Lin-28B expression promotes transformation and invasion in human hepatocellular carcinoma

Yun-Chu Wang1, Yu-Ling Chen1, Ray-Hwang Yuan2, Hung-Wei Pan2, Wan-Ching Yang1,3, Hey-Chi Hsu1,3 and Yung-Ming Jeng1,3,*

1Graduate Institute of Pathology, National Taiwan University, Taipei 100, Taiwan and 2Department of Surgery and 3Department of Pathology, National Taiwan University Hospital, Taipei 100, Taiwan

*To whom correspondence should be addressed. Tel: +886 2 23123456; Fax: +886 223934172; Email: mrna0912@yahoo.com.tw

MicroRNAs (miRNAs) play critical roles in embryonic development and are frequently deregulated in human cancers. The let-7 family members are tumor-suppressing miRNAs and are frequently downregulated in cancer cells. Lin-28 and Lin-28B are RNA-binding proteins highly expressed in embryonic tissues. Lin-28 proteins block let-7 precursors from being processed to mature miRNAs by inducing terminal uridylation and degradation of let-7 precursors. Here, we report that Lin-28B, but not Lin-28, is highly expressed in hepatocellular carcinoma (HCC). Lin-28B expression was more frequently noted in high-grade HCCs with high α-fetoprotein levels. Knockdown of Lin-28B by RNA interference in the HCC cell line HCC36 suppressed proliferation in vitro and reduced in vivo tumor growth in NOD/SCID mice. In contrast, overexpression of Lin-28B in the HCC cell line HA22T enhanced tumorigenicity. Overexpression of Lin-28B also induced epithelial-mesenchymal transition in HA22T cells and hence, invasion capacity. Large-scale real-time PCR array analysis revealed that, among 380 miRNAs, only let-7/mir-98 family members were regulated by Lin-28B. Lin-28B overexpression enhanced the expression of the known let-7 targets c-myc and HMGA2. It was also found that Lin-28B enhanced the expression of type 1 insulin-like growth factor receptor in a let-7-dependent manner. These results indicate that Lin-28B regulates tumor formation and invasion in HCC through coordinated repression of the let-7/mir-98 family and induction of multiple oncopgenic pathways.

Introduction

Hepatocellular carcinoma (HCC) is one of the most common fatal malignancies in Taiwan and many other countries in Asia and Africa (1). In Western countries, the incidence of HCC is increasing mainly because of the prevalence of chronic hepatitis C infection (2). The major risk factors for HCC are hepatitis B and hepatitis C virus infections, liver cirrhosis of any etiology and aflatoxin exposure (3). Molecular approaches have revealed the involvement of p53 and β-catenin mutations in hepatocarcinogenesis (4,5). These mutations account for ~50% of HCCs; somatic mutations of other known oncogenes and tumor suppressor genes in HCC are rare. Hence, the molecular mechanisms of HCC, particularly in the early stage of disease, remain largely unclear.

MicroRNAs (miRNAs) are small, non-coding RNAs composed of ~21 nucleotides that regulate gene expression by either impediment translation or destabilizing messenger RNA (mRNA). miRNAs play critical roles in diverse biological processes such as development and differentiation, control of cellular proliferation, stress response and metabolism (6). Dysregulations of miRNAs are frequently seen in cancer cells and contributes to tumorigenesis (7). The let-7 miRNAs are highly conserved in bilateral animals and controls the timing of stem cell division and differentiation (8). Let-7 also functions as a tumor suppressor by targeting Ras (9), high mobility group AT-hook 2 (HMGA2) (10) and c-Myc (11) and inhibits cell proliferation and tumorigenesis when ectopically expressed (12,13). Low expression levels of let-7 have been observed in a variety of cancers, including HCC (14). Recently, it was shown that the processing of pri-let-7 miRNA is selectively blocked by Lin-28, which recruits TUTase 4 and induces uridylation of precursor let-7 at the 3’ end (15–17). In turn, the uridylated let-7 precursor fails to be processed by Dicer and undergoes degradation (16).

Lin-28 was initially described as a regulator of developmental timing in Caenorhabditis elegans (18). It consists of two domains that contain RNA-binding motifs: the N-terminal cold shock domain and a pair of retroviral-type CCHC zinc fingers near the C-terminus. Two orthologues of Lin-28 exist in the genome of mammals: Lin-28 and Lin-28B. Both proteins have high homology to the Lin-28 protein from C.elegans in RNA-binding domains, indicating functional conservation. Lin-28 is highly expressed in embryonic stem cells and in early embryo development (19) and is one of the four genes used to produce inducible pluripotent stem cells (20). Lin-28 plays a central role in the generation and/or maintenance of stem cells. In contrast, Lin-28B is abundantly expressed in placenta, testis and fetal liver (22), but its function in development remains unclear.

Deregulation of Lin-28 expression was reported in germ cell tumors (21) and ovarian cancers (23). Lin-28 is overexpressed in ~15% of cancer cell lines and this overexpression enhances transformation (24). While overexpression of Lin-28B has been reported in HCC (22), the clinicopathological and functional significance is unknown. In this report, we demonstrate that Lin-28B, but not Lin-28, is frequently overexpressed in HCC and the expression of Lin-28B contributes to tumorigenesis and invasion of HCC through activation of multiple let-7-regulated oncopgenic pathways.

Materials and methods

Liver samples

From 1982 to 1998, 187 patients with surgically resected, unifocal primary HCCs were included in this study. Resected tumors underwent detailed pathological assessment and patients underwent regular follow-up at the National Taiwan University Hospital. The study was conducted according to the regulations of Ethics Committee. The resected specimens were anonymous and analyzed in a blinded manner. The patients included 141 men and 46 women with a mean age of 55.17 years (range 15–88 years). Serum hepatitis B surface antigen (HBsAg) and anti-hepatitis C virus (anti-HCV) antibody was detected in 121 and 53 patients, respectively, including 14 cases that were positive for both HBsAg and anti-HCV. Liver cirrhosis was found in 68 cases (36%). None of the included patients had received transhepatic arterial embolization or chemotherapy prior to surgical resection of the HCC.

Histology study and tumor staging

Surgically resected specimens were formalin-fixed and paraffin embedded. Histologic sections cut at 5 μm thickness were stained with hematoxylin and eosin and reviewed by one of the authors (H.C.H.) to determine tumor grade and stage. The tumor grade was based on the criteria proposed by Edmondson and Steiner (25) and tumors were staged according to the American Joint Commission on Cancer system (26). The surgical margins of each specimen were inked and analyzed microscopically. Only completely resected specimens were included in this study.

© The Author 2010. Published by Oxford University Press. All rights reserved. For Permissions, please email: journals.permissions@oxfordjournals.org
RNA isolation and reverse transcription–polymerase chain reaction
Total RNA was isolated from tissue samples and cell lines using the Trizol reagent (Life Technologies, Invitrogen, Carlsbad, CA), according to the manufacturer’s instructions. Reverse transcription–polymerase chain reaction (PCR) was used to determine the mRNA level of Lin-28 and Lin-28B in the paired HCC and non-tumorous liver samples. S26 ribosomal protein mRNA, a housekeeping gene, was used as an internal control (27). PCR was arrested during the exponential phase for each gene (i.e., 32 cycles for Lin-28 and Lin-28B and 22 for S26). PCR was performed in an automatic DNA thermal cycler (PerkinElmer, Wellesley, MA) with initial heating at 94°C for 2 min followed by cycles at 94°C for 30 s, 55°C for 1 min, 72°C for 1 min and finally, 72°C for 10 min. The primers for Lin-28 were Lin-28-F (5'-CGCTTGAATGACGAGACG-3') and Lin-28-R (5'-GGTGCTTCTGGTCGACT-3'). The primers for Lin-28B were Lin-28B-F (5'-GACCCAAAAGGAGACGAC-TA-3') and Lin-28B-R (5'-TTCTCATCTGGACTCGGGC-3'). The primers for S26 were S26-F (5'-CCGAGCCAGGATGACAAAA-3') and S26-R (5'-GTTGGTCTGTCGTTGACT-3').

Real-Time PCR
Real-time PCR was performed with the ABI PRISM 7000 Sequence Detection System (Applied Biosystems, Foster City, CA) using the SYBR Green method. Housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as a control. The primers for Lin-28 were Lin-28-F2 (5'-CTCTGGCAGAGTATCAGTGC-3') and Lin-28-R2 (5'-GGTGGCTGTCGTCGACT-3'). The primers for GAPDH were GAPDH-F (5'-AGGCCTCAAGATCAGACCTGC-3') and GAPDH-R (5'-TTGGTCTGATGAGTTCAC-3').

Cell culture
All cell lines were grown in Dulbecco's modified Eagle's medium (DMEM) (36). Cell culture was performed in a humidified atmosphere composed of 95% air and 5% CO2. The cells were passaged with trypsin/ethylenediamine-tetracetic acid at 80–90% confluence.

Plasmid, transfection and retroviral infection
The open reading frame of Lin-28B was amplified in Hep3B cells by reverse transcription–PCR and cloned to the expression vector pCMV-Tag2C (Stratagene, La Jolla, CA) to generate a pCMV-Lin-28B vector. FLAG-tagged Lin-28B was cut from the pCMV-Lin-28B construct and subcloned into the NotI-Pacl site of pQXCIPl (Clontech, Mountain View, CA) to generate pQXCILin-28B that was subsequently cotransfected with pSVS-G (Clontech) into the retroviral packaging cells 293-GP2 to produce retrovirus using the Lipofectamine 2000 reagent (Invitrogen). After incubating with medium containing containing retroviral particles for 2 days, the target cells were treated with puromycin (2 μg/ml; Clontech) for 2 weeks to select the cells with stable integration of retroviral vectors.

RNA interference
For the knockdown of the endogenous Lin-28B, the following target sequences were constructed in the small hairpin RNA (shRNA) vector pLKO.1: Lin-28B-1: 5'-GCAGCCGATAAAGACGTTA-3'; Lin-28B-2: 5'-GCCTTGAGGTCTGGACT-3'; Lin-28B-3: 5'-CATTCTGATCATGCCATG-3'; and Lin-28B-4: 5'-GCCATAGTATTGGTCCTGTTA-3'. The shRNA vector pLKO.1-shLuc was used as a negative control. For lentivirus production, 293T cells were transfected with 4 μg pLKO.1 lentiviral vectors along with 0.4 μg of envelope plasmid pMD.G and 3.6 μg of packaging plasmid pCMVΔR8.91. Virus was collected 24 and 48 h after transfection. To prepare Lin-28B-knockdown cells, HCC36 cells were infected with lentivirus for 24 h. Fresh medium containing 2 μg/ml puromycin was replaced every 3 days for 2 weeks to select cells with stable integration of retroviral vectors.

Western blot
Protein samples (60 μg each) were subjected to 10% sodium dodecyl sulfate–polyacrylamide gel electrophoresis and then electrotransferred onto nitrocellulose membranes (Amersham, Buckinghamshire, UK). The membranes were allowed to react with primary and secondary antibodies at the optimum dilutions and the immunoreactive signals were detected using an enhanced chemiluminescence kit (Amersham). The antibodies used included E-cadherin (1:400; Santa Cruz Biotechnology, Santa Cruz, CA), N-cadherin (1:400; Abcam, Cambridge, MA), HMG2 (1:1000; Abcam), Snail (1:1000; Abcam), Twist (1:1000; Santa Cruz), vimentin (1:3000; Dako Cytomation, Carpinteria, CA), c-Myc (1:1000; Cell Signaling Technologies, Beverly, MA) and type 1 insulin-like growth factor I receptor (IGF1R, 1:1000; Cell Signaling Technologies).

Cell proliferation assay
To measure cell survival and proliferation, the 3-(4,5-dimethylthiazole-2-yi)-2,5-bisiphenyl tetrazolium bromide (MTT) assay was performed using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazoliumbromide. Cells (2000 each) were seeded into 96-well plates and incubated at 37°C in a humidified atmosphere with 5% CO2. At the appropriate time interval, MTT (2 mg/ml in phosphate-buffered saline) was added and incubated for 4 h. The colored formazan product, MTT formazan, was extracted with dimethyl sulfoxide and the absorbance at 540 nm measured.

Tumorigenicity assay
NOD/SCID mice (male, 4–6 weeks old) were purchased from the National Taiwan University Laboratory Animal Center and accommodated for 7 days for environmental adjustment prior to experimentation. Cells were trypsinized, resuspended in serum-free DMEM and injected subcutaneously (2 × 106) cells in a total volume 0.1 ml into both flanks. Animals were observed weekly for tumor development for 5–8 weeks, the final tumor weights at the time of animal killing were recorded.

Boydren cell assay
For the invasion assays, a modified Boydren chamber with filter inserts (pore size, 8 μm) coated with Matrigel (40 μg; Collaborative Biomedical, Becton Dickinson Labware, Bedford, MA) in 24-well dishes (Nucleopore, Pleasanton, CA). Cells (2 × 105) in 100 μl of serum-free DMEM were placed in the upper chamber and 0.5 ml of DMEM with 10% fetal bovine serum was placed in the lower chamber. After 24 h in culture, cells were fixed in 3.7% formaldehyde for 15 min and then stained with 0.05% crystal violet and 4'-6-diamidino-2-phenylindole in phosphate-buffered saline for 15 min. Cells on the upper side of the filters were removed with cotton-tipped swabs and the filters were washed with phosphate-buffered saline. Cells on the underside of the filters were viewed and counted under a fluorescent microscope. Each group was placed in triplicate in each experiment and each experiment was performed twice. The cell motility assay was done in the same way as invasion assay except for the absence of Matrigel coating and the incubation time is 20 h.

Wounding assay
HA22T cells were grown to confluence in six-well culture plates. Cell layers were scraped with a sterile pipet tip and reincubated at 37°C. Photographs were taken at indicated time points. Migration from the edge of the injured monolayer was quantified by measurement of the distance between the wound edges. Experiments were done in triplicate and repeated thrice.

miRNA quantification
miRNA profiling was performed with the 7900HT Fast Real-Time PCR System (Applied Biosystems) using the MegaplexTM Pool A for miRNA expression assays (Applied Biosystems) according to the manufacturer’s protocol. Selected miRNAs targets were verified using the TaqMan Micro individual RNA assays (Applied Biosystems).

Reporter assay
The three putative let-7-binding sites in the 3′-untranslated region (UTR) of IGFR1 was amplified by PCR (site 1: nt4155-4801, site 2: 6522-7149 and site 3: 10466-11077; refseq ID NM_000875) and cloned downstream to the open reading frame of firefly luciferase vector pMD.P-Report (Ambion, Austin, TX). Mutations were introduced by site-directed mutagenesis in the putative let-7-binding sites using the QuickChange Kit (Stratagene). Transient transfection was performed using the Lipofectamine 2000 (Invitrogen) reagent. Reporters were cotransfected with pQCXIP-Lin-28B or control plasmid pQCXIP, and the luciferase activity was quantified using the Dual-Glo Luciferase Assay System (Promega, Madison, WI) in an Orion II luminometer (Berthold Detection Systems, Pforzheim, Germany). Relative luciferase activity was compared using the QuickChange Kit (Stratagene). Transient transfection was performed using the Lipofectamine 2000 (Invitrogen) reagent. Reporters were cotransfected with pQCXIP-Lin-28B or control plasmid pQCXIP, and the Renilla luciferase plasmid TK-Renilla into HA22T cells grown in 12-well plates at 70% confluence. Twenty-four hours after transfection, cell extracts were prepared and the luciferase activity was quantified using the Dual-Glo Luciferase Assay System (Promega, Madison, WI) in an Orion II luminometer (Berthold Detection Systems, Pforzheim, Germany). Relative luciferase activity was determined by comparing the firefly luciferase activity to Renilla luciferase activity. All experiments were performed in triplicate.

Statistical analysis
The data analyses were carried out using Epi Info (version 3.3.2; Centers for Disease Control and Prevention) software. Correlation between Lin-28B expression and clinicopathological parameters was evaluated using χ2 and Fisher’s exact tests. Two-tailed P < 0.05 was considered statistically significant.

Results
Lin-28B is frequently overexpressed in HCC
Reverse transcription–PCR was employed for semiquantitative measurement of Lin-28 and Lin-28B mRNA expression in HCC. Initial screening of surgically resected HCCs from 22 patients revealed...
that Lin-28B was overexpressed in 10 cases, but Lin-28 was overexpressed in only one case (Figure 1). Therefore, we focused on Lin-28B in the subsequent studies. To determine the clinicopathological significance of Lin-28B expression, 162 unifocal HCCs were analyzed by real-time PCR. Lin-28B was expressed in 60 tumors (37.0%). In contrast, none of the 102 paired non-tumorous liver RNA samples available for examination expressed Lin-28B. Lin-28B expression correlated with high-serum α-fetoprotein level (P = 0.04) and high tumor grade (P = 0.007), but not with age, size, tumor stage or early tumor recurrence (Table I). Lin-28B expression did not appear to be a prognostic factor for HCC.

**Lin-28B enhances growth and tumorigenicity of HCC**

To investigate the role of Lin-28B in the tumorigenesis of HCC, five different lentivirus constructs carrying Lin-28B shRNA obtained from the National RNAi Core Facility (Academia Sinica, Taipei, Taiwan) were used to transduce HCC cell line HCC36. Cells transduced with shRNAs #1, #2 and #5 resulted in a dramatic reduction of Lin-28B mRNA expression (Figure 2A). ShRNAs #1 and #5 were selected for use in subsequent studies.

Stable knockdown of Lin-28B by both shRNAs #1 and #5 inhibited HCC36 cell proliferation in vitro, as measured by the MTT assay (Figure 2B). The role of Lin-28B in tumor formation was assessed using the HCC36 parental, negative control and sh-Lin-28B cells in NOD/SCID mice. The tumor weights in both Lin-28B-knockdown groups (i.e. using small interference RNAs #1 and #5) were dramatically reduced compared with both the parental HCC36 cell and vector control groups (Figure 2C and D). In contrast, retroviral transduction of Lin-28B in HA22T cells, which have low endogenous Lin-28B, enhanced cell proliferation (Figure 2E).

After subcutaneously injecting HA22T cells overexpressing Lin-28B into NOD/SCID mice, the vector control group had no tumors after 8 weeks, but large tumor masses were found in the mice injected with Lin-28B-overexpressing HA22T cells (Figure 2F and G).

**Lin-28B enhances epithelial-mesenchymal transition and tumor invasion**

After culturing cells for 2 weeks, the morphology of Lin-28B-overexpressing HA22T cells changed from cobblestone-like epithelium to spindle-shaped single cells or clusters without well-defined cell–cell contacts in low-density cultures. When grown to confluence, the Lin-28B-overexpressing HA22T cells were arranged in a whorl-like pattern with loss of contact inhibition instead of the pavement-like monolayer pattern noted in vector control cultures (Figure 3A). This morphological change was reminiscent of epithelial-mesenchymal transition (EMT).

Western blot analysis showed induction of the mesenchymal markers vimentin and N-cadherin and reduced expression of the epithelial marker E-cadherin (Figure 3B). Transcription repressors Snail and Twist are major EMT inducers in HCC (28). Therefore, we tested whether Lin-28B enhances the expression of these two EMT inducers and found Lin-28B induced the expression of Twist, but not Snail in HA22T (Figure 3B). The migration and invasive capacity of Lin-28B-expressing HA22T cells were upregulated, as demonstrated by the wounding assay (Figure 3C) and modified Boyden chamber assays (Figure 3D).

**Let-7/mir-98 family is the only target (among 380 miRNAs) of Lin-28B in HCC**

Previous studies using let-7 to identify interacting proteins found that Lin-28 and Lin-28B are associated with let-7 precursors (15–17). To determine if the let-7 family is either the most important or the only target of Lin-28B, the miRNA expression profiles of HA22T cells with Lin-28B and vector controls were determined using a large-scale real-time PCR array containing 380 miRNA targets. Of the 380 miRNAs analyzed, 111 were expressed significantly in HA22T (at a cycle threshold of < 32). Of these 111 miRNAs, only let-7/mir-98 family members (let-7a, let-7b, let-7d, let-7e, let-7f, let-7g and mir-98) and mir-15a were reduced by >2-fold by Lin-28B overexpression (Figure 4A). The let-7/mir-98 family members, but not mir-15a, were confirmed to be reduced in independent experiments (Figure 4B).

Knockdown of Lin-28B enhanced the expression of let-7/mir-98 family members, but not mir-15a (Figure 4C). These results indicate that, among 380 miRNAs analyzed, the let-7/mir-98 family is the only target of Lin-28B in HCC.

**Lin-28B activates several oncogenic pathways**

To elucidate the mechanisms of Lin-28B in carcinogenesis, known targets of let-7 were evaluated. Lin-28B overexpression potently increased the protein abundance of c-Myc and to a lesser extent, HMGA2 (Figure 5A). For identification of additional proteins affected by the Lin-28B/let-7 pathway, we searched TargetScan microRNA-binding site prediction program (www.targetscan.org) and found IGFlR has three putative binding sites of let-7/mir-98 family in the 3’-UTR (Figure 5B) that are highly conserved among vertebrates. IGFlR is a high affinity receptor of insulin-like growth factor (IGF)-1 and IGF-II. Activation of IGFlR is a critical event in the malignant transformation and progression of HCC (29). Overexpression of Lin-28B enhanced the expression of IGFlR (Figure 5A).

### Table I. Clinicopathological significance of Lin-28B expression in HCC

<table>
<thead>
<tr>
<th>Age (years old)</th>
<th>+, N = 60</th>
<th>–, N = 102</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>≤55</td>
<td>20</td>
<td>47</td>
<td>Not significant</td>
</tr>
<tr>
<td>&gt;55</td>
<td>40</td>
<td>55</td>
<td></td>
</tr>
<tr>
<td>HBsAg (–)</td>
<td>14</td>
<td>31</td>
<td>Not significant</td>
</tr>
<tr>
<td>(+)</td>
<td>43</td>
<td>64</td>
<td></td>
</tr>
<tr>
<td>α-Fetoprotein (ng/ml) ≤200</td>
<td>26</td>
<td>62</td>
<td>0.04</td>
</tr>
<tr>
<td>&gt; 200</td>
<td>32</td>
<td>39</td>
<td></td>
</tr>
<tr>
<td>Size (cm) ≤5</td>
<td>29</td>
<td>55</td>
<td>Not significant</td>
</tr>
<tr>
<td>&gt; 5</td>
<td>31</td>
<td>47</td>
<td></td>
</tr>
<tr>
<td>Grade I</td>
<td>3</td>
<td>20</td>
<td>0.007</td>
</tr>
<tr>
<td>II</td>
<td>33</td>
<td>44</td>
<td></td>
</tr>
<tr>
<td>III–IV</td>
<td>21</td>
<td>29</td>
<td></td>
</tr>
<tr>
<td>Stage I</td>
<td>27</td>
<td>35</td>
<td>Not significant</td>
</tr>
<tr>
<td>II</td>
<td>13</td>
<td>20</td>
<td></td>
</tr>
<tr>
<td>III</td>
<td>18</td>
<td>38</td>
<td></td>
</tr>
<tr>
<td>Early recurrence No</td>
<td>27</td>
<td>39</td>
<td>Not significant</td>
</tr>
<tr>
<td>Yes</td>
<td>20</td>
<td>35</td>
<td></td>
</tr>
</tbody>
</table>
The three putative let-7-binding sites of human IGF1R were fused downstream to a luciferase reporter gene. These three constructs were transfected with either Lin-28B or vector control into HA22T cells. The reporter activities of site 1 and 3, but not site 2, were enhanced by Lin-28B overexpression (Figure 5C). To test whether the activations were due to the derepression of the let-7-binding sites, the let-7-binding sites were mutated. The mutated site 1 was unresponsive to Lin-28B overexpression. Mutation of site 3 partially abolished the response to Lin-28B expression (Figure 5C). One noteworthy observation was that the mutated constructs had higher luciferase activity than the wild-type constructs in the vector control group, indicating an insensitivity to inhibition by endogenous let-7.

Discussion
This study confirmed that Lin-28B is overexpressed in HCC and that this expression is associated high serum α-fetoprotein level and high tumor grade indicating Lin-28B is more probably to be expressed in poorly differentiated tumors. Consistent with a recent publication (24), overexpression of Lin-28B enhances cell growth and tumor formation in SCID mice. Furthermore, shRNA knockdown of Lin-28B in HCC36 cells slows cell growth in vitro and dramatically reduces tumor growth in SCID mice. Together, these results indicate that Lin-28B is a bona fide oncogene.

This study also found that Lin-28B induced EMT and hence, tumor migration and invasion. A similar phenotype is seen in breast cancer cell line MB-231 (data not shown) indicating this phenomenon is not cell type specific. Until now, no direct evidence of let-7 in preventing EMT is reported. Although the EMT inducer Twist is induced by Lin-28B overexpression, no let-7 binding is identifiable in the 3′-UTR or coding region of Twist. Of the known let-7 targets, HMGA2 was reported to elicit EMT by transcriptional activation of other EMT inducers, including Twist (30). Myc and Ras induces features of EMT in breast cancer cell lines by activation of Twist promoter activity (31). IGF signaling also drives EMT and tumor invasion via upregulation of Zeb1 and Snail in different types of cancers (32,33). Therefore, in
addition to its role in cell growth, the Lin-28B/let-7 pathway enhances EMT and tumor invasion through coordinated activation of multiple
pathways.

Let-7/mir-98 family members are well-known targets of Lin-28 and Lin-28B. It is still controversial whether Lin-28 and Lin-28B regulate the biosynthesis of other miRNAs. Chang et al. (34) reported enforced Lin-28B expression reduces the expression level of miR-146a, mir-150 and mir-210. Lin-28 binds to pre-let-7 by recognizing a tetra-nucleotide sequence motif (GGAG) in the terminal loop (17). Similar motifs are present in the terminal loops of mir-107, mir-143, mir-200c, mir-324 and mir-363 and it was reported that Lin-28 and TUT4 bind to these pre-miRNAs and induces terminal uridylation in vitro (17). In our large-scale real-time PCR analysis, all let-7/mir-98 family members tested were repressed by Lin-28B expression and enhanced by knockdown of Lin-28B. No other miRNA was significantly affected by Lin-28B expression. These observations indicate that the let-7/mir-98 family is the most important, if not the only target of Lin-28B in HCC. Interestingly, Lin-28B itself is negatively regulated by let-7 (22). These observations suggest that Lin-28B and let-7 form a positive feedback circuit in mediating oncogenesis.

The IGF signaling pathway plays a pivotal role in cell proliferation (35), prevention of apoptosis (36) and initiation and maintenance of oncogenesis (37). Overexpression of IGF1R has been observed in many different types of cancers, including HCC. One study analyzing mRNA levels of 15 pairs of HCC and non-tumorous liver parenchyma showed that the mRNA level of IGF1R was not significantly overexpressed in the tumor (38). However, another study showed that the protein level was increased in ~30% of the HCCs (39), indicating that regulation occurs at the posttranscriptional level. The transcript of IGF1R has an unusually long 3'-UTR (7088 nucleotides), which contains three putative let-7-binding sites that are conserved among vertebrates. This current study demonstrates that Lin-28B enhances the expression of IGF1R through derepression at these let-7-responsive sites. Interestingly, putative let-7-binding sites are also present in the 3'-UTR of both IGF1 and insulin receptor substrate 2. Besides, Lin-28 was found to bind IGF-II mRNA directly and enhance the translation efficiency of IGF-II in developing skeletal muscle cells (40). In our experiments, mutation of site 3 did not completely abolish the effect of Lin-28B on the luciferase activity. Further study will be done to determine whether Lin-28B also regulates the translation efficiency of IGF1R by direct binding. It is probably that Lin-28B increases the expression of the ligand, the receptor and downstream signal molecules of IGF-II pathway through both let-7-dependent and independent mechanisms to enhance tumor growth.

In conclusion, aberrant expression of Lin-28B in HCC enhances tumor growth and invasion through activation of multiple oncogenic pathways in a let-7-dependent manner. Together, the restricted expression of Lin-28B in normal, mature tissues, its frequent expression in HCC, and the regulation of multiple oncogenic pathways make it a potential target of cancer therapy, particularly for HCC, which is one of the most common fatal malignancies worldwide and refractory to chemotherapy.
Fig. 4. miRNA targets of Lin-28B. (A) Real-time PCR array analysis identified that let-7/mir-98 family members and mir-15a were repressed by Lin-28B in HA22T cells. (B) Independent analysis showed that let-7/mir-98 family members, but not mir-15a, were repressed by Lin-28B. (C) let-7/mir-98 family members, but not mir-15a, were upregulated by knockdown of Lin-28B.

Fig. 5. Oncogenic pathways activated by Lin-28B overexpression. (A) Western blot analysis showed protein levels of c-myc, HMGA2 and IGF1R were increased in HA22T cells with Lin-28B overexpression. (B) The location of three putative let-7-binding sites in the 3'-UTR of IGF1R. (C) The luciferase assay showed site 1 and site 3 were responsive to Lin-28B expression and that the responsiveness was abolished by mutation of the putative let-7-binding sites (Mu).
Funding
National Science Council (97-2320-B-002-044-MY3 to Y.-M. J.); National Science Council (96-2628-B-002-054-MY3 to H.-C.H); National Research Program for Genomic Medicine grants of the National Science Council (NSC 97-3112-B-001-016).

Acknowledgements
RNAi reagents were obtained from the National RNAi Core Facility located at the Institute of Molecular Biology/Genomic Research Center, Academia Sinica.

Conflict of Interest Statement: None declared.

References

Received March 27, 2010; revised May 12, 2010; accepted May 25, 2010