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miR-181a-5p is downregulated in hepatocellular carcinoma and suppresses motility, invasion and branching-morphogenesis by directly targeting c-Met

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ABSTRACT

c-Met receptor tyrosine kinase has been regarded as a promising therapeutic target for hepatocellular carcinoma (HCC). Recently, microRNAs (miRNAs) have been shown as a novel mechanism to control c-Met expression in cancer. In this study, we investigate the potential contribution of miR-181a-5p dysregulation to the biology of c-Met overexpression in HCC. Herein, we found an inverse expression pattern between miR-181a-5p and c-Met expression in normal, cirrhotic and HCC liver tissues. Luciferase assay confirmed that miR-181a-5p binding to the 3′-UTR of c-Met downregulated the expression of c-Met in HCC cells. Overexpression of miR-181a-5p suppressed both HGF-independent and -dependent activation of c-Met and consequently diminished branching-morphogenesis and invasion. Combined treatment with miR-181a-5p and c-Met inhibitor led to a further inhibition of c-Met-driven cellular activities. Knockdown of miR-181a-5p promoted HGF-independent/-dependent signaling of c-Met and accelerated migration, invasion and branching-morphogenesis. In conclusion, our results demonstrated for the first time that c-Met is a functional target gene of miR-181a-5p and the loss of miR-181a-5p expression led to the activation of c-Met-mediated oncogenic signaling in hepatocarcinogenesis. These findings display a novel molecular mechanism of c-Met regulation in HCC and strategies to increase miR-181a5p level might be an alternative approach for the enhancement of the inhibitory effects of c-Met inhibitors.

1. Introduction

HCC is one of the most common and aggressive cancers worldwide [1,2]. Although many advances have been made in the diagnosis and management of HCC, the prognosis of patients with HCC remains poor due to metastasis, recurrence and development of resistance to conventional chemotherapy and radiotherapy [1–3]. Intense efforts have been made over the past decade to understand molecular mechanisms of HCC, and HGF/c-Met signaling pathway has become one of the most promising targets for the molecular therapy of HCC [4–6].

Classically, upon binding to HGF, c-Met becomes activated and drives complex biological activities including proliferation, survival, migration, invasion, and morphogenesis [4–8]. Abnormalities in HGF/c-Met signaling were reported to be linked to an unfavorable clinicopathological status, including high proliferation index, low degree differentiation, vascular invasion and metastasis in several cancer types [4–6]. We and others have identified ligand independent kinase activity for c-Met in HCC, occurring by several mechanisms including gene amplification, activating point mutations, receptor crosstalk, receptor overexpression, and non-coding RNAs [7,8]. Recent studies have shown that miRNAs regulating c-Met expression are down-regulated in HCC and directly contributes to cell migration and invasion of HCC cells [7,9–11].

miRNAs are evolutionary endogenous regulatory small non-coding RNAs that play critical roles in post-transcriptional gene expression through base pairing with the 3′untranslated region (3′UTR) of target mRNAs [12,13]. miRNAs regulate gene expression by directly degrading messenger RNA (mRNA) or repression translation [12,13]. It is now known that one miRNA can simultaneously control the expression of hundreds of different genes and over 60% of human protein-coding genes are predicted to contain miRNA-binding sites within 3′UTRs [12]. These properties of miRNAs make them a powerful regulator in human physiology and pathology, including cancer [14,15]. Therefore, miRNAs represent a novel and attractive therapeutic strategy for human diseases.

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Several studies have explored that dysregulated miRNAs possess important roles in HCC progression and directly contribute to proliferation, apoptosis and metastasis of HCC [15–20]. Recently, we have identified HCC Epithelial-to-Mesenchymal-Transition (EMT)-associated miRNAs. Among these miRNAs, miR-181a-5p has been computationally predicted to target c-Met. In this study, we investigated the potential role of miR181a5p in the regulation of c-Met expression and c-Met induced motility, invasion and branching-morphogenesis in HCC.

2. Material and methods

2.1. Cell culture

Human HCC cell lines Mahlavu (MV), and SNU-449, were cultivated as described [8]. Hepatocyte growth factor/scatter factor (HGF) was from R&D Systems (MN, US). HGF (40 ng/mL) was used at specific time points after overnight starvation in DMEM with 2% FBS. For the inhibition of c-Met, SU11274 (Calbiochem 448101, US) was added to the culture medium in the indicated doses and times. DMSO (Applichem, US) was used as solvent for SU11274.

2.2. The prediction of candidate miRNAs targeting c-Met

To investigate the target genes and the conserved sites bound by the seed region of miR-181a-5p, the miRDB, and microRNA.org-Targets and Expression, algorithms were used.

2.3. miRNA transfection

MV and SNU-449 cells were transiently transfected with miR-181a-5p-mimics (Ambion, MC10421, US, 100 nM,) or miR-181a-5p-inhibitor (Ambion, MH10421, 100 nM) or respective negative-controls pre-miR miRNA precursor (Ambion, AM:17010, 100 nM, control), or anti-miR miRNA (Ambion, AM:17010, 100 nM, inhibitor control) using "Fugene HD-Promega (US)" following the manufacturer's protocol. After transfection for 24 h, the cells were harvested for further experiments.

2.4. Plasmid constructs and luciferase activity assay

Luciferase vector pEX-MT01 with wild type c-Met miTarget microRNA 3′UTR target clones (product ID HmiT011181-MT01) and mutant with c-Met 3′-UTR with deletions of 7 bp from the site of perfect complementarity of miR-181a-5p (product ID CS-HmiT011181-MT01-01) were purchased from GeneCopoeia (US). Cells were co-transfected with luciferase constructs (100 ng/ml) and miR-181a-5p-mimics or mimic-control or miR-181a-5p-inhibitor or inhibitor-control using Fugene. After 48 h, renilla luciferase activity was measured using a dual-luciferase reporter system (Promega, WI, USA) (For detail see Supplementary Material). The renilla luciferase activities were normalized with the internal firefly luciferase activity.

2.5. Real-time RT-PCR

The total RNA including miRNA was extracted from cell lines using a mirVANA miRNA isolation kit (ambion/RNA by life technologies, USA) according to the manufacturer's instructions. RNA concentration was detected by Nanodrop (Thermo, US). The expression of mature miR-181a-5p was quantified using TaqMan microRNA Assay Kit (Applied Biosystems, US) with specific primers for miR-181a-5p (Applied Biosystems, 000480). miR-181a-5p expression was normalized to RNU6B (Applied Biosystems, 001093) using the $2^{-\Delta Ct}$ method.

2.6. Western blotting

Total protein was prepared by using modified RIPA buffer as described previously [8]. Antibodies against phospho-Met (Y-1234/1235) (cell signaling (cs)-3129, US), phospho-p44/42 ERK1/2 (p-MAPK) (Thr202/Tyr204) (cs-9101), p44/42 ERK1 (MAPK) (C-16) (santa cruz (sc) 93, USA), Calnexin (sc-11397) as described [10]. Equal loading and transfer were confirmed by repeat probing for calnexin (house-keeping gene). Band intensities were quantified as pixels by using ImageJ software (NIH).

2.7. Motility and invasion assay

In vitro motility and invasion assays were performed as described previously [8]. Briefly, cells were transiently transfected with miRNA-mimic or mimic-control or inhibitor or inhibitor-control in 2%DMEM. For c-Met inhibition, cells were pretreated with SU11274 overnight and were placed into upper chambers. DMEM with 2% FBS with/without HGF and/or SU11274 was added to the lower chambers. After 24 h incubation at 37 °C, the medium was removed; cells were fixed and stained with Diff Quick (Siemens Healthcare Diagnostics, UK). Cells that had traversed through the membrane were counted using a bright-field inverted microscope. Total cell numbers were counted for each chamber. Experiments were performed in at least triplicates and repeated at least three times.

2.8. Branching-morphogenesis assays

miRNA, miRNA control treated cells were embedded in three-dimensional collagen I gels (BD 354236, US) that contain SU11274 and/or HGF as previously described [21]. For the quantitation of the morphogenic response, the entire area per experimental condition in each of the 4 independent cultures were photographed using 20X phase contrast objective using Olympus (US) CKX41. All colonies were analyzed and scored on the ability to form branching tubules. Data were expressed as mean ± standard error (SE) for at least four independent experiments.

2.9. Immunofluorescence staining and miRNA LNA in situ hybridization of formalin-fixed, paraffin-embedded tissue section

Human liver tissue microarray (TMA) samples containing 12 normal and 19 cirrhotic liver tissues, and 48 HCC tissues were purchased from US Biomax (catalog no: BC03117 and T031a) to detect miR-181a-5p and c-Met expression levels in HCC, cirrhotic and normal liver tissues by in situ hybridization (ISH) and immunofluorescence (IF), respectively. IF with c-Met was performed on the TMA section after deparaffinization followed by antigen retrieval using proteinase K. After the secondary fluorochrome-conjugated antibodies (Alexa Fluor 488, invitrogen A-21206) treatment, slides were mounted with fluorescence mounting medium (Dako, S3023, Denmark). miR-181a-5p detection was performed on the TMA cores by ISH as described in the Supplementary Material section. DAPI (Invitrogen, US) was used to stain the cell nuclei. The entire TMA section was analyzed by acquisition of c-Met, miR-181a-5p, U6 and DAPI signals by Immunofluorescence Microscopy using Olympus BX50 fluorescence microscope. A score for both miR-181a-5p and c-Met protein expression was given according to the percentage of positively stained cores. Images were generated using Adobe Photoshop (US) and ImageJ software (NIH, US).

2.10. Statistical analysis

Statistical analysis was performed using the GraphPad Prism (US). $p < 0.05$(*), $p < 0.001$(**) and $p < 0.0001$(***) were considered statistically significant.

3. Results

3.1. miR-181a-5p expression is inversely correlated with c-Met expression in HCC tissues

LNA staining results showed that the expression of miR-181a-5p had significantly decreased during the progression from normal to cirrhotic liver, further decreasing with the development of HCC ($p < 0.0001$) (Fig. 1A). Staining of the serial sections of the liver microarray from the same patient with a U6, or scramble were negative (Fig. S1) However, as we previously reported [8], there was a significant increase in c-Met expression towards normal to cirrhosis and to HCC (Fig. 1B). Almost 90% of the normal tissues were positive for miR-181a-5p expression (Fig. 1A), however no demonstrable c-Met staining was observed in normal liver cores (Fig. 1B). Positive staining for miR-181a-5p was observed in 42% of cirrhotic liver tissue cores and 21% of HCC tissue cores (Fig. 1A). To strengthen the validity of this expression profile, we further evaluated the same core for the expression of c-Met and miR-181a-5p (Fig. 1C and D). In HCC, 75% of miR-181a-5p positive cores were determined as c-Met negative. Spearman Correlation analysis in the miR-181a-5p positive group showed an inverse correlation between miR-181a-5p and c-Met expression in HCC ($r = -075$, $p < 0.001$). Taken together, these data suggest that while miR-181a-5p expression was frequently down-regulated through hepatocarcinogenesis, its target potential c-Met expression was up-regulated.

3.2. Overexpression of miR-181a-5p decreases c-Met expression

Having identified the inverse correlation between miR-181a-5p and c-Met in HCC tissue samples, we next examined if miR-181a-5p expression affects the expression of c-Met at the protein level. We used chemically synthesized miR-181a-5p-mimics or inhibitors, which acts either as an enhancer or silencer of the effects of endogenous miRNA, and facilitates the detection of miRNA effects on a target gene. Based on the endogenous expression levels of miR-181a-5p in the five different HCC cell lines examined (Fig. S2), we chose the MV-cells with a low expression of miR-181a-5p for the transfection of miR-181a-5p-mimics or mimic-control (Fig. 2A). Correspondingly, we chose SNU-449 cells with a high expression of miR-181a-5p for depletion by transfection of miR-181a-5p-inhibitor or inhibitor-control (Fig. 2B). As expected, while forced expression of miR-181a-5p reduced c-Met expression significantly (Fig. 2C), the knockdown of endogenous miR-181a-5p resulted in a significant increase in the protein content of c-Met (Fig. 2D). Collectively, these results suggest that c-Met expression is regulated by miR-181a-5p.

3.3. c-Met 3′-UTR is a novel target of miR-181a-5p in HCC cells

To clarify the molecular mechanism involved in the overexpression of c-Met due to the downregulation of miR-181a-5p, we performed a computational analysis using miRNA target databases. We identified that 3′-UTR of c-Met mRNA contains a complementary site for the seed region of miR-181a-5p (Fig. 2E). To determine whether or not the predicted target sites for miR-181a-5p in the 3′-UTR of c-Met mRNA is responsible for c-Met downregulation, we performed luciferase reporter assays with vectors containing the wild type c-Met 3′-UTR and the mutant c-Met 3′-UTR downstream of the luciferase reporter gene. We co-transfected the wild type and mutant plasmids with either miR-181a-5p-mimics (Fig. 2F), or miR-181a-5p-inhibitor (Fig. 2G) or with their corresponding controls. As expected, we observed that the overexpression of miR-181a-5p resulted in the downregulation of luciferase fused to c-Met 3′-UTR ($p < 0.05$). Whereas this effect was abolished when the miR-181a-5p binding site of the c-Met 3′-UTR were mutated ($p > 0.05$) (Fig. 2F). Also, we found that miR-181a-5p inhibition caused a significant rise in luciferase activity ($p < 0.05$), while luciferase activity was not increased in the mutant 3′-UTR group ($p > 0.05$) (Fig. 2G). These data provide evidence that miR-181a-5p directly binds to the 3′-UTR of c-Met to control its expression.

3.4. miR-181a-5p regulated branching-morphogenesis, cell migration, and invasion through targeting c-Met signaling

With the aim of elucidating the impact of miR-181a-5p on c-Met-mediated signaling pathways, we first analyzed the phosphorylation level of c-Met and activation status of its downstream target, p44/42-MAPK, in the absence or presence of miR-181a-5p. Parallel to the downregulation of c-Met expression by miR-181a-5p-overexpression, c-Met phosphorylation was decreased (Fig. 3A). Up-regulation of c-Met due to miR-181a-5p-depletion resulted in the activation of c-Met (Fig. 3B). The expression and phosphorylation of p44/42-MAPK, a downstream effector molecule of c-Met, showed a similar pattern to that seen with c-Met expression and activation after miR-181a-5p mimics or inhibitor transfections. These data suggested that miR-181a-5p regulates HGF-independent activation of c-Met in a manner that was dependent upon c-Met expression.

c-Met is involved in the progression of HCC by playing a key role in EMT, angiogenesis, invasion and metastasis [5–8]. Considering the importance of miR-181a-5p as a regulator of c-Met signaling, we performed branching-morphogenesis assay which is a powerful *in vitro* model for testing angiogenesis, involving sequential cell attachment, differentiation, invasion and morphogenesis, all of which contributes to the aggressive nature of cancer. We observed an increase in regularly shaped cystic structures with very short outgrowths in control MV cells (Fig. 3C(a and b)), whereas MV cells transfected with the miR-181a-5p-mimics failed to form tubular structures ($p < 0.05$) (Fig. 3C(a and c)). On the other hand, the inhibition of miR-181a-5p expression significantly promoted branching-morphogenesis in SNU-449-cells ($p < 0.05$) (Fig. 3D(a–c)). Furthermore, we performed migration and invasion assays, which are the most common in *in vitro* assays used for testing the metastatic capacity of cancer cells. The migration and invasion of MV-cells were reduced by more than 50% ($p > 0.05$) (Fig. 3E) and 60% ($p < 0.05$) (Fig. 3F) respectively, by miR-181a-5p overexpression compared to the control cells (Fig. 3E and F). In contrast, the knockdown of miR-181a-5p expression significantly enhanced migration (Fig. 3G) and invasion (Fig. 3H) of SNU-449 cells ($p < 0.05$) (Fig. 3G and H). Furthermore, a specific inhibitor for c-Met kinase, SU11274, was able to suppress c-Met activation and downstream signaling, and consequently inhibited c-Met-dependent responses, including branching-morphogenesis, migration and invasion (Fig. S3). We also tested whether miR-181a-5p has a role in HCC cell proliferation and found that miR-181a-5p has no effect on the proliferation of HCC cells (Fig. S4). These data suggest that miR-181a-5p is a negative regulator of the c-Met signaling pathway and c-Met driven branching-morphogenesis, migration, and invasion in HCC cells.

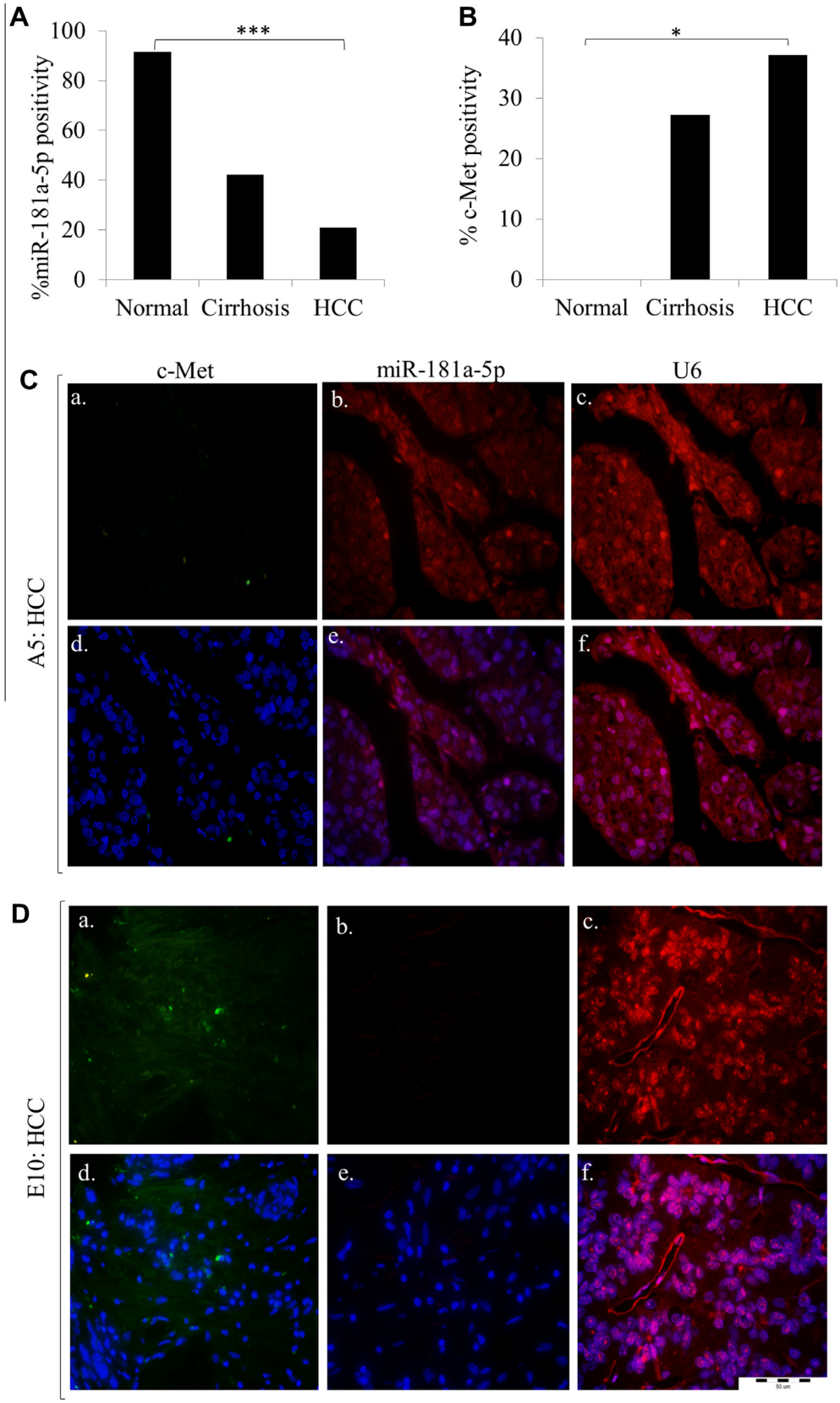

Fig. 1. Expression analysis of miR-181a-5p and its target gene c-Met in normal, cirrhotic and HCC liver tissues. Each column represents histologically classified normal, cirrhotic, HCC liver tissues with the percentage of positive stained samples for (A) miR-181a-5p, and (B) c-Met. Trend in χ^2-test was performed to determine the trend between groups ($^{*}p < 0.05$, $^{***}p < 0.0001$). Representative images from two HCC cores A5 and E10 shows the expression of c-Met (green) (C.a., D.a.); miR-181a-5p (red) (C.b., D.b.); U6 (red) (C.c., D.c.); and DAPI (blue nuclear staining). Merged images of c-Met and DAPI (C.d., D.d.), miR-181a-5p and DAPI (C.e., D.e.), U6 and DAPI (C.f., D.f.) were shown. U6 was chosen as the reference in ISH studies as it is stably expressed in cell (Bar = 200 μm).

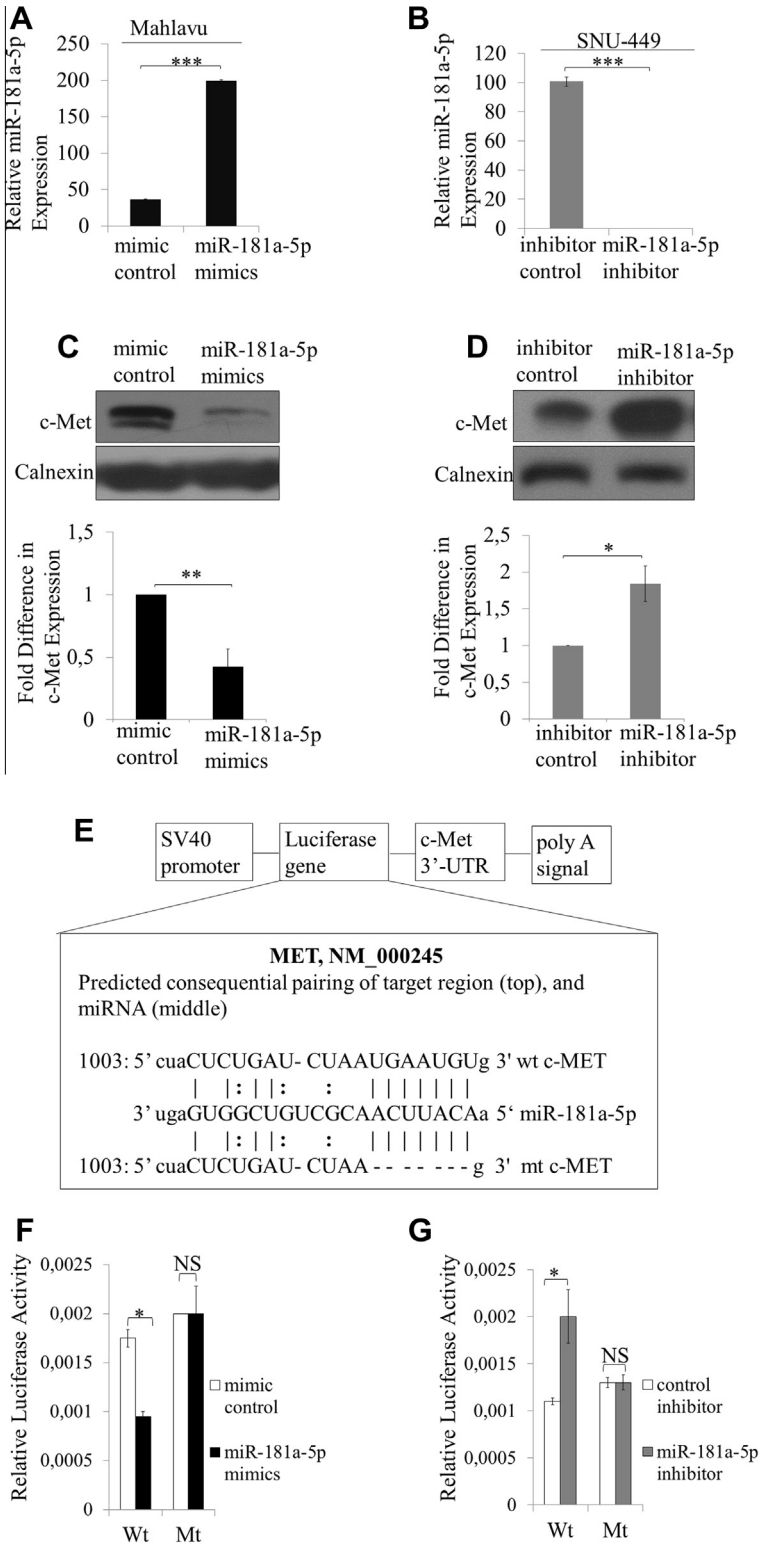

Fig. 2. Direct interaction of miR-181a-5p with c-Met 3′-UTR. The expression analysis of miR-181a-5p and c-Met was performed after transfecting MV-cells for 24 h with miR-181a-5p mimics or mimic-control and SNU-449-cells with miR-181a-5p-inhibitor or inhibitor-control. miR-181a-5p expression in (A) MV and (B) SNU-449 was analyzed by RT-PCR. The results were normalized to RNU6B. The protein content of c-Met was determined by WB in (C) MV and (D) SNU-449. Calnexin was used as an internal standard. The graph depicting results from the densitometry quantitation of the c-Met band, which was normalized to calnexin. Values from the control mimic/inhibitor were set at 1. (E) Schematic representation describing the 3′-UTR luciferase reporter assay. Sequence alignments of miR-181a-5p with 3′UTR of c-Met. Deletion was generated on the c-Met 3′-UTR sequence in the complementary site for the seed region of the miR-181a-5p. SNU-449 cells were co-transfected with either miR-181a-5p (F) mimics or (G) inhibitor or with their corresponding controls, and plasmid reporter containing wild type (wt) or mutant (mt) 3′UTR. Activity of plasmids co-transfected with mimic-control or inhibitor-control was used as control. The results were normalized to firefly luciferase activity. Values from control mimic/inhibitor were set at 1. Data are represented as ±S.E. ($n = 4$). $^{***}p < 0.0001$, $^{**}p < 0.001$, $^{*}p < 0.05$.

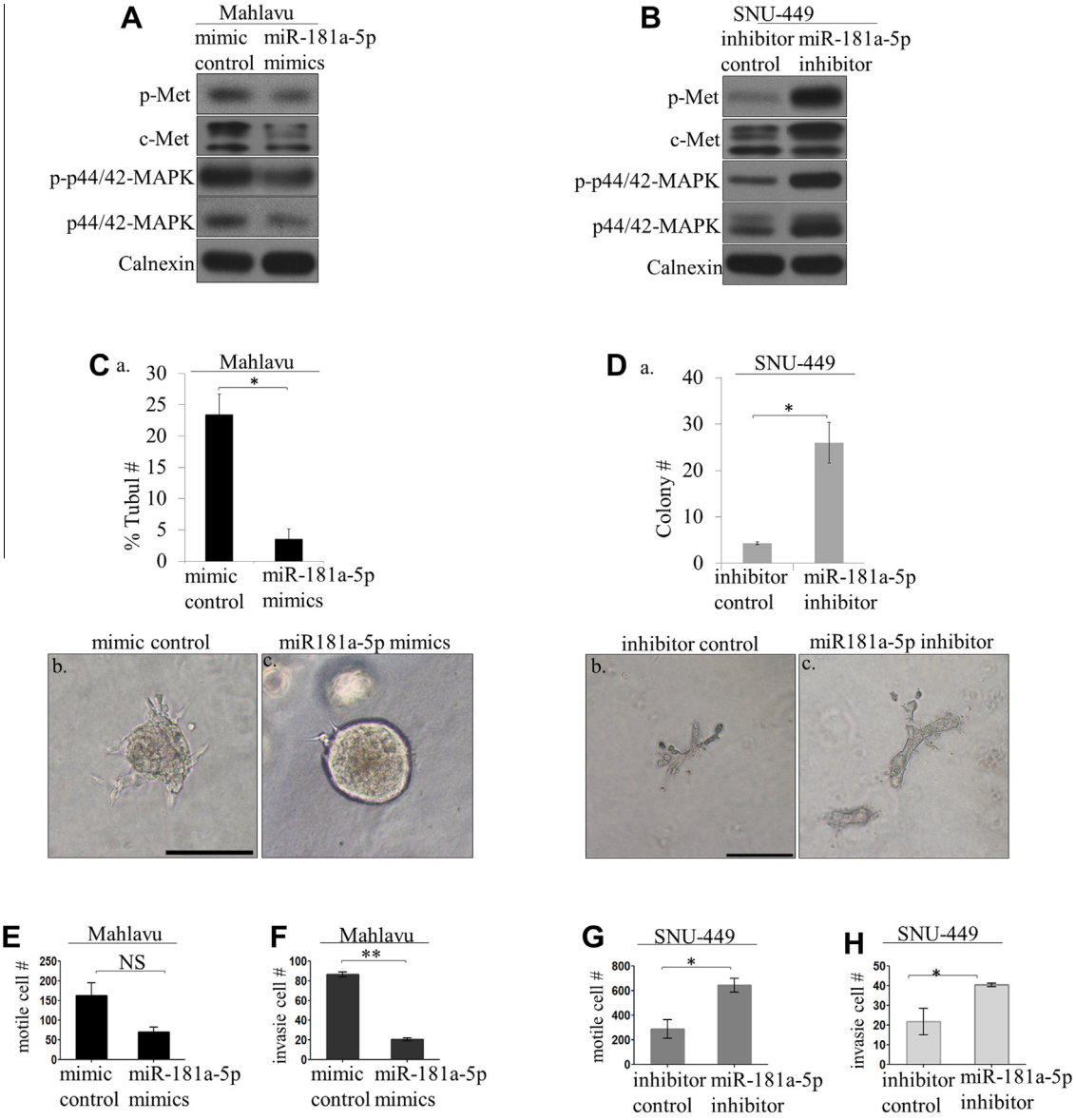

Fig. 3. Impact of differential expression of miR-181a-5p on c-Met signaling pathway. (A–H) MV-cells transfected with miR-181a-p-mimics or mimic-control and SNU-449-cells transfected with miR-181a-p-inhibitor or inhibitor-control were subjected to WB analysis, branching-morphogenesis, migration and invasion assays. WB analysis was performed with the indicated antibodies and calnexin was used as loading control. (C) The results obtained from MV were plotted as the average number of cysts able to undergo morphogenesis per culture per 100 for MV cells. (D) The results for SNU-449 were calculated as the average colony number form per culture. Images show a representative experiment that had been performed in quadruplicate. (E–H) Migration (E and G) and invasion (F and H) assays were performed in triplicates with mean ± S.E. (NS: not significant, $^{**}p < 0.001$, $^{*}p < 0.05$; Bar: 200 μm.)

3.5. miR-181a-5p expression modulated HGF-dependent c-Met signaling

To confirm the importance of miR-181a-5p as a functional regulator of the c-Met signaling pathway, we treated miR-181a-5p-overexpressed MV-cells and miR-181a-5p-knockdown SNU-449-cells with HGF and SU11274. We demonstrated that MV and SNU-449 are HGF responsive cell lines (Fig. S4). The forced expression of miR-181a-5p decreased HGF-induced phosphorylation of both c-Met and MAPK (Fig. 4A). However, knockdown of miR-181a-5p upregulated HGF-induced phosphorylation of both c-Met and MAPK (Fig. 4B). Predictably, SU11274 inhibited HGF-induced phosphorylation of c-Met and MAPK in all cell types (Fig. 4A and B). Thus, these data suggest that miR-181a-5p may modulate the c-Met signaling pathway at multiple points, including affecting c-Met protein and activation levels and affecting the levels of the downstream effector MAPK.

We and others have shown that upon HGF binding, activated c-Met triggers multiple signaling pathways, including p44/42-MAPK, to promote cell migration, invasion and branching-morphogenesis, and SU11274 abrogated the biological responses induced by HGF [8]. Using the branching-morphogenesis assay, we observed that under HGF induction, ~72% of control cells form tubular structures, whereas ~53% of miR-181a-5p overexpressed cysts could undergo branching-morphogenesis with simple tubular structure and less branching than was seen with the control group (Fig. 4C(a)–(c)) ($p < 0.05$). In the presence of SU11274, only ~54% of control cells (Fig. 4C(a) and (d)) and ~38% of miR-181a-5p overexpressed cells (Fig. 4C(a) and (e)) underwent morphological changes in response to HGF. These results suggest that the combination of miR-181a-5p and SU11274 had additive inhibitory effects on branching-mor-

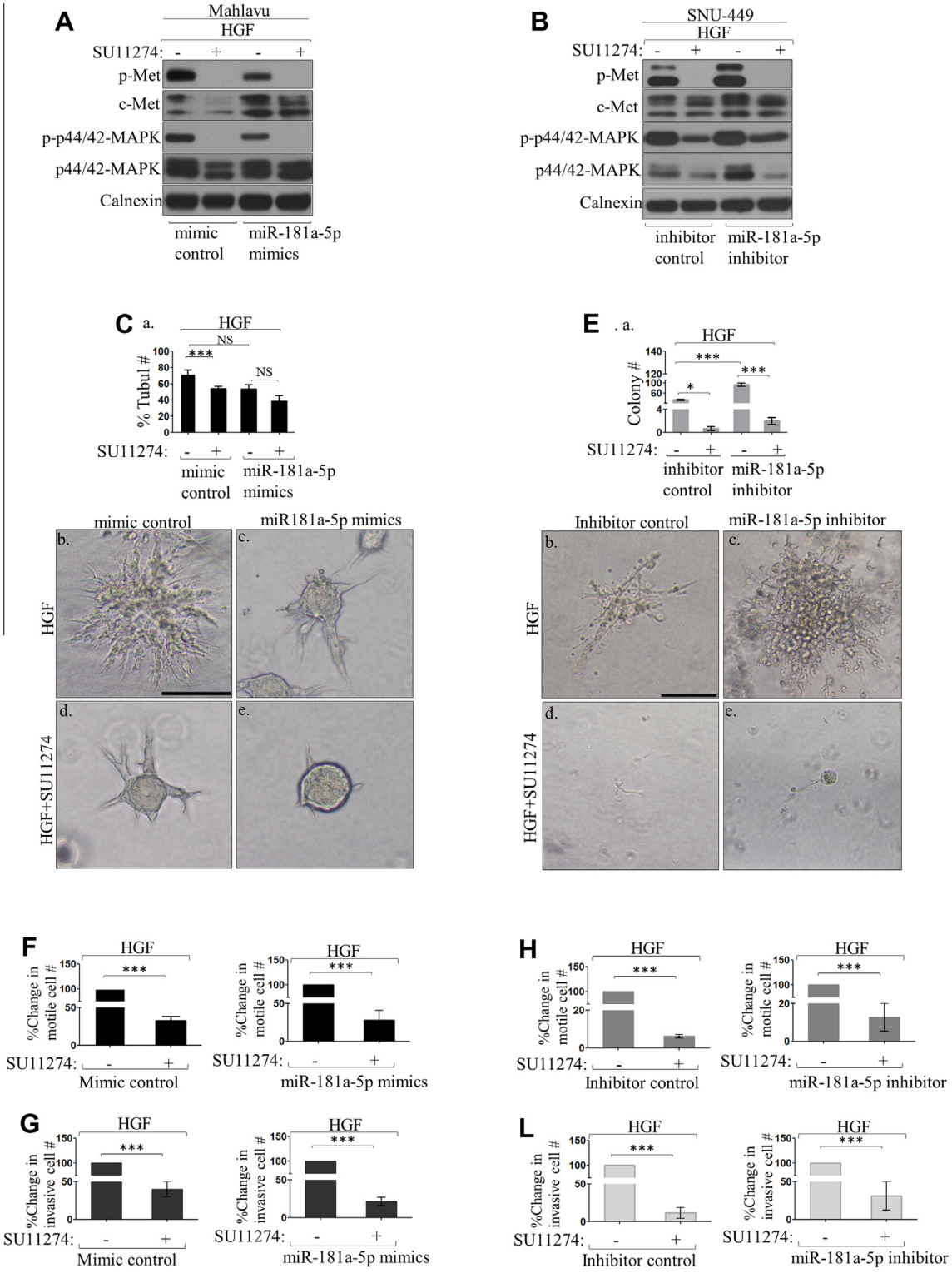

Fig. 4. Regulation of HGF-dependent cell migration, invasion, and branching-morphogenesis by miR-181a-5p. MV-cells transfected with miR-181a-5p-mimics or mimic-control and SNU-449-cells transfected with miR-181a-5p-inhibitor or inhibitor-control were pretreated with or without SU11274 in the presence of HGF. (A and B) WB analysis was performed with the indicated antibodies and calnexin was used as loading control. (C) Columns show the mean number of cysts able to undergo morphogenesis. (D) The results show the average colony number. Images show a representative experiment that had been performed in quadruplicate. Changes in the (E–G) migrative (F and H) and invasive (F and H) cell number. Data represented with mean ± S.E. ($n = 4$; NS: not significant, $^{*}p < 0.05$, $^{***}p < 0.05$; Bar: 200 μm.)

phogenesis (Fig. 4C(a) and (e)). In the presence of HGF, miR-181a-5p knockdown cells formed more branching tubules with highly branched and dense structures than observed with the HGF-treated control group (Fig. 4E(a)–(c)) ($p < 0.0001$). Strikingly, the co-incubation of HGF with SU11274 prevented a tubule formation in both the control and miR-181a-5p-inhibitor-treated group ($p < 0.05$). However more colony formed in miR-181a-5p-treated group compared to control when cells co-treated with SU11274

and HGF (Fig. 4E(a), (d), and (e)). This data indicates that SU11274 was less effective in the miR-181a-5p-inhibitor-treated group compared to the respective control. Consistently, the inhibitory effect of SU11274 on HGF-induced migration and invasion was increased by the restoration of miR-181a-5p expression in MV-cells (reduction in migration: ~67% vs ~75%; reduction in invasion: ~54% vs ~77% reduction) (Fig. 4F and G). Moreover, the knockdown of miR-181a-5p expression diminished the effectiveness of SU11274 against HGF-induced migration and invasion (reduction in migration: ~95% vs ~87%; reduction in invasion: ~89% vs ~69%) (Fig. 4H and I). These results suggested that the overexpression of miR-181a-5p sensitized cells to SU11274 and the loss of miR-181a-5p expression might improve HGF-dependent c-Met signaling and consequently increase HGF/c-Met-dependent migration, invasion and branching-morphogenesis.

4. Discussion

The activation of c-Met signaling is important for the promotion of tumor growth, angiogenesis, and metastasis and is the major cause of poor prognosis for HCC patients. Thus, the determination of the molecular mechanisms of c-Met dysregulation is important for the development of effective therapeutic approaches in HCC. In this study, we identified that c-Met is a functional target gene of miR-181a-5p and the loss of miR-181a-5p expression led to the activation of c-Met-mediated oncogenic signaling in hepatocarcinogenesis.

miR-181a-5p belongs to the miR-181s family, which contains four highly conserved mature miR-181s (miR-181a, miR-181b, miR-181c, miR-181d) [15–20]. miR-181a-5p functions both as oncogenes and tumor suppressors depending upon the origin of the relevant tissues and cellular context [15–20]. HCC versus healthy liver miRNA expression profiling studies revealed that miR-181a-5p has no unique expression pattern in HCC. While some studies showed that miR-181a-5p is down-regulated, others reported the up-regulation of miR-181a-5p during hepatocarcinogenesis [15–20]. In the light of previous microarray studies and bioinformatic analyses, we identified that miR-181a-5p directly binds to the 3′-UTR of c-Met to regulate its expression in HCC cells, and this conclusion was supported by the following reasons: complementary sequence of miR-181a-5p is identified in the 3′-UTR of c-Met mRNA; overexpression of miR-181a-5p led to a significant reduction in c-Met 3′-UTR-luciferase hybrid; depletion of miR-181a-5p caused a significant rise in c-Met 3′UTR luciferase activity and these effects were abolished by the deletion mutation of the miR-181a-5p seed binding site. Relevantly, the forced expression of miR-181a-5p decreased c-Met expression and miR-181a-5p depletion caused a significant rise in the c-Met protein content. More importantly, we showed a significant decreased expression trend of miR-181a-5p and increase trend of c-Met expression toward HCC compared to cirrhotic and normal liver tissues. The development of HCC is a multi-step process from chronic hepatitis, to cirrhosis, to dysplastic nodules, and to malignant tumors [22]. In our knowledge, there is no study that analyzes the expression of miR-181a-5p in normal, cirrhotic and HCC liver samples. This study will be the first study reporting the inverse correlation between c-Met and miR-181a-5p expression during hepatocarcinogenesis.

miRNAs are known to achieve functional specificity by targeting a core network of genes that belong to the same pathway and this interaction is highly dependent on the relative abundance of multiple mRNA targets. Based on this information, we evaluated the influence of miR-181a-5p on the c-Met signaling pathway. In human carcinomas, the up-regulation of c-Met has been found to cause receptor dimerization and transactivation even in the absence of HGF [23]. Consistently, we established that the loss of miR-181a-5p expression resulted in the gain of c-Met protein content, which in turn induced HGF-independent c-Met activation, and consequently accelerated cellular motility, invasion and branching-morphogenesis. Strikingly, we further supported our findings on the suppressive function of miR-181a-5p on c-Met signaling with the observation that miR-181a-5p overexpression reduced HGF-mediated cellular activities similar to phenotypes observed when the c-Met inhibitor is used. More importantly, the restoration of miR-181a-5p expression sensitized cells to anti-migratory, anti-invasive, and anti-morphogenetic activities of the c-Met inhibitor. Overall, our data emphasized that miR-181a-5p is a repressor of HGF/c-Met-driven cellular activities of HCC cells.

In conclusion, our study provides mechanistic insight into c-Met overexpression as a consequence of miR-181a-5p downregulation during hepatocarcinogenesis. miR-181a-5p exerted its functional role by targeting c-Met expression and the loss of miR-181a-5p expression was connected with the acquisition of an aggressive phenotype. These findings will facilitate a better understanding of the molecular pathogenesis of HCC and suggest that the propagation of miR-181a-5p expression may be utilized as a novel strategy in combination with the c-Met inhibitor.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2014.06.142.

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