhou, China (Supplementary Table 2).

Luciferase reporter assay

Intact putative miR-125b recognition sequence from the 3'-UTR of placental growth factor (PIGF) (pMIR-PIGF-wt-3'-UTR) or with random mutations (pMIR-PIGF-mut-3'-UTR) were cloned downstream of the firefly luciferase reporter gene. Luciferase assays were performed 72 hours after transfection using the Dual Luciferase Reporter Assay system (Promega, Madison, WI).

RNA isolation, real-time PCR and western blots

Total RNA was extracted from cells and tissues using Trizol (Invitrogen, Carlsbad, CA). The miRNome MicroRNA PCR Array Kit was purchased from SBI (System Biosciences, Mountain View, CA) and used for miRNA profiling. Real-time PCR analysis of mRNA and miRNAs was performed as described [22]. Western blot analysis was performed as described previously [22]; the membranes were blotted with antibodies for PIGF and β -actin (both from Santa Cruz Biotechnology, Santa Cruz, CA).

In vitro proliferation, migration, invasion and angiogenesis assay

Commercial available kits were used for proliferation, migration, invasion and angiogenesis assay in normal and malignant hepatic cells. Cell migration and invasion index was further normalized with proliferation index under the same conditions to rule out the impact factor of altered cell growth rate.

Statistics

A double-sided Student t test was performed to compare two groups ($p < 0.05$ was considered significant) unless otherwise indicated.

Please see supplementary for more detailed information of this section.

Results

miR-125b is aberrantly expressed in HCC tissues and cell lines

Aberrant expression of selected miRNAs has been observed in HCC. To identify miRNAs that are differentially decreased in expression in tumor tissues, we analyzed miRNA expression in three pairs of HCC tumor and normal liver tissues using SBI miRNome MicroRNA Profiling PCR Array. Among 318 of the 379 human miRNAs detected by this PCR array, the expression of 37 miRNAs was significantly altered relative to normal tissues. Of these, the majority of aberrantly expressed miRNAs were increased in expression. However, the expression of two miRNAs, miR-125b and miR-122 was markedly decreased in expression (<4 -fold) in tumor tissues compared to normal tissues (Figure 1). The expression of miR-125b was decreased in malignant hepatocytes (HepG2, and PLC/PRF-5) compared to that of normal human hepatocytes (Figure 2). By the real time-PCR confirmation, miR-125b expression was decreased by 3-fold or more in all 3 samples compared with the normal liver tissues (Figure 2D). Furthermore, among additional 19 Paired HCC patients and adjacent non-tumor liver tissues, reduced miR-125b expression was observed in 16 out of 19 HCC tumor and positively correlated with HCC patients' survival time after surgery (Figure 2 E&F and Supplementary Table 2; $p < 0.01$). These results show that the aberrant expression of miR-125b is a frequent event in human primary HCCs.

Modulation of miR-125b alters cell migration and invasion in HCC cell lines

The ability of cells to migrate into adjacent tissues and invade ECM is a key determinant of tumor progression, spread and metastases. We began by first verifying the efficacy of transfection and target effects by assessing the expression of mature miR-125b by real-time PCR in HepG2 cells (transfected with miR-125b precursor) as well as normal human hepatocytes with anti-miR-125b inhibitor (Figure 3A). Next, we assessed vertical cell migration and cell invasion. Pre-miR-125b decreased cell migration as well as invasion in HepG2 and PLC/PRF-5 HCC cell lines, as well as in human liver cancer derived endothelial cells (T-LECs) when compared to relative controls (Figure 3B). These results support a functional role for miR-125b in mediating cell migration and invasion in malignant

hepatocytes and hepatic tumors endothelial cells, and provide a mechanism by which down-regulation of miR-125b may contribute to tumor spread.

Inhibition of miR-125b increases HCC growth in vitro

We then assessed cellular proliferation in HepG2, PLC/PRF-5 and T-LEC cell lines. In cells transfected with Pre-miR-125b, there was a reduction in proliferation compared to cells transfected with control PremiRNA (Figure 4A). Moreover, there was a significant change in anchorage-independent growth following the modulation of Pre-miR-125b (Figure 4B). Overexpression of miR-125b in PLC/PRF-5 and T-LECs also significantly increased the fractions of early and late apoptotic cell populations (Figure 4C). These observations indicate a role for miR-125b in growth regulation of malignant human hepatic cells.

Identification of PIGF as a target for miR-125b

The target prediction program miRNA Viewer database (<http://cbio.mskcc.org/cgi-bin/mirnaviewer/mirnaviewer.pl>) indicated the presence of a highly conserved binding site for miR-125b that is present in the 3'-UTR region of PIGF, an angiogenic and survival cytokine in cancer biology. To demonstrate PIGF expression pattern in human HCC, 24 human HCC and matched noncancerous liver tissues were analyzed by immunohistochemistry (Figure 5A). The human liver cancer tissue array showed the signal intensity was strong (+++) or positive (++) in 18 out of 24 HCC tissues; whereas the weak (+) or negative (++) signals were seen in only 4 out of 24 samples of HCC tissues. Therefore, the PIGF protein expression was much higher in the HCC tissues as compared with the non-cancerous tissues ($p < 0.01$). We have also checked miR-125b and PIGF expression in 6 pairs of HCC tumor/control tissues and PIGF is overexpressed in 4 of 5 tumor tissues with reduced expression of miR-125b. PIGF is also significantly up-regulated in malignant hepatocytes and hepatic endothelial cells (Figure 5B and Figure 6A). Both *in vitro* and *in vivo* data analysis has demonstrated that the expression of PIGF is reversely correlated with miR-125b expression (Supplementary Figure 2 A&B). However, no negative correlation could be observed between PIGF expression and HCC patients' survival time after surgery (Supplementary Figure 2C). To verify that PIGF is a direct target of miR-125b, we performed studies using luciferase reporter constructs containing the miR-125b recognition sequence (Figure 6B) from the 3'-UTR of PIGF inserted downstream of the luciferase gene. Transfection with miR-125b precursor decreased the reporter activity in HepG2 HCC cells. However, when these studies were repeated with reporter constructs containing random mutations in the recognition sequence, the effects of reporter deactivation by miR-125b precursor were abolished (Figure 6C). Moreover, a decrease in PIGF expression occurred in HCC cells and tumor endothelial cells after 2 days incubated with miR-125b precursor. Concomitant with enhanced PIGF expression, there was a decrease of MMP-9 expression, an established downstream mediator in PIGF signaling pathway (Figure 6D). Among all the other confirmed targets of miR-125b including E2F3, LIN28B, Mucin 1, Bcl 3 and Bak 1, only E2F3 could be verified in HCC cells (Figure 6D and Supplementary Figure 2D). In contrast, transfection with miR-125a precursor, which is also silenced in HCC tumors and modulates cell growth in malignant hepatocytes, did not alter the expression of PIGF with a relative expression of 1.04 ± 0.11 -fold of controls. To evaluate the contribution of PIGF to miR-125b mediated cell invasion and migration, we assessed the impact of PIGF through transient transfection, and hence PIGF expression on miR-125b dependent cell proliferation, migration and invasion in HepG2, PLC/PRF-5 and T-LEC cells. Co-transfection experiments with wild-type PIGF constructs and Pre-miR-125b are also performed and the recovery effects of PIGF re-introduction on migration inhibition and growth suppression induced by miR-125b are verified (Supplementary Figure 3). Taken together, these findings indicate that PIGF is a biologically relevant target of miR-125b.

miR-125b expression can be epigenetically regulated

To evaluate for potential mechanisms by which miR-125b expression was deregulated in HCC, we assessed the effect of the methylation inhibitor, 5-Aza-CdR, on miR-125b expression in malignant hepatic cells. The dramatic increases in miR-125b were noted in HepG2 cells and T-LECs compared to relative control after 5-Aza-CdR treatment (Supplementary Figure 4A). We selected 2000 bps of sequence extending from the region of the miR-125b. Analysis of the specific region revealed the presence of CpG islands ~300 base pairs close to the miR-125b sequence (Supplementary Figure 5). These results suggest that the expression of miR-125b could be potentially regulated by the modulation of promoter methylation. Of note, the short arm of chromosome 11 is a “hot spot” for hypermethylation in human neoplasia [6]. Thus, we examined the methylation status of specific region of miR-125b by methylation-specific PCR (MSP), a bisulfite conversion based PCR technique for the study of DNA CpG methylation. Using a real-time MSP assay, we found that the specific region of miR-125b was hypermethylated in HepG2 cells and TLECs, but not normal hepatocytes and LSECs (Supplementary Figure 4B). Moreover, miR-125b is also epigenetically modified in HCC tissues detected by MSP assay (Supplementary Figure 6A). 5-Aza-CdR induced de-methylation of miR-125b CpG islands was also confirmed in HepG2 cells and T-LECs (Supplementary Figure 6B). These results suggest that gain of the methylation mark in the miR-125b region could be associated with the deactivation of miR-125b expression in HCC cells.

Regulation of angiogenesis by miR-125b in normal and malignant hepatic endothelial cells

Since PIGF is the member of VEGF family and exerts pleiotrophic functions in promoting tumor growth and angiogenesis, we next examined the alterations of angiogenesis following the overexpression of miR-125b in hepatic endothelial cells for 72 h. Significant overexpression of PIGF was observed in T-LECs relative to LSECs (Figure 6A), along with the substantial reduction of miR-125b (Supplementary Figure 4A). De-methylation treatment by 5-Aza-CdR significantly reduced angiogenesis index in T-LECs but not LSECs (Supplementary Figure 4C). Changes in angiogenesis in T-LECs become apparent as well after 72 h transfection of Pre-miR-125b, whereas only moderate reductions were noticed in LSECs group (Supplementary Figure 4D). Modulation of miR-125b also affected invasion potentials in T-LECs but not in LSECs (Figure 3D). Taken together, these results suggest that miR-125b dependent regulation of PIGF contributes to tumor endothelial cell angiogenesis and invasion.

miR-125b regulates matrix metalloproteinase mRNA expression

Invasion through basement membrane and interstitial ECM involves a series of proteolytic enzymes named matrix metalloproteinases. Alterations of the PIGF–VEGF complexes have been mechanistically linked to decreased expression of MMPs and cell invasion [9]. We therefore examined the expression of selected MMPs involved in cell invasion in HCC/normal liver tissues and cell lines. Compared with expression in normal liver tissue, the expression of MMP-2 and MMP-9 is increased in HCC tumors (Supplementary Figure 7A). Enhanced expression of MMP-2 and MMP-9 was also seen in HepG2 cells and T-LECs compared to normal hepatocytes and LSECs (Supplementary Figure 7B). To confirm the functional impact and relevance of miR-125b dependent modulation of PIGF, we assessed the effect of modulation of miR-125b and demethylation on MMPs expression. Inhibition of DNA methylation by 5-Aza-CdR significantly up-regulated MMP-2 and MMP-9 expression in hepG2 and T-LEC cells (Supplementary Figure 7C). Furthermore, the expression of both MMP-2 and MMP-9 mRNA was significantly reduced after transfection with Pre-miR-125b in HepG2, PLC/PRF-5 and T-LEC cell lines (Supplementary Figure 7D). These findings link epigenetic regulator, miR-125b and putative mediators of cell invasion in malignant

hepatic cell lines and suggest that de-regulated expression of miR-125b can contribute to tumor spread.

Discussion

In this study we demonstrated the role of alterations of miR-125b in contributing to cellular phenotypic changes that are associated with tumor progression. We show that miR-125b is silenced in malignant hepatocytes and tumor endothelial cells compared to relative normal hepatic cells (controls), and that it may contribute to tumor spread by modulating cell proliferation, migration, invasion and angiogenesis. Some of these effects are mediated through PIGF, a member of the VEGF sub-family and an important cytokine of cancer cell survival and tumor angiogenesis. Decreased expression of miR-125b was shown by *in situ* hybridization and PCR analysis in human HCCs and a similar role for miR-125b has been postulated in breast, bladder as well as head and neck carcinogenesis [2, 15, 17-18, 20-21, 26-28]. The concomitant silencing of miR-125b-dependent activation of metalloproteinases in tumor cells can facilitate tissue invasion. These findings support the oncogenic role for silencing of miR-125b in contributing spread of liver cancers.

miRNA mediated mechanisms are being increasingly implicated in tumor progression. Deregulation of miR-125b can occur as a result of chemotherapy of cancer patients. Likewise, the ectopic expression of miR-125b can modulate expression of genes that are known to be involved in cancer cell survival such as Bak1, C-Raf, E2F3 and Bcl2 modifying factor (BMF) [13, 29, 31]. These and other studies [11, 13, 24] support a tumor suppressor role for miR-125b. Tumors with low expression of miR-125b lack its pro-apoptotic stimulus and acquire the capability to proliferate and grow. Variable expression of miR-125b has been reported in different organ systems and diseases states. In breast and bladder cancer cells miR-125b is a potent suppressor of cell proliferation but it supports cell growth in prostate carcinogenesis [25]. These diverse observations necessitate a clear definition of tissue-specific expression and function of miR-125b expression. It is likely that targeted therapeutic approaches involving miR-125b may result from defining tissue and disease-state specific roles of miR-125b.

PIGF is one of endothelial growth polypeptides with structural similarities to VEGF. It is found to be specifically expressed in the placenta and in some tumor cell types. PIGF is expressed during embryonic vasculogenesis; nevertheless, PIGF is not required for embryonic vessel formation because mice lacking PIGF develop normally. In contrast, PIGF seems to contribute to pathologic angiogenesis. For example, in PIGF-deficient mice, tumor growth and tumor angiogenesis are markedly reduced. Recently, it has been shown that circulating hematopoietic progenitor cells and macrophages contribute to tumor angiogenesis, and that PIGF might induce tumor angiogenesis by the recruitment of these cells to the growing tumors [4]. Overexpression of PIGF has been correlated with early recurrence of HCC, suggested that PIGF may be an important prognostic indicator in HCC [12, 23]. Moreover, up-regulation of PIGF has been observed in hepatitis C cirrhosis patients [16].

Our findings identify a previously unrecognized mechanism for direct regulation of PIGF, involving non-coding microRNA in HCC. Epigenetic mechanisms of regulation of expression involving PIGF promoter methylation are well recognized [30]. However, we did not observe a correlation between promoter methylation and reduced PIGF immunoreactivity in human HCC tissues and cells supporting the argument that there may be additional mechanisms. The relationship between miRNA-dependent pathways and other mechanisms of regulation during tumor spread warrants further detailed study. Epigenetically mediated gene silencing through promoter hypermethylation can contribute

to cancer initiation and progression [5, 7-8]. Many studies have shown that CpG island methylation changes can play a significant role in human HCC and other cancers. Genomic scanning approaches to identify epigenetically modified targets in HCC are lacking, but such strategies could identify other novel targets that could be epigenetically modified in HCC.

The current effective treatments available for HCC are only applicable in a relatively small proportion of early stage cases. We postulate that therapeutic strategies based on targeting miR-125b may be useful to consider in the adjuvant setting to limit intrahepatic metastases. The presence of therapeutic strategies targeting miRNA, with diverse mechanisms, makes them interesting starting points in the search for potential strategies for prevention of spread in patients who have undergone resection or locoregional therapies for advanced HCC.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

5-aza-CdR	5-aza-2'deoxyctidine
PIGF	placenta growth factor
HCC	hepatocellular cancer
miRNA	microRNA
MMP	matrix metalloproteinase

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Figure 1. Aberrant miRNA expression in human HCC

(A) miRNA expression patterns in human HCC samples were analyzed using a self-organizing tree algorithm. A dendrogram showing 3 clusters was generated. miRNA expression in HCC tumor tissues is shown on the right axis relative to normal liver controls on the left axis. Cluster 3 comprised of 2 miRNA that were decreased in expression, including miR-125b and miR-122. (B) Relative miRNA expression profile between HCC Tumor vs. Normal Control tissues is shown. The expression of a panel of diverse updated miRNAs was evaluated by qPCR array (SBI). miR-125b and miR-122 are the most down-regulated miRNAs among the 318 miRNAs detected in HCC tumors.

Figure 2. miR-125b is silenced in human HCC

Total RNA was isolated from either normal liver and HCC tumors (Panel A), or from normal human hepatocytes, HepG2 and PLC/PRF-5 (Panel B), as well as normal and liver tumor endothelial cells (Panel C). The expression of a group of selected miRNAs from each cluster (miR-221 from cluster 1, miR-96 from cluster 2 and miR-125b & miR-122 from cluster 3) was assessed using real-time PCR. *Panel D:* Total RNA was isolated from HCC (T) and normal control (N). Real-time PCR analysis was performed, and the ratio of miR-125b to U6 small RNA expression in HCC samples was determined. The PCR products

were verified by 1.8% agarose gel electrophoresis. Panel E: The expression of miR-125b is reduced HCC tissues. Expression of miR-125b in 19 HCC patients was examined by qPCR. The y axis indicates the fold change in the miR-125b level in HCC (T) and matched adjacent non-tumor tissue (N) relative to the median value of adjacent non-tumor tissue. The horizontal line indicates the median and interquartile range (25th to 75th percentiles). Cases are divided into two groups (x axis): HCC (T) and matched adjacent non-tumor tissue (N). Significant reduction of miR-125b in HCC tumors were detected ($p=0.0058$). Panel F: Positive correlation between miR-125b expression and HCC patients' survival time. The survival time of 19 HCC patients were recorded through the follow-up in 19 HCC patients after surgery. The mature miR-125b level in HCC tumors was examined by real-time qPCR analysis and normalized to adjacent control liver tissues. Statistical analysis was performed using Pearson's correlation coefficient.

Figure 3. Modulation of miR-125b expression altered cell migration and invasion

Panel A: miR-125b expression was assessed by real-time PCR in normal human hepatocytes transfected with either control or miR-125b precursors, or in HepG2 cells transfected with control or anti-miR-125b inhibitors. The ability of these constructs to modulate miR-125b expression was verified using miR-125b precursor in normal hepatocytes and with anti-miR-125b in HCC cells. **Panel B&C:** HCC cells were transfected with Pre-miR-125b (■) or with control precursor (□) (Panel B). Meanwhile, human liver sinusoidal endothelial cells were transfected with control and anti-miR-125b inhibitors (Panel C). Cell migration was assessed. **Panel D:** Cells were transfected with miR-125b or control precursor, and cell invasion was assessed after 72 hours using the QCM 96-well cell Invasion assay kit. * $p < 0.05$ relative to controls.

Figure 4. Overexpression of miR-125b reduces HCC cell growth

Panel A: Cell proliferation was assessed using a viable cell assay and the proliferation index was assessed after 72 hours. **Panel B:** Cells were plated in agar wells in 96-well plates, and anchorage independent growth assessed fluorometrically after 7 days. * $p < 0.05$ relative to controls. **Panel C:** Flow cytometric analysis of control and miR-125b overexpressed malignant hepatic cells to demonstrate the basis for the gating of viable, apoptotic and necrotic cells. The lower left quadrant shows the viable cells, the lower right quadrant represents the early apoptotic cells. The upper right quadrant represents nonviable, late apoptotic/necrotic cells, positive for Annexin V and APOTEST-FITC staining. The upper left quadrant shows nonviable necrotic cells/nuclear fragments.

Figure 5. Aberrant expression of PIGF in human HCC tumor sections and cells

Panel A: Tissue microarrays of paraffin-embedded HCC tumors with adjacent controls were stained with *PIGF* antibody and visualized by 3, 30-diaminobenzidine (Zymed Laboratories Inc.). Left panels, two representative adjacent control staining specimen with semi-quantitative scores negative (-); right panels, two representative HCC tumor specimen with strong-PIGF staining (semi-quantitative score +++). Figures are 100 magnifications. **Panel B:** Immunocytochemistry for PIGF was performed in normal human hepatocytes (N-Hep), HepG2 cells, LSECs and T-LECs. An increase in PIGF expression is observed in HepG2 and T-LECs when compared to N-Hep and LSECs respectively.

Figure 6. miR-125b regulates expression of PIGF

(A) Western blot analysis and densitometric analysis of relative expression levels of PIGF and β-actin were performed in normal human hepatocytes and liver sinusoidal endothelial cells, as well as in HepG2 and T-LEC cell lines. The increase in PIGF was observed in both malignant cell lines. * $p < 0.05$ relative to expression in normal controls. (B) Schematic of predicted miR-125b site in the 3'UTR of human PIGF. (C) Luciferase reporter constructs containing the miR-125b recognition sequence from the 3'-UTR of PIGF inserted downstream of the luciferase gene were generated. * $p < 0.05$. (D) Cells were transfected with miR-125b or control precursor. Cell lysates were obtained after 48 hours, and western blots performed for PIGF and β-actin. Conditioned medium was collected, concentrated 20-fold by lyophilization and 10 μg analyzed by zymography to detect MMP-9 activity.