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ORIGINAL ARTICLE – TRANSLATIONAL RESEARCH AND BIOMARKERS

Silencing of Glucose-Regulated Protein 78 (GRP78) Enhances Cell Migration Through the Upregulation of Vimentin in Hepatocellular Carcinoma Cells

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ABSTRACT

Background. Glucose-regulated protein 78 (GRP78) plays an important role in embryonic development and cancer progression. However, there is little information regarding the regulation of GRP78 in hepatocellular carcinoma (HCC) metastasis.

Methods. We used RNA silencing and cDNA expression vectors to manipulate target gene expression in HCC cells. The transwell migration assay and xCelligence biosensor system were applied to determine the proliferatory and migratory ability of the HCC cells.

Results. In this study, we found that GRP78 silencing enhanced cell migration in both HepJ5 and Mahlavu cells. Overexpressed GRP78 in skHep1 cells suppressed the migratory ability. In the insight mechanism dissection for GRP78-mediated cancer migration, we found that downregulation of GRP78 caused the increase of vimentin expression on HCC cells. Suppressed vimentin expression also decreased the migratory ability on HCC, indicating that vimentin expression levels modulated the cell migratory ability.

Conclusion. We found that silencing GRP78 in HCC cells may enhance cell migration through the increase of vimentin expression.

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Glucose-regulated protein 78 (GRP78) is an endoplasmic reticulum chaperone molecule that plays a key role in cell survival.[1–3] In vivo knockout mouse models have demonstrated that GRP78 is required for cell proliferation and protects the cell mass from apoptosis during early embryonic development.[4] In many cancers, high expression of GRP78 has been correlated with carcinogenesis and tumor progression.[5–7] Immunohistochemistry (IHC) studies of different types of tumors have revealed that GRP78 expression is markedly higher in tumors compared with the levels observed in adjacent, nontumorigenic, tissue.[5,6,8,9] Additionally, expression of GRP78 is also correlated with the level of therapeutic resistance in the tumor, which leads to a higher risk for clinical recurrence and reduced survival.[8,10–13]

Metastasis is an important issue for general cancer therapy and is an important concern for hepatocellular carcinoma (HCC) therapy. The level of GRP78 expression

is correlated (positive) with the metastatic status of many different types of cancer.[7,9,14,15] However, downregulation of GRP78 expression has been shown, by IHC staining, to occur during oral squamous cell cancer metastasis.[16] In HCC, GRP78 has been linked with the capability of tumors to infiltrate the vasculature system. However, heat shock protein 70 (HSP70) has no association with the pathologic outcome.[17] HSP upregulation strongly suggests vascular invasion and intrahepatic metastasis; thus, the upregulation of the expression of several HSPs (GRP78, GRP94, and HSP90) in hepatitis B virus (HBV)-related HCCs may be an important prognostic marker.[18] This indicates that the role of GRP78 in cancer metastasis may be organ specific.

The epithelial to mesenchymal transition (EMT) is considered to be a major process for cell migration.[19] Several biomarkers correlate with the process of EMT, including E-cadherin, N-cadherin, vimentin, and fibronectin.[20,21] Increased levels of vimentin, N-cadherin, or fibronectin may contribute to enhance the ability of cells to migrate.[22–25] In addition, reduced expression of E-cadherin or beta-cadherin may increase cancer metastasis.[25–27] However, there is little information about GRP78 and EMT processes in HCC. Our previous reports indicate that silencing GRP78 enhances the migratory ability of cells in HCC but does not affect cell growth.[28] In this study, we further dissect the mechanism of GRP78-mediated cancer migration and the regulation of GRP78 during EMT processes in HCC. We believe that more information will improve HCC therapy.

MATERIALS AND METHODS

Chemicals, Reagents, and Cell Culture

Triton X-100, Tris–HCl, neomycin, and Trypan blue EDTA were obtained from Sigma Chemical Co. (St. Louis, MO). Anti-GRP78, anti-vimentin, and anti-β-actin antibodies were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). The HCC cell lines were grown in DMEM medium (Gibco BRL, Grand Island, NY) with 2 mM L-glutamine that was adjusted to contain 1.5 g/L sodium bicarbonate and supplemented with 10% fetal bovine serum (Gibco BRL, Grand Island, NY) and 2% penicillin–streptomycin (10,000 U/mL penicillin and 10 mg/mL streptomycin). Cell lines were cultured in a 5% CO_2 humidified incubator at 37°C as previously described.[28,29]

Generation GRP78 Knockdown HCC Cells

The expression of GRP78 was knocked down in HCC cells with small interfering RNA (siRNA), as previously described.[28–30] Briefly, the target sequence for the human GRP78 mRNA was 5′-AAGGTTACCCATGCAGTTGT T-3′. The scrambled siRNA sequence was 5′-AAGGT GGTTGTTTTGTTCACT-3′. The GRP78 siRNA and scrambled siRNA were inserted into the pSUPERIOR vector and transfected into the cells. Cells that were successfully transfected were selected by antibiotic resistance, as previously described.[28,29,31,32]

Silenced Vimentin Expression in HCC

The expression of vimentin was ablated in Mahlavu cells using shRNA clones purchased from National RNAi Core Facility (Taiwan, ROC). The target sequence for the human vimentin mRNA (NM_003380) gene was 5′-GCTAAC TACCAAGACACTATT-3′. The nontarget shRNA control vector (SHC002) was purchased Sigma Chemical Co. (St. Louis, MO), and the sequence of scrambled-shRNA was 5′-CAACAAGATGAAGAGCACCAA-3′. Briefly, 1.5×10^5 cells were washed twice with phosphate-buffered saline (PBS) and mixed with 0.5 μg plasmid. One pulse was applied for 20 ms under a fixed voltage of 1.4 kV on a pipette-type microporator Neon (Life Technologies). After 48 h, the cells were harvested for detecting the protein level of vimentin by western blot analysis and performing the transwell migration assay.

Overexpression of GRP78 in HCC Cells

The full-length GRP78 cDNA was generated by polymerase chain reaction (PCR) and subcloned into the pCMV6-XL5 expression vector (OriGene Technologies, Inc). Full-length sequencing was performed by automatic sequencing (ABI). The pCMV6-XL5-GRP78 plasmid was transfected into cells by electroporation. After 48 h, the cells were harvested to determine the expression level of GRP78 by real-time PCR and western blotting.

Protein Extraction and Western Blot Analysis

The abundance of proteins was determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and western blot, as previously described. The cells were washed with cold PBS and lysed using cell lysis buffer, which contained protease inhibitors (Complete Protease Inhibitor Tablets, Boehringer Mannheim, Indianapolis, IN). Then, 30 microgram proteins were separated by 10% SDS-PAGE under reducing conditions and electrotransferred onto PVDF membranes (Bio-Rad Laboratories). The membranes were subsequently blotted using anti-GRP78 or anti-actin antibodies and horseradish peroxidase (HRP)-conjugated secondary antibodies (1:5000), visualized with enhanced chemiluminescence reagent (GE Healthcare,

Piscataway, NJ), and were detected using a VersaDoc 5000 system (Bio-Rad Laboratories).[28,29]

RT-PCR and Quantitative RT-PCR Analysis

Total RNA was extracted from HCC cells using the TRIZOL reagent, according to the manufacturer's instructions (Invitrogen Life Technologies). The cDNA was amplified from 2 μg of total RNA, in a final volume of 20 μL, using Moloney murine leukemia virus (M-MLV) reverse transcriptase at 37°C for 90 min. For validation, real-time RT-PCR was performed using a Power SYBR-Green real-time RT-PCR system and an ABI StepOne detection system (Applied Biosystems, Foster City, CA). The primers for real time PCR were: GRP78 (forward: 5′-GCCTGTATTTCTAGACCTGCC-3′; reverse: 5′-TTCA TCTTGCCAGCCAGTTG-3′), vimentin (forward: 5′-GG CTCAGATTCAGGAACAGC-3′; reverse: 5′-CTG AATC TCATCCTGCAGGC-3′), *β*-actin (forward: 5′-AGCGCG GCTACAGCT-3′; reverse: 5′-GGCCATCTCTTGCTCG AAGT-3′).[28,29] The quantitative RT-PCR reaction was carried out using ABI SYBR Green Master Mix. Thermal cycling was performed using an ABI StepOne system (Applied Biosystems), and the quantitative PCR conditions were: 95°C for 10 min, followed by 40 cycles of 95°C for 15 s, and 60°C for 1 min.

Cell Proliferation and Migration Assay by x'Celligence Biosensor System

Experiments were carried out using the RTCA DP instrument (Roche Diagnostics GmbH, Germany), which was placed in humidified incubator maintained at a 5% CO_2 humidified incubator at 37°C. Growth curves were constructed using 16-well plates (E-plate 16, Roche Diagnostics GmbH). For proliferation assay, cells were seeded in E-plates 16 at 10,000 cells/well in fetal calf serum (FCS)-containing medium, and the plate was then monitored once every 30 s for 4 h, then once every half hour. Cell migration was assessed using specifically designed 16-well plates (CIM-plate 16, Roche Diagnostics GmbH) with 8 μm pores. These plates are similar to conventional transwells with the microelectrodes located on the underside of the membrane of the upper chamber. The 10% FCS medium was added in the lower chamber, and cells were seeded into the upper chamber at 20,000 cells/well in serum-free medium. The CIM-plate 16 was monitored every 10 s for 40 min then once every hour. Data analysis was carried out using RTCA software 1.2 supplied with the instrument.

Transwell Migration Assay

In vitro cell migration studies were performed using a BD Falcon cell culture insert (BD Biosciences) as previously described.[33–35] Briefly, we suspended 1×10^5 cells in 500 μL of serum-free DMEM, and they were seeded into the upper part of each chamber. The lower compartments of each chamber were filled with 1 mL of DMEM that contained the 10% FCS serum gradient. After incubation for 24 h at 37°C in 5% CO_2, the nonmigrating cells were removed from the upper surface of the membrane by scrubbing. Cells on the reverse side of the membrane were stained with 0.1% crystal violet. The migrating cells were counted under a microscope at 100-fold magnification.

Statistical Analysis

All experiments were repeated a minimum of three times. All of the data are expressed as the means ± SD. The data presented in some figures are from representative experiments, which were quantitatively similar to the experimental replicates. Statistical significance was determined with a *t* test (two-tailed) comparison between two groups of data.

RESULTS

GRP78 Expression Levels and Silenced GRP78 Expression on HCC Cells

GRP78 was highly expressed, ectopically, in HepJ5 cells and Mahlavu cells compared with skHep-1 cells (Fig. 1a). Our previous study indicated that silence of GRP78 in HepJ5 cells enhanced cell migratory ability, indicating that GRP78 may correlate with the migratory ability in HCC.[28] In order to realize the molecular mechanism of GRP78 in HCC migration, we further silenced GRP78 expression by siRNA in HepJ5 cells and Mahlavu cells. The western blotting and real-time PCR results demonstrated that GRP78 expression levels were suppressed successfully in HepJ5 cells and Mahlavu cells (Fig. 1b).

Silenced GRP78 in HepJ5 Cells Had No Influence on Cell Growth Rate, But Enhanced the Migratory Ability in xCelligence Biosensor System

We further characterized the cell growth rate between GRP78 knockdown (GRP78KD) HepJ5 cells and scrambled control cells by xCelligence biosensor system. As shown in Fig. 2a, the proliferation rate was similar between

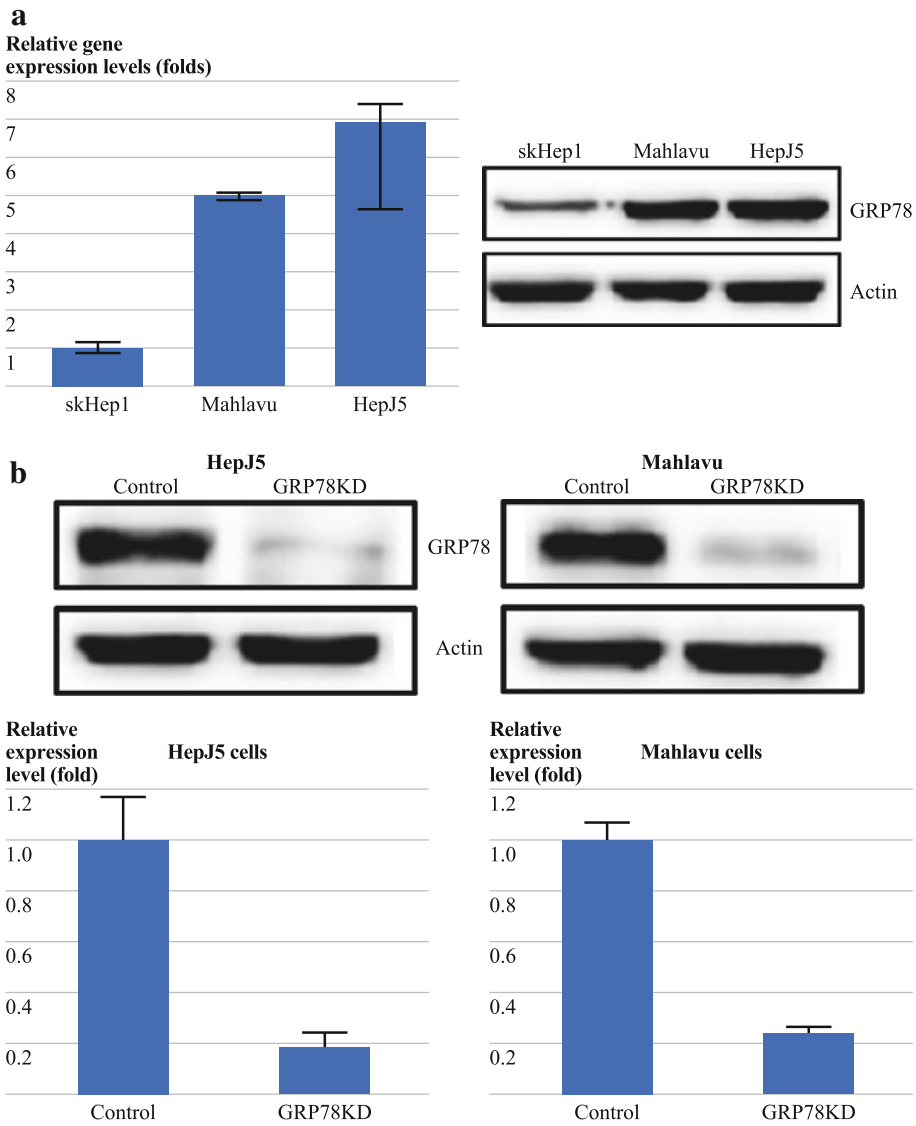

FIG. 1 Determined and silenced GRP78 expression in HCC cells. The expression of GRP78 in HCC cells lines (skHep-1, Mahlavu, and HepJ5 cells) were determined by **a** real-time PCR and western blotting. **b** The expression of GRP78 was knocked down by GRP78 specific siRNA in Mahlavu cells and HepJ5 cells. After antibiotic selection, the cells colonies were pick-up and the expression levels of GRP78 were determined by western blotting and real-time PCR. All the experiments were repeated at least three times independently. $*P < .05$

GRP78KD HepJ5 cells and scrambled-control HepJ5 cells. This is consistent with our previous finding.[28] We further detected the migration assay between GRP78KD and scrambled control HepJ5 cells by xCelligence system. The migratory ability was increased significantly in GRP78KD HepJ5 cells compared with scrambled control cells (Fig. 2b).

Silenced GRP78 Enhanced the Cell Migration in HepJ5 and Mahlavu Cells in Transwell Migration Assay

We further confirmed the migration assay results by Transwell migration system and found that knockdown of GRP78 enhanced the cell migration in HepJ5 cells (Fig. 3a, b). We further confirmed this finding in GRP78KD Mahlavu cells and scrambled control cells. Interestingly, the ability of Mahlavu cells to migrate was increased after GRP78 knockdown (Fig. 3c, d). Those results indicate that GRP78 may medicate the cell migratory ability in HCC cells.

Overexpressed GRP78 Suppressed the Migratory Ability of skHep-1

To determine if GRP78 mediates that ability of cells to migrate, we overexpressed GRP78 in skHep-1 cells. As shown in Fig. 4a, GRP78 expression increased dramatically after the GRP78 expression vector was transfected into skHep-1 cells. The ability of skHep-1 cells to migrate following GRP78 overexpression was determined by transwell assay. Our data shows that overexpressed GRP78 resulted in decreased skHep-1 cell migration compared with the control cells (Fig. 4b, c). These data indicate that high GRP78 expression levels may decrease the migratory ability in HCC cells.

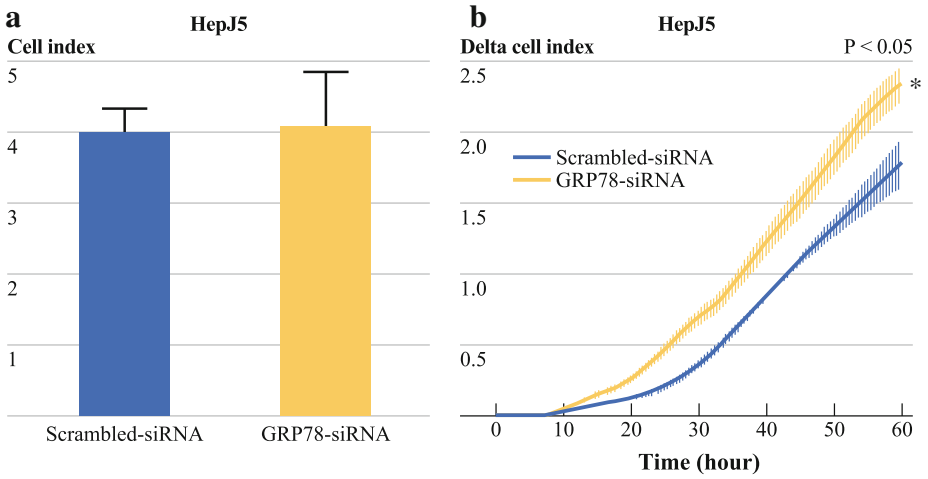

FIG. 2 Silenced of GRP78 expression did not influence the proliferation ability, but increased the cell migratory ability. **a** For proliferation assay, cells were seeded in E-plates 16 at 10,000 cells/well in FCS-containing medium and the plate was then monitored once every 30 s for 4 h, then once every half hour by the RTCA DP instrument. **b** Cell migration was assessed using specifically designed 16-well plates (CIM-plate 16) with 8 μm pores. The 10% FCS medium was added in the lower chamber and cells were seeded into the upper chamber at 20,000 cells/well in serum-free medium. The CIM-plate 16 was monitored every 10 s for 40 min then once every hour. Data analysis was carried out using RTCA software 1.2 supplied with the instrument. All the experiments were repeated triplicate and two times independently. *$P < .05$

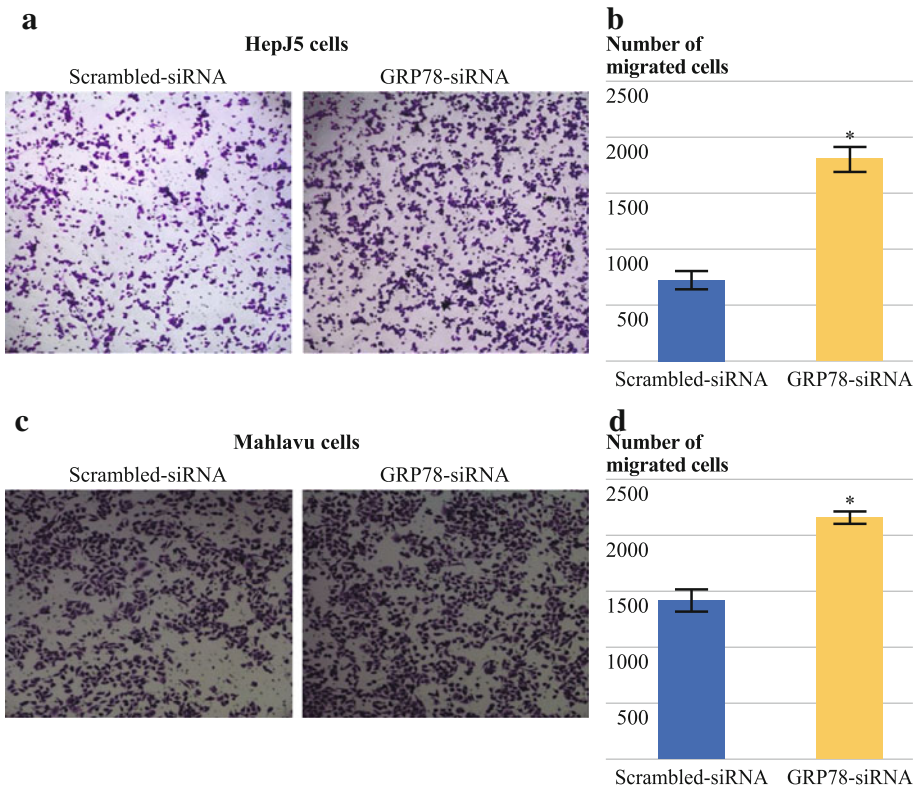

FIG. 3 Knockdown GRP78 enhanced the migratory ability on HepJ5 and Mahlavu cells. The transwell migration system was used to evaluate migration ability. The Transwell migration system demonstrated enhanced migration ability after knockdown of GRP78 expression in HepJ5 (**a**, **b**) and Mahlavu cells (**c**, **d**). The pictures of migratory cells were taken by phase contrast microscopy under 100× amplification. **b**, **d** The quantitative of Transwell migration assay. Y-axis represents the number of migrated cells. The data are the average number of cells that migrated in a representative experiment measured in triplicate and presented as means ± SD. All the experiments were repeated at least three times independently. *$P < .05$

Knockdown of GRP78 Stimulated the Expression of Vimentin

The epithelial-to-mesenchymal transition (EMT) is a major step that occurs during cell migration. E-cadherin, N-cadherin, fibronectin, and vimentin are the key molecules that are involved in the switch from epithelial to mesenchymal status, which is the hallmark of EMT. Thus, we determined the expression pattern of E-cadherin, N-cadherin, fibronectin, and vimentin in scrambled control and GRP78KD cells. Interestingly, the expression level of vimentin was stimulated in GRP78KD Mahlavu cells compared with scrambled control cells (Fig. 5a).

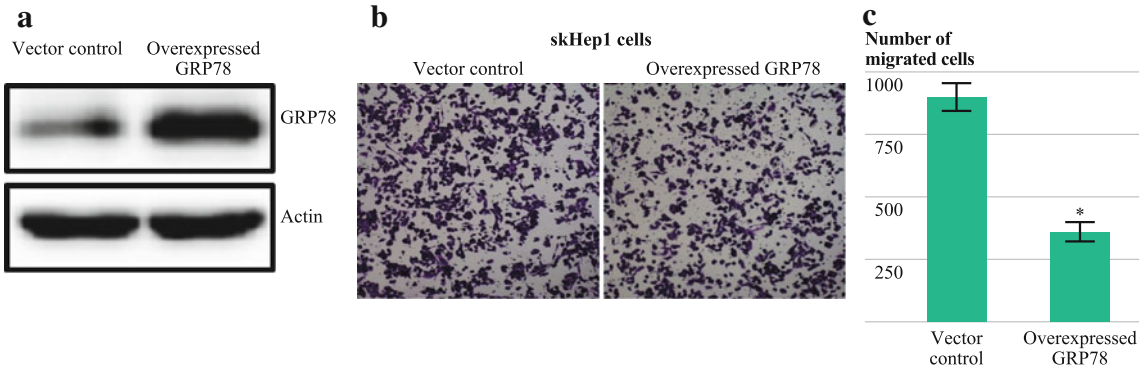

FIG. 4 Overexpression of GRP78 suppresses cancer migration in skHep-1 cells. The transwell migration system was used to evaluate migration ability between skHep-1 cells transfected with pCMV6-XL5 or pCMV6-XL5-GRP78 (**a**) GRP78 protein expression was assessed by western blot analysis to confirm its overexpression. **b** The transwell migration system demonstrated that overexpression GRP78 caused the reduction of migrated cell numbers in skHep-1 cells. The migratory cells were taken by phase contrast microscopy under 100× amplification. **c** The quantitative of transwell migration assay. Y-axis represents the number of migrated cells. The data are the average number of migrated cells in a representative experiment measured in triplicate and presented as means ± SD. All the experiments were repeated at least three times independently. *$P < .05$

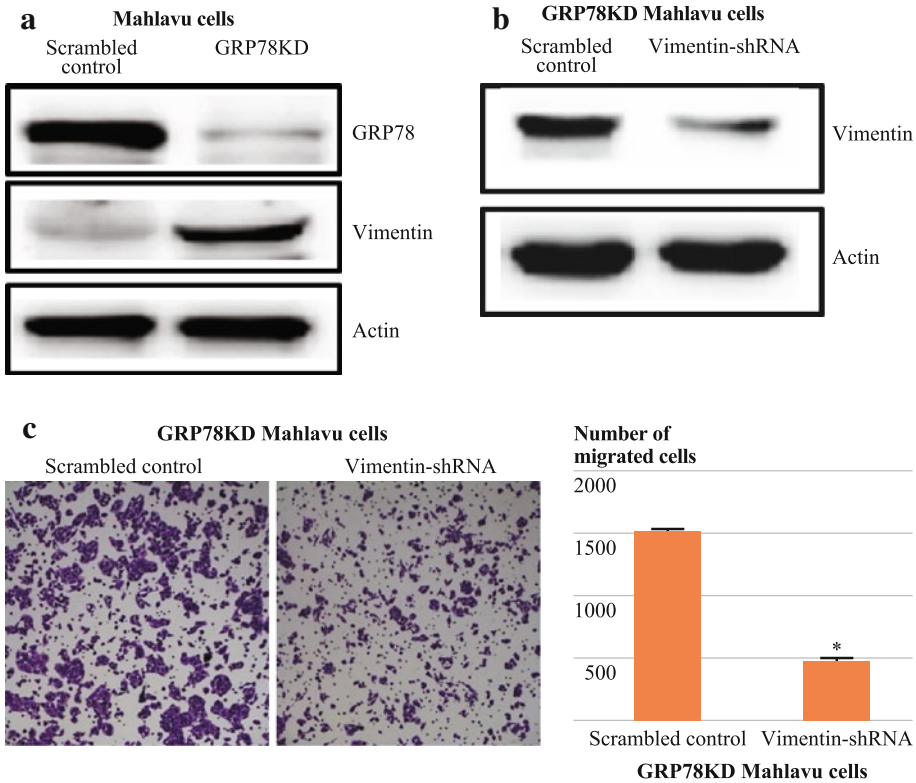

FIG. 5 Knockdown of GRP78 resulted in change of vimentin expression. **a** Knockdown of GRP78 expression in Mahlavu cells resulted in increased expression of vimentin in Western blotting. **b**, **c** We knocked down the vimentin by transient transfected vimentin-specific shRNA into GRP78KD Mahlavu cells. After 48 h, the expression levels were checked by western blotting (**b**) and migration assay (**c**) was performed. We found that silenced vimentin expression in Mahlavu cells reduced the migration ability. All the experiments were repeated at least three times independently. *$P < .05$

Silenced Vimentin Expression Reduced Migratory Ability

In order to further confirm whether the increase of vimentin expression may contribute to the enhancement of migratory ability, we knocked down the vimentin by transient transfected vimentin-specific shRNA into GRP78KD Mahlavu cells. After 48 h, the expression levels were checked by western blotting, and migration assay was performed. As shown in Fig. 5b, c, the expression level of vimentin was reduced dramatically by specific shRNA. The migration results showed that silenced vimentin expression reduced the migratory ability of GRP78KD cells compared with scrambled control cells, indicating that GRP78 modulates cell migration through the regulation of vimentin.

DISCUSSION

GRP78 is a protein that belongs to the Hsp70 family of proteins. Many studies have shown that GRP78 plays an important role in cancer progression and metastasis.[7–9,15,36] High levels of GRP78 expression was found in several different cancer tissues compared with the normal tissue.[5] The expression levels of GRP78 may correlate with the progression and the therapeutic response in breast cancer, head-and-neck cancer, prostate cancer, and melanoma.[7,8,10,36] Interestingly, recent results have revealed an opposite role for GRP78 in oral squamous cell carcinoma.[16] Decreased levels of GRP78 protein expression is a potential prognostic marker for various types of cancer.[16] However, the role of GRP78 is still limited to information that has been obtained from HCC. HCC is a complex disease, and there are currently many obstacles that are obstructing HCC prognosis, diagnosis, and therapy.

Our previous report indicated that decreased GRP78 expression enhanced the therapeutic efficiency of sorafenib therapy.[29] Further, we also found that silencing GRP78 did not influence cell growth.[28] However, our current study suggests that cell migration can be dramatically enhanced after silencing GRP78 expression in HepJ5 and Mahlavu cells (Figs. 2, 3). Overexpressed GRP78 showed the suppression effect in skHep-1 cells (Fig. 4), indicating that GRP78 expression level may correlate with the migratory ability. We dissected the downstream mechanism and found that GRP78 silencing caused the stimulation of vimentin expression (Fig. 5a). Knockdown vimentin caused the decrease of migratory ability (Fig. 5b, c). These findings are consistent with the enhanced migration phenotype that we observed in these cells. This is the first report to describe a correlation between GRP78 and the EMT pathway.

However, Su et al.'s published results demonstrated that GRP78 can promote cell invasion, which contradicts our previous data.[14] A possible explanation for these conflicting results may be due to the different experimental resources used in the two papers. Clinically, only about 10–30% of the patients with HCC are suitable for operation.[37–39] The sample in the report of Su et al. is relatively small, and there was still certain portion of highly expressed GRP78 HCC without portal and intrahepatic invasion. In our study, although three cell lines (HepJ5, skHep-1, and Hahlavu) were used to explore the relationship between GRP78 and invasion ability of HCC, it is still difficult to fully answer the issue. In the era of personalized medicine, the conflict between the two articles makes pretreatment liver tumor biopsy important, which is still controversial.

Vimentin is one of intermediate filaments that all share a common tripartite structure consisting of a highly conserved central α-helical rod domain and variable N-terminal head and C-terminal tail domains.[40] Focal contacts participate in the movement of cells on a substratum. The architecture of focal contacts is disturbed in vimentin-deficient fibroblasts and vimentin regulates focal contact and helps to stabilize cell-ECM adhesions in endothelial cells, which all support a role for vimentin in regulating focal adhesions.[41,42] Vimentin becomes one of the epithelial-to-mesenchymal transition markers, and vimentin is considered as a prerequisite for EMT induction recently.[43] Vimentin-positive HCCs were frequently observed in patients with moderately and poorly differentiated HCCs and had more vascular invasion ability.[44,45] Besides, Lahat and colleagues published recently an interesting article on the use of vimentin as novel anticancer therapeutic cancer target.[46] We believe that it is worthwhile to further dissect the correlation between GRP78 and vimentin in clinical cancer progression and therapeutic responses.

In conclusion, our study indicates that GRP78 may play a suppressor role in HCC cell migration. GRP78 silencing can enhance the expression of vimentin in HCC. We believe that these results can provide additional insight into the mechanism associated with cancer progression and regulation in HCC.

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