Krüppel-like factor 4 (KLF4) plays important roles in development, stemness and tumorigenesis; however limited information is available on the detailed function of KLF4 in hepatocellular carcinoma (HCC). The objective of the present study was to examine the functional roles of KLF4 in the metastasis of HCC cells. KLF4 was overexpressed and knocked down by lentiviral transduction method in highly metastatic HCC cells. KLF4 overexpression in HCC cells led to inhibition of cell migration and invasion. These inhibitory effects were associated with the upregulation of tissue inhibitors of metalloproteinase (TIMP)-1 and TIMP-2 by KLF4. Treatment with recombinant TIMP-1 decreased the migratory ability of HCC cells. Moreover, myeloperoxidase (MPO)-TIMP-1/TIMP-2 inactivator counteracted the KLF4-induced inhibition of cell migration/invasion. Consistently, KLF4 knockdown in HCC cells downregulated TIMP-1 and TIMP-2 expression, consequently promoting cell migration and invasion. Furthermore, we found that KLF4 regulated E-cadherin and epithelial-mesenchymal transition (EMT)-related proteins such as snail, vimentin and Bmi1 to modulate the cell migration ability. These results together demonstrated for the first time that KLF4 plays an important role in inhibiting the aggressiveness of HCC cells via upregulation of TIMP-1 and TIMP-2.

Introduction

Hepatocellular carcinoma (HCC) is the fifth most common malignancy associated with a high mortality worldwide (1). To date, surgical resection and liver transplantation are considered the optimal option for the treatment of HCC (2). However, not all HCC patients are suitable for partial hepatectomy or liver transplantation (3). Therefore, to develop more effective therapeutic strategies that directly target key molecules in signaling pathways of tumorigenesis (4) and/or metastasis in HCC is urgently needed.

Krüppel-like factor 4 (KLF4) is mainly found in differentiated epithelial cells of the skin and the gastrointestinal tract (5). KLF4 is a zinc-finger transcription factor that inhibits cell proliferation and maintains the differentiation of epithelial cells by regulating gene expression (6). Moreover, KLF4 expression is also involved in the tumorigenesis and tumor progression in different types of cancer cells (7). KLF4 has been identified as a tumor-suppressor gene, since reduction in KLF4 expression has been reported in esophageal, gastric, colon and lung cancers (8-11). Conversely, KLF4 may also serve as an oncogene since it was found to be increased in oral cancer (12). Thus, these findings suggest that KLF4 has various functions in different type of cancer cells.

Recently, reduction in KLF4 has been found in HCC samples and was significantly correlated with reduced survival time of patients (13). Several reports indicated that KLF4 is involved in the tumor metastasis of HCC and breast cancer cells through suppression of the expression of E-cadherin and epithelial-mesenchymal transition (EMT) inducers such as Slug and Snail (13-15). Using animal model, it has also been demonstrated that KLF4 inhibits the tumorigenic progression of HCC (13). In addition, KLF4 is positively regulated by PPARγ via binding directly to the PPAR response element within the KLF4 promoter in colorectal cancer cells (16). PPARγ agonist, troglitazone, has been found to upregulate KLF4 expression, and this KLF4 induction can be prevented by pretreatment with PPARγ antagonist, GW9662 (16). However, the detailed mechanisms of KLF4 in the modulation of cell motility and invasiveness in hepatoma cells remain unclear.
Tissue inhibitors of metalloproteinases (TIMPs) control the functions and activities of matrix metalloproteinases (MMPs), which play a crucial role in extracellular matrix degradation involved in tumor cell invasion, metastasis and angiogenesis. For example, increased MMP9 and MMP2 associated with tumor aggressiveness and poor prognosis in HCC (17–20) are regulated by TIMP-1 and TIMP-2, respectively. Additionally, the levels of TIMP-1 and TIMP-2 were also used as prognostic factors for predicting the metastasis of HCC (21,22). In the present study, we examined the effects of KLF4 on HCC cell migration and invasion. In addition, the relationship between KLF4 and TIMP-1/TIMP-2 was also investigated.

Materials and methods

Cell culture and reagents. Four HCC cell lines (HepG2, Hep3B, HA22T/VGH and Mahlavu) and HEK293T were maintained in Dulbecco's modified Eagle's medium (DMEM; Invitrogen, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (FBS; HyClone, Logan, UT, USA), 0.1 mM non-essential amino acids, 2 mM L-glutamine and 1% penicillin/streptomycin in a humidified atmosphere containing 5% CO2 at 37°C. Rosiglitazone, troglitazone and GW9662 were obtained from Cayman Chemical (Ann Arbor, MI, USA). Recombinant TIMP-1 protein and myeloperoxidase (MPO) were purchased from Enzo Life Sciences (Plymouth Meeting, PA, USA) and Millipore (Temecula, CA, USA), respectively.

Overexpression and knockdown of KLF4. KLF4 was amplified from human cDNA using PCR method with primers: 5'-GTCTAGAACCACCATGGCTGACCCGCACGGCTG-3' and 5'-GGGATCGTTTAAAATGCCTCTTCATG-3'. KLF4 was cloned into the lentiviral expression vector pLV-EF1α-MCS-IRE-Bsd (pLV-Bsd; Biosettia, San Diego, CA, USA) at the BamHI and NheI restriction enzyme sites. Construct pLV-Bsd-KLF4 was confirmed by DNA sequencing. The lentiviral vector pLV0.1-shKLF4 used for KLF4 knockdown was purchased from RNAi core of Academia Sinica (Taiwan). The oligonucleotide targeting to human KLF4 was 5'-CTGGGACTTTATTTCTCTCTCAAT-3'. The lentiviruses were generated by co-transfecting HEK293T cells with the lentiviral expression vectors (pLV-Bsd, pLV-Bsd-KLF4 or pLV0.1-shKLF4) and packaging plasmids (pCMVΔR8.91 and pCMV-VSVG) using T-Pro NTR II (Ji-Feng Biotechnology, Taipei, Taiwan). Supernatants containing the lentivirus were collected 72 h after transfection. HCC cells were infected with lentivirus in the presence of 8 µg/ml Polybrene (Sigma, St. Louis, MO, USA). KLF4-overexpressing and KLF4-knockdown HCC cells were selected by blastidin S and puromycin (Sigma).

Proliferation assay. The tetrazolium salt 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) assay was used to determine the effects of KLF4 overexpression on cell growth. Lenti-Ctrl, Lenti-KLF4, shLuc and shKLF4 cells were seeded at a density of 5,000 cells/well in 96-well plates and incubated for 24, 48 and 72 h. MTT reagent (Sigma-Aldrich) was added into each well of the plates and incubated for another 2 h at 37°C. After incubation, formazan crystals were dissolved in dimethylsulfoxide (DMSO), and then optical absorbance at a wavelength of 570 nm was measured by a microplate reader (SpectraMax 250; Molecular Devices, Sunnyvale, CA, USA).

Wound healing, Transwell migration and Transwell invasion assays. For the wound-healing assay, the cells were seeded into each well of the culture insert (Ibidi GmbH, Martinique, Germany) and incubated overnight. After cells achieved a confluent layer, the culture insert was gently removed. Bright field images were captured after 14 h using an inverted microscope. The changes in wound area were quantified by ImageJ software.

Table I. DNA sequence of the primers for real-time PCR analysis.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Sequence (5’–3’)</th>
</tr>
</thead>
<tbody>
<tr>
<td>E-cadherin</td>
<td>F TGA AGG TGA CAG AGC CTC TGG AT</td>
</tr>
<tr>
<td>R TGG GTG AAT TCG GGC TTG TT</td>
<td></td>
</tr>
<tr>
<td>KLF4</td>
<td>F CGA ACC CAC ACA GGT GAG AA</td>
</tr>
<tr>
<td>R TAC GGT AGT GCC TGG TCA GTT C</td>
<td></td>
</tr>
<tr>
<td>Vimentin</td>
<td>F CCT TGA ACG CAA AGT GGA ATC</td>
</tr>
<tr>
<td>R GAC ATG CTC TCT CTG AAT CTG AG</td>
<td></td>
</tr>
<tr>
<td>Bmi1</td>
<td>F ACA TCC GAA GCC ACA CGC TGC</td>
</tr>
<tr>
<td>R CGC ACG TTT GAG CCG TCA GC</td>
<td></td>
</tr>
<tr>
<td>Snail</td>
<td>F ACA TCC GAA GCC ACA CGC TGC</td>
</tr>
<tr>
<td>R CGC ACG TTT GAG CCG TCA GC</td>
<td></td>
</tr>
<tr>
<td>MMP2</td>
<td>F TTG ACG GTG AGC AGC GC</td>
</tr>
<tr>
<td>R ACT TGC AGT ACT CCC CAT CG</td>
<td></td>
</tr>
<tr>
<td>MMP9</td>
<td>F TTG ACA GCG ACA AGA AGT GG</td>
</tr>
<tr>
<td>R CCC TCA GTG AAG CAG TAC AT</td>
<td></td>
</tr>
<tr>
<td>TIMP-1</td>
<td>F AAG GCT CTG AAA AGG GCT TC</td>
</tr>
<tr>
<td>R GAA AGA TGG GAG TGG GAA CA</td>
<td></td>
</tr>
<tr>
<td>TIMP-2</td>
<td>F CCA AGC AGG AGT TTC TCG AC</td>
</tr>
<tr>
<td>R GAC CCA TGG GAT GAG TGT TT</td>
<td></td>
</tr>
<tr>
<td>β-actin</td>
<td>F TGG CAT TCG CCA GAG GAT</td>
</tr>
<tr>
<td>R GCT CAG GAG GAG CAATG A TCT</td>
<td></td>
</tr>
</tbody>
</table>

KLF4, Krüppel-like factor 4; MMP, matrix metalloproteinase; TIMP, tissue inhibitors of metalloproteinases.
Carlsbad, CA, USA). The alterations in gene expression were obtained using the ΔΔCt method in which all samples were first normalized to the level of β-actin in each sample. Relative normalized units were then compared between the control and KLF4-overexpressing cells.

**Western blot analysis.** Proteins were separated on 10% SDS-PAGE and then transferred onto a nitrocellulose membrane. After blocking the membrane in Tris-buffered saline, 0.1% Tween-20 (TBST) buffer with 5% skim milk, primary antibodies: E-cadherin, Snail (Cell Signaling, Danvers, MA, USA), KLF4 (Santa Cruz Biotechnology, Santa Cruz, CA, USA), vimentin, β-actin (Sigma), Bmi1 (Upstate Biotechnology, Lake Placid, NY, USA), MMP2, TIMP-1 (Millipore) and TIMP-2 (NeoMarkers, Fremont, CA, USA) were used to probe the proteins on the membrane at 4˚C overnight. After incubation with the horseradish peroxidase-conjugated secondary antibody, the probed proteins were detected using the enhanced chemiluminescence system (Millipore) according to the manufacturer's instructions.

**Enzyme-linked immunosorbent assay (ELISA).** The concentrations of TIMP-1 and TIMP-2 in the cell culture supernatants were analyzed using the ELISA kit (R&D Systems, Minneapolis, MN, USA) according to the manufacturer's instructions.

**Statistical analysis.** The means and standard deviation (SD) were calculated from three independent experiments. The Student's t-test was used for comparing the means of two treatment groups. Differences were considered to indicate a statistically significant result when P<0.05.

**Results**

**KLF4 overexpression inhibits the migratory and invasive abilities in the HCC cells.** In order to assess the functional role of KLF4 in HCC cells, we examined the expression of KLF4 in 4 HCC cell lines. It was found that HepG2 cells had the highest level of KLF4 and also the highest level of E-cadherin. In contrast, Hep3B and HA22T/VGH cells had lower levels of KLF4, and Mahlavu cells had the lowest level of KLF4 and undetectable E-cadherin (Fig. 1A). We adopted a lentiviral transduction method to overexpress KLF4 in the Mahlavu cells. Cells were infected by lentivirus with pLV-Bsd-KLF4 (indicated as Lenti-KLF4), and overexpression of KLF4 expression was confirmed by western blot analysis as compared to cells infected by the lentivirus with the pLV-Bsd vector (indicated as Lenti-Ctrl) (Fig. 1B). Cell proliferative ability was examined by the MTT assay, and the results showed that KLF4 overexpression did not inhibit the growth of the Lenti-KLF4 cells (Fig. 1C). However, KLF4 overexpression led to morphological change in the Lenti-KLF4 cells which showed a round shape and aggregated form as compared to the Lenti-Ctrl cells (Fig. 1D).

We next used wound healing, Transwell migration and Transwell invasion assays to evaluate the migratory and invasive abilities of the Lenti-KLF4 cells. In the wound-healing assay, the Lenti-Ctrl cells almost filled the gap after a 14-h culture, while the Lenti-KLF4 cells showed a 30% decrease in the migratory ability (P<0.001, Fig. 2A). Consistent results were obtained using the Transwell migration assay showing that the Lenti-KLF4 cells had a 50% decrease in the migratory ability (P<0.001, Fig. 2B). In the Transwell invasion assay, a thin layer of commercial Matrigel was used as extracellular
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matrix. Compared to the Lenti-Ctrl cells, the Lenti-KLF4 cells showed a 35% decrease in the invasive ability (P<0.001, Fig. 2C). These results clearly demonstrated that KLF4 overexpression significantly suppressed the migratory and invasive abilities but not the growth of HCC cells.

**KLF4 overexpression modulates MMPs and TIMPs.** To clarify the interaction between KLF4 and proteins involved in cell migration, we examined the effects of KLF4 expression on regulation of the expression of MMPs and TIMPs. The results showed that KLF4 overexpression led to significant upregulation of both the mRNA and protein levels of TIMP-1, and TIMP-2 in the Lenti-KLF4 cells as compared to the Lenti-Ctrl cells. Messenger RNA levels of MMP2 and MMP9 were significantly decreased, yet the protein expression of MMP2 was slightly increased in the Lenti-KLF4 cells (Fig. 3A and B).

To further examine the functional importance of the PPARγ pathway in relation to KLF4 in modulation of TIMP-1 and TIMP-2, we used PPARγ antagonist GW9662 to block the PPARγ pathway. The results showed that GW9662 treatment for 48 h inhibited the expression of KLF4 in both the Lenti-Ctrl and Lenti-KLF4 cells as compared to the untreated control (Fig. 3B). Additionally, the expression levels of MMP2, TIMP-1, and TIMP-2 were also concomitantly downregulated in both the Lenti-Ctrl and Lenti-KLF4 cells after GW9662 treatment for 48 h.

As newly synthesized TIMPs are secreted and function in extracellular space, we used ELISA assay to determine the concentrations of secreted TIMP-1 and TIMP-2 in the cell culture supernatants. Compared to the Lenti-Ctrl cells, KLF4 overexpression upregulated the concentrations of secreted TIMP-1 and TIMP-2 in the Lenti-KLF4 cells (Fig. 3C and D). However, GW9662 treatment for 48 h had no effect on the levels of secreted TIMP-1 and TIMP-2 in both the Lenti-Ctrl and Lenti-KLF4 cells.

To clarify the role of KLF4-induced TIMP-1/TIMP-2 expression in relation to migration, we found that treatment with recombinant protein TIMP-1 showed markedly decrease in the migratory ability of the Lenti-Ctrl cells as compared to the untreated cells (Fig. 4A). In addition, we used MPO (50 nM treatment at 37°C for 60 min) to block the activities of TIMP-1/TIMP-2 in the Lenti-KLF4 cells. The results showed that the migratory and invasive abilities of the MPO-treated Lenti-KLF4 cells were significantly increased as compared to the untreated Lenti-KLF4 cells (Fig. 4B and C). These results together clearly indicate that KLF4 modulates the expression of TIMP-1 and TIMP-2, leading to changes in migration and invasion of HCC cells.

**KLF4 silencing inhibits TIMP-1 and TIMP-2 while increasing migration and invasion.** In order to further validate the importance of KLF4 in the regulation of cell migration and

Figure 2. Effects of KLF4 overexpression on the migratory and invasive abilities of HCC cells. Mahlavu cells were infected by a recombinant lentivirus carrying the empty vector or KLF4 (indicated as Lenti-Ctrl or Lenti-KLF4, respectively). (A) Wound-healing, (B) Transwell migration and (C) invasion assays were used to examine the migratory and invasive abilities of the control vs. KLF4-overexpressing cells at the indicated time points. *P<0.001, indicates significantly different from the Lenti-Ctrl cells. KLF4, Krüppel-like factor 4.
invasion, we used lentiviral vector-mediated shRNA to create KLF4-knockdown HA22T/VGH cells (indicated as shKLF4). Compared to the control (indicated as shLuc), the mRNA and protein levels of KLF4 were significantly decreased in the shKLF4 cells (Fig. 5A and B). KLF4 knockdown led to significant upregulation of MMP2 and MMP9 but...
downregulation of TIMP-1 and TIMP-2 expression in the shKLF4 cells (Fig. 5A and B).

We next evaluated the effects of KLF4 knockdown on cell proliferation, migratory and invasive abilities. In the cell proliferation assay, the result showed that KLF4 knockdown did not alter the growth of the shKLF4 cells (Fig. 5C). Compared to the shLuc cells, the migratory and invasive abilities showed a 1.2-fold (P<0.001, Fig. 5D) and 1.7-fold (P<0.01, Fig. 5E) increase in the shKLF4 cells, respectively. These results clearly demonstrated the critical role of KLF4 in cell migration and invasion by regulating TIMP-1 and TIMP-2 expression.

KLF4 regulates E-cadherin and EMT-related proteins. As EMT has been reported to be highly associated with cell migration in HCC, we also evaluated the expression levels of several EMT-related proteins. We found that KLF4 overexpression in the Mahlavu cells resulted in increased E-cadherin and decreased Snail mRNA, yet had no effect on the levels of vimentin and Bmi1 mRNA (Fig. 6A). Western blot results showed that KLF4 overexpression not only increased the expression of E-cadherin but also inhibited the protein expression of vimentin, Snail and Bmi1 (Fig. 6B). Moreover, KLF4 knockdown in the HA22T/VGH cells resulted in decreased E-cadherin and increased vimentin protein expression (Fig. 6D). Taken together, these results suggest that KLF4 also plays an important role in regulating E-cadherin and EMT-related proteins to modulate cell migration ability.

Discussion

It has been reported that KLF4 demonstrates functions to inhibit migration and invasion in several types of cancer (14,24 -26); however, the detailed molecular mechanisms in HCC remain unclear. The present study showed that KLF4 overexpression inhibited the migratory and invasive abilities of highly metastatic Mahlavu cells with elevated expression levels of TIMP-1/TIMP-2 (Figs. 2 and 3). Knockdown of KLF4 in the HA22T/VGH cells by shRNA also correlated with increased migration/invasion and reduced TIMP-1/TIMP-2 levels (Fig. 5). Using TIMP inactivator MPO (27), the effects of KLF4 on migration/invasion were partially blocked, suggesting that KLF4 can inhibit cell migration and invasion via upregulation of TIMP-1/TIMP-2 expression (Fig. 4B and C). It has been reported that inhibition of TIMP-1 enhanced the migration of microvascular endothelial cells (28); TIMP-2 overexpression also significantly inhibited migration/invasion of ras-transformed breast epithelial cells (29). It is notable that the expression levels of MMP2, TIMP-1 and TIMP-2 were increased simultaneously in the Lenti-KLF4 cells; however, the increased fold-change of TIMP-1 and TIMP-2 was higher than that of MMP2 (Fig. 3B), consequently resulting in migration/invasion inhibition (Fig. 2). Consistent with our findings,
Wang et al also indicated that KLF4 directly regulates TIMP-2 expression and inhibits migration/invasion in prostate cancer cells (30). Taken together, we demonstrated for the first time that KLF4 suppresses HCC cell migration/invasion through upregulation of TIMP-1 and TIMP-2 expression.

KLF4 has also been reported to inhibit EMT through regulation of E-cadherin expression in breast cancer cells (14). EMT, a process defined by acquisition of mesenchymal phenotype with reduced cell adhesion and increased mobility, plays a key role not only in development but also in malignant tumor progression and metastasis (31). Reduction or loss of E-cadherin expression is considered as an early and critical step to disrupt intercellular contacts and induce the EMT process (32). Moreover, in various types of cancers, the expression of E-cadherin was obviously repressed through the direct binding of transcription factors Snail or Bmi1 on the E-cadherin promoter (33,34). Li et al found that overexpressed KLF4 induced E-cadherin and reduced Snail in HepG2 and SK-Hep1 cells (13). Consistent with our findings, our results showed that KLF4 overexpression led to an increase in E-cadherin and concomitant decrease in Snail and Bmi1 in Mahlavu cells (Fig. 6B). On the other hand, it has been reported that TIMP-2 upregulates E-cadherin expression in lung cancer cells (35). Thus, it is most likely that KLF4 not only directly upregulates E-cadherin expression through the transcription level but also indirectly induces TIMP-2 to increase the expression of E-cadherin in HCC cells. These data suggest that KLF4-induced TIMP-1 and TIMP-2 may be the most important factors to regulate HCC migration/invasion in HCC cells.

Vimentin, an intermediate filament, is a marker of mesenchymal cells and has been linked to aggressive tumors. It has been reported that increased vimentin is significantly correlated with the metastasis of HCC (36). Reduction in vimentin by the PPARγ antagonist GW9662 inhibited the migration and invasion of HCC cells (37). This corresponded well with our observation that GW9662 inhibited the expression of KLF4 and TIMP1/TIMP2 (Fig. 3B). Moreover, consistent with our findings, downregulation of vimentin by KLF4 overexpression (Fig. 6B) inhibited the migration and invasion of Mahlavu cells. Moreover, upregulation of vimentin by KLF4 knockdown promoted the migration and invasion of HA22T/VGH HCC cells (Fig. 5). Taken together, our results suggest that the overexpression of vimentin, as a result of loss of KLF4, significantly contributes to HCC progression.

Our findings provide important insights into the mechanism of the KLF4-TIMP-1/TIMP-2 signaling pathway in HCC progression, which inhibits the migration, invasion and metastasis of HCC cells. These unique findings strongly suggest that KLF4 may serve as a potential molecular target in cancer therapy for HCC patients.

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