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HEF1 Promotes Epithelial Mesenchymal Transition and Bone Invasion in Prostate Cancer Under the Regulation of microRNA-145

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ABSTRACT

The principal problem arising from prostate cancer (PCa) is its propensity to metastasize to bones, and it's crucial to understand the mechanism of tumor progression to metastasis in order to develop therapies that may reduce the morbidity and mortality of PCa patients. Although we had identified that microRNA(miR)-145 could repress bone metastasis of PCa via regulating epithelial–mesenchymal transition (EMT) in previous study, it is still unknown how miR-145 regulated EMT. In the present study, we constructed a luciferase reporter system and identified HEF1 as a direct target of miR-145. More importantly, HEF1 was shown to promote migration, invasion and EMT of PC-3 cells, a human PCa cell line originated from a bone metastatic PCa specimen. And HEF1 was also shown to partially mediate miR-145 suppression of EMT and invasion. Furthermore, inhibition of HEF1 repressed bone invasion of PC-3 cells in vivo. Expression of HEF1 was negatively correlated with miR-145 in primary PCa and bone metastatic specimens, but HEF1 was higher in samples which were more likely to commit to bone metastasis or those with higher free prostate-specific antigen (fPSA) levels and Gleason scores. Taken together, these findings indicate that HEF1 promotes EMT and bone invasion in prostate cancer by directly targeted by miR-145, and miR-145 suppresses EMT and invasion, at least in part, through repressing HEF1. J. Cell. Biochem. 114: 1606–1615, 2013.

KEY WORDS: PROSTATE CANCER; BONE METASTASIS; MICRORNAS; EPITHELIAL–MESENCHYMAL TRANSITION; HUMAN ENHANCER OF FILAMENTATION 1

In male patients, prostate cancer (PCa) is the most frequently diagnosed malignant tumor and the second leading cause of cancer-related deaths in Western countries [Nelson et al., 2003]. The principal problem arising from PCa is its propensity to metastasize to bones, which occurred in as many as 90% of men with advanced PCa [Carlin and Andriole, 2000]. Once tumors metastasize to bones, they are virtually incurable and result in significant morbidity prior to a patient's death. Therefore, it is crucial to understand the mechanism of tumor progression to metastasis in order to develop therapies that may reduce the morbidity and mortality of PCa patients.

The epithelial–mesenchymal transition (EMT) is a key step of the progression of tumor cell metastasis, which includes consecutive

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processes of cell detachment, migration, invasion, dispersion, and final attachment at a distant site [Voulgari and Pintzas, 2009; Hanahan and Weinberg, 2011]. This process has been identified as a hallmark of metastasis in multiple tumors and also as an important step in bone metastasis of PCa [Sethi et al., 2010]. Many oncogenic proteins have been established as positive regulators of EMT, including human enhancer of filamentation 1 (HEF1; also known as NEDD9 or Cas-L), a cytoplasmic scaffolding protein. Recent studies have described the involvement of HEF1 in cell cycle processes, apoptosis, and oncogenic signal transduction [Singh et al., 2007], as well as its critical role in driving migration, invasion, and metastasis of cancer cells [O'Neill et al., 2007]. More importantly, HEF1 has been shown to be implicated to TGF-β signaling pathway [Giampieri et al., 2009], which is important in EMT. In breast cancer, HEF1 was been found to be overexpressed in clinical samples of aggressive tumors and to promote EMT in cell lines through modulating E-cadherin [Kong et al., 2011; Tikhmyanova and Golemis, 2011]. However, it is not known whether HEF1 regulates PCa bone metastasis.

Our previous results have identified microRNA-145 (miR-145) as one of the miRNAs that may repress bone metastasis of PCa by regulating EMT [Peng et al., 2011], but it is unknown how miR-145 regulates EMT and bone invasion in PCa. In this study, we demonstrated that HEF1, which is directly targeted by miR-145, played a role in regulating EMT and promoting invasion of PC-3 cells derived from PCa bone metastasis in vitro and in vivo. We also demonstrated that HEF1 expression positively correlated with clinicopathology of PCa patients. Therefore, a new link was identified between miR-145 and HEF1 in regulation of the EMT process and bone invasion of PCa.

MATERIALS AND METHODS

CELL CULTURE

The bone metastasis PCa cell line PC-3 was purchased from American Type Culture Collection (ATCC, Manassas, VA) and grown in Ham's F-12 culture medium (Hyclone) supplemented with 10% fetal bovine serum (Hyclone, Logan, UT). Stably transfected cells were maintained in media in the presence of puromycin (Sigma–Aldrich, St. Louis, MO). Cells were maintained at 37°C in humidified atmosphere with 5% CO_2.

PLASMIDS

miR-145 mimics and inhibitor were purchased from RiboBio Co. Ltd. (Ribo, China). The pSuper plasmid expressing shRNA-HEF1 (5′-GATCCCCGGATGGTGGCTGTGCTCATTATTCAAGAGATAATGAG-CACAGCCACCATCCTTTTTA-3′), psiCHECK-2 plasmid containing the 3′-UTR w/o mutant of HEF1 (Promega, Madison, WI) and pMSCV plasmid expressing miR-145 were constructed as described previously [Peng et al., 2011]. A plasmid expressing HEF1 was purchased from GeneCopoeia (Rockville, MD). The luciferase reporter constructs were generated by introducing the HEF1 3′-UTR carrying a putative miR-145 binding site into the psiCHECK-2 vector (Promega; 5′-AGAGGAAGGGGACTGCGTTAACGGTTAC-TAAGGAAAACTGGAAATACTGTC-3′), and the plasmid containing an alteration in the miR-145 binding site in the HEF1 3′-UTR was constructed simultaneously by site-directed mutagenesis as a control vector (5′-AGAGGAAGGGGACTGCGTTAACGGTTAC-TAAGGAATAGCATGAATACTGTC-3′).

TRANSIENT TRANSFECTION

Before transfections, 2×10^5 cells were seeded per well of 6-well plates. After 24 h of incubation in growth medium, the cells were transiently transfected with plasmids and/or synthetic miRNAs using Lipofectamine 2000 (Invitrogen, Carlsbad, CA). The transfection medium was removed after 4–6 h, and the cells were incubated for an additional 48 h in culture medium.

WESTERN BLOTTING

Immunoblotting was carried out to analyze protein expression. All cells were seeded in 100-mm tissue culture dishes, then harvested after 24 h when the confluence reached 60–70%. Equal amounts of protein were loaded per lane and resolved by SDS–PAGE, then transferred onto PVDF membrane (Millipore). Block membrane by 5% nonfat milk and probed it with primary antibodies (1:1,000) overnight at 4°C, including mouse anti-E-cadherin (BD Biosciences, San Jose, CA), mouse anti-fibronectin (BD Biosciences), mouse anti-Vimentin (BD Biosciences), mouse anti-HEF1 (Abcam, Cambridge, MA), mouse anti-c-Myc (Abcam) and mouse anti-ZEB2 (Sigma). Membranes were washed three times in TBS-T buffer and incubated for 40 min at room temperature with anti-mouse secondary antibodies. Blots were washed again and developed using an enhanced chemiluminescence (ECL) system (KeyGEN Biology Co. Ltd., Nanjing, China). Protein loading was normalized by reprobing the blots with mouse anti-β-actin or rabbit anti-GAPDH antibody (Abcam).

QUANTITATIVE REAL-TIME PCR (QRT-PCR)

The procedure was carried out according to the manual of the All-in-One™ miRNA qRT-PCR Detection Kit (GeneCopoeia). Briefly, total RNA was extracted from cells and reverse transcribed by adding a poly-A sequence. The qRT-PCR analysis was performed with a specific primer to hsa-miR-145 (GeneCopoeia). Each sample was analyzed in triplicate and negative controls were included. U6 snRNA was used for normalization. Relative expression levels from three independent experiments were calculated following the $2^{-\Delta\Delta C_t}$ method of Livak and Schmittgen [2001].

WOUND HEALING ASSAY

PC-3 cells were trypsinized and seeded equivalently into 6-well tissue culture plates, reaching almost total confluency within 24 h. After serum starvation for 24 h, an artificial homogenous wound was scratched in the cell monolayer with a sterile 100 μl tip (Axygen, Union City, CA). Images of PC-3 cells migrating into the wound were captured at time points of 0, 6, 12, and 24 h under an inverted microscope (40×). Each test was repeated in triplicate.

INVASION ASSAY

The invasion assay was performed using Transwell chambers (Corning, Corning, NY) coated with Matrigel (BD Biosciences) as previously described [Rieger-Christ et al., 2004]. Briefly, cells were trypsinized and resuspended in serum-free medium after serum

starvation for 24 h. Thereafter, 1.5×10^5 cells were added to the upper chamber, while the lower chamber was filled with medium containing 10% FBS. After incubation for 48 h, cells that had invaded through the coated membrane to the lower surface were fixed and stained. Each test was repeated in triplicate, and the cell count was performed under a microscope (100×).

ADHESION ASSAY

The adhesion assay was performed as described previously [Peng et al., 2011]. Briefly, suspended cells which had been serum starved for 24 h were seeded into plate that was blocked with 1% BSA, at the density of 1.5×10^4 cells per well. After incubation for 30 min, the plate were gently washed twice with PBS to remove non-adherent cells. Adherent cells were fixed and stained, and counted under an inverted microscope (100×). Each test was repeated in triplicate.

LUCIFERASE ASSAY

Luciferase assays were carried out in 293FT cells. Cells were co-transfected with miRNA and a luciferase reporter plasmid. After culturing for 48 h, the cells were harvested and lysed for luminescence detection. The following procedure and detection were performed using a luciferase assay kit (Promega) according to the manufacturer's protocol. *Renilla* luciferase was activated to emit the primary luminescence, and firefly luminescence was used for normalization. Each test was repeated in triplicate.

GENERATION OF STABLE CELL LINES

The sequence of shRNA-HEF1 was cloned into a pSuper-puromycin retroviral vector, and the resulting plasmid was transfected into 293FT cells to generate virus as described previously [Mo and Beck, 1999]. After incubation at 37°C for 6 h, the transfected cells were cultured in fresh media overnight. In the following days, media were collected three times a day to gather the produced virus until the 293FT cells reached total confluency. Media containing viruses were used to infect PC-3 cells for 24 h. After removal of the inoculum and replacement with fresh media, infected cells were selected by adding puromycin. Stable cell lines were verified by Western blotting.

IN VIVO MODEL OF PROSTATE CANCER BONE METASTASIS

We used an intra-tibial injection model to detect whether inhibition of HEF1 could reduce the bone metastatic capacity of PCa cells. Six male severe combined immunodeficient (SCID) mice at 4 weeks old were purchased from HFK Bio-Technology Co., Ltd. (Beijing, China). All mice were injected with stably selected PC-3/HEF1-shRNA cells on the right tibias and PC-3/vector cells on the left tibias as matching controls. The inoculation procedure was performed as previously described [Peng et al., 2011]. Bone lesions were evaluated and analyzed using the following scoring criteria based on X-ray examination: 0, no lesion; 1, minor lesions; 2, small lesions; 3, significant lesions with minor break of margins; 4, significant lesions with major break in peripheral lesions [Yang et al., 2006].

HUMAN PCA TISSUE SAMPLES AND RNA EXTRACTION

Tissue samples were collected from 22 primary PCa patients and 14 skeletal metastatic patients. The methods for collection of data and RNA extraction were previously described [Tikhmyanova and Golemis, 2011]. The expression of miR-145 was detected using qRT-PCR. The study was approved by the Institutional Review Board (IRB) in the First Affiliated Hospital of Sun Yat-sen University.

IMMUNOHISTOCHEMISTRY (IHC)

Paraffin sections were used for IHC analysis. Staining of HEF1 in primary and bone metastatic PCa tissues was carried out using Histostain-Plus kit according to the manufacturer's instructions (Invitrogen). Briefly, a 1:500 dilution of mouse anti-HEF1 antibody (Abcam) was used. Slides were incubated with the primary antibody overnight at 4°C. Staining was visualized and photos were taken with an Olympus image processing system. The percentage of positively stained area was estimated and expressed as a proportional score (PS; $<10\% = 1$, $10–25\% = 2$, $26–75\% = 3$, $>75\% = 4$). A 4-tiered scale was also used for scoring the staining intensity and presented as an intensity score (IS; negative = 0, weak = 1, moderate = 2, strong = 3). The total score (TS) = PS × IS (range 0–12).

STATISTICAL ANALYSES

Statistical significance of the studies was analyzed by Student's *t*-test or one-way ANOVA, and $P < 0.05$ was considered significant. The correlation coefficient (Spearman rank correlation test) was calculated to estimate the linear correlation between HEF1 expression and the miR-145 level or extent of clinicopathology in clinical specimens.

RESULTS

MIR-145 NEGATIVELY REGULATES EXPRESSION LEVELS OF HEF1, ZEB2, C-MYC, AND MIR-9 IN PC-3 CELLS

First, we determined by Western blotting whether miR-145 regulates the expression of HEF1 and ZEB2, which are putative targets of miR-145 based on bioinformatic analysis, as well as the expression of c-Myc, an identified target of miR-145 [Sachdeva et al., 2009]. The expression of miR-145 in PC-3 cells was detected by qRT-PCR (Fig. 1A). As shown in Figure 1B, expression levels of HEF1, ZEB2 and c-Myc were decreased in PC-3 transfected with miR-145 and increased in PC-3 cells treated with miR-145 inhibitor, demonstrating that miR-145 negatively regulated expression of these three proteins. Furthermore, miR-145 was shown by real-time PCR to negatively regulate the expression of miR-9 (Fig. 1C; $^{**}P < 0.01$), which directly targets E-cadherin [Ma et al., 2010].

MIR-145 DIRECTLY TARGETS HEF1 BY BINDING TO ITS 3′-UTR

To identify whether miR-145 directly targets HEF1, dual-luciferase reporter gene assays were carried out. Luciferase reporter plasmids containing the wild-type 3′-UTR (Luc-HEF1-wt) or mutant 3′-UTR (Luc-HEF1-mt) of HEF1 (Fig. 1D) were constructed to determine the targeted region. As shown in Figure 1E, luciferase activities were significantly higher in cells transfected with either Luc-HEF1-mt or scrambled miRNA, with 2.02- and 1.81-fold higher levels, respectively, than that in cells transfected with Luc-HEF1-wt. Meanwhile, the scrambled miRNA base sequence did not suppress Luc-HEF1-wt, and miR-145 could not bind to the mutant HEF1-3′-

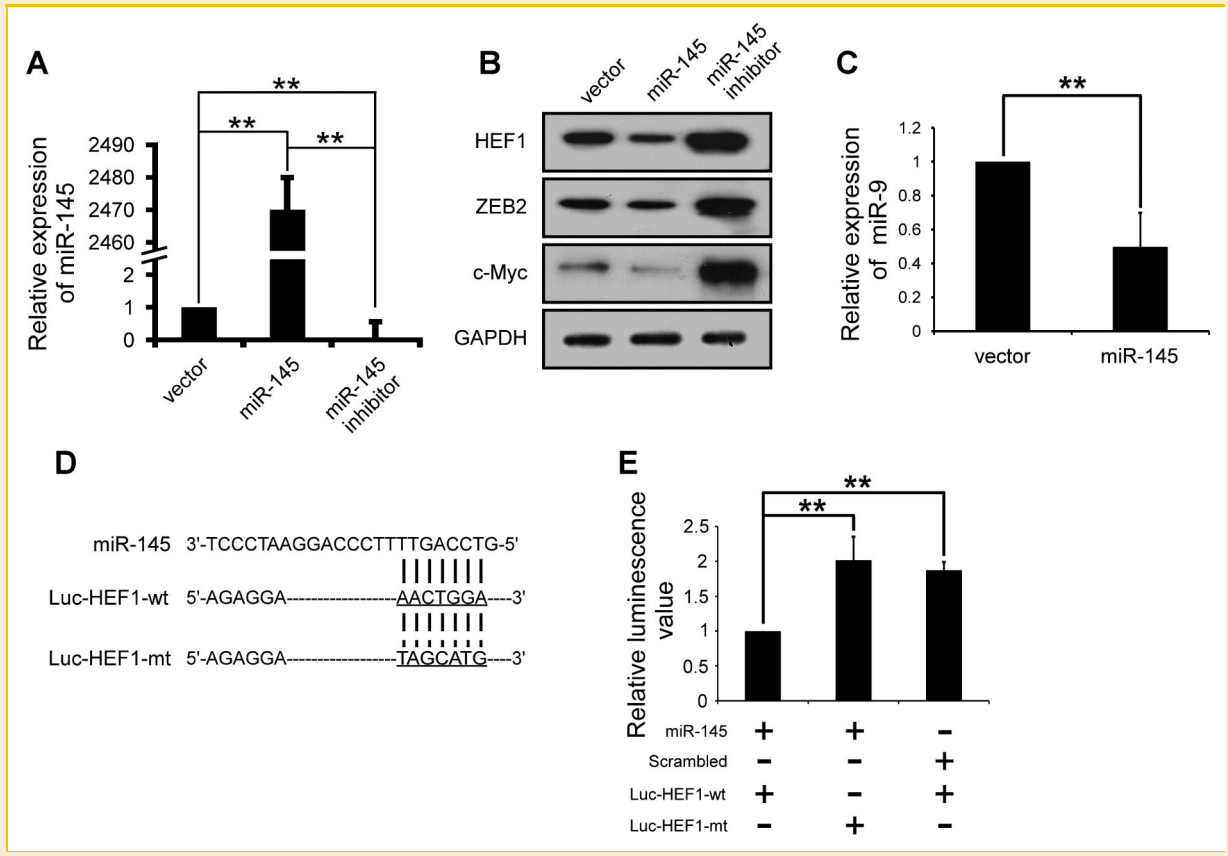

Fig. 1. miR-145 negatively regulates expression of HEF1 by directly binding to its 3′-UTR. A: miR-145 expression was detected by real-time PCR. B: Total cell lysates of PC-3 with different treatments were subject to Western blotting and probed with antibodies to HEF1, ZEB2, and c-Myc. C: miR-9 expression was detected under different transfection conditions. D: wild-type 3′-UTR sequence or mutant 3′-UTR sequence of HEF1 was cloned into luciferase plasmids (Luc-HEF1-wt or Luc-HEF1-mt, respectively). Binding sites are indicated by solid lines and mutant binding sites by dotted lines. E: Relative luminescence in groups transfected with different factors in luciferase assay are presented as the mean ± SEM of each group. $^{**}P < 0.01$.

UTR. Thus, these observations indicated that miR-145 directly targeted HEF1 through interacting with the 3′-UTR.

HEF1 IS A POSITIVE REGULATOR OF EMT AND AGGRESSIVENESS IN PC-3 CELLS

To investigate whether HEF1 regulates metastasis by promoting EMT, we knocked down the endogenous HEF1 with a specific shRNA in PC-3 cells. As expected, down-regulation of HEF1 promoted the epithelial phenotype of metastatic PCa cells. As shown in Figure 2A, PC-3 cells treated with the vector control displayed typical fibroblastic morphology, which was consistent with a very low level of E-cadherin expression. Knockdown of HEF1 with shRNA produced a dramatic shift in morphology, from a stick-like or long spindle-shaped mesenchymal cell population to a short spindle-shaped or round and flat epithelial cell population. Moreover, Western blotting analysis was performed to detect protein expression of E-cadherin, fibronectin, and vimentin in PC-3 cells. The results showed that HEF1 knockdown increased E-cadherin in PC-3 cells, while HEF1 overexpression decreased it. Furthermore, fibronectin and vimentin levels were decreased in PC-3 cells with HEF1 down-regulation, while they were promoted in PC-3 cells with HEF1 up-regulation (Fig. 2B and quantification in Fig. S1). These findings implicated HEF1 as a positive regulator of EMT in PCa PC-3 cells.

To further investigate the role of HEF1 in the development and progression of PCa metastasis, we examined the function of HEF1 on migration, invasion and adhesion in PC-3 cells. As shown in Figure 2C, downregulation of HEF1 decreased the invasive ability of PC-3/HEF1 stable cells to 68.2%, and promoted the adhesive ability to 150%, compared with that of PC-3/vector stable cells (Fig. 2D). Finally, HEF1 knockdown decreased the rate of cell migration compared with the vector control (Fig. 2E). These results suggest that inhibition of HEF1 can suppress the metastatic potential of PCa PC-3 cells.

HEF1 PARTIALLY MEDIATES REGULATORY FUNCTIONS OF MIR-145 ON EMT AND AGGRESSIVENESS IN PC-3 CELLS

To determine whether HEF1 mediates the functions of miR-145 in the regulation of EMT and aggressiveness in PC-3 cells, HEF1 was overexpressed by transfecting PC-3 cells with the pHEF1 plasmid, while it was attenuated with shRNA (shHEF1). The expression levels of miR-145 under these different conditions were confirmed by real-time PCR (Fig. 3A). Interestingly, expression of miR-145 could be lowered by ectopic HEF1 when its expression vector was

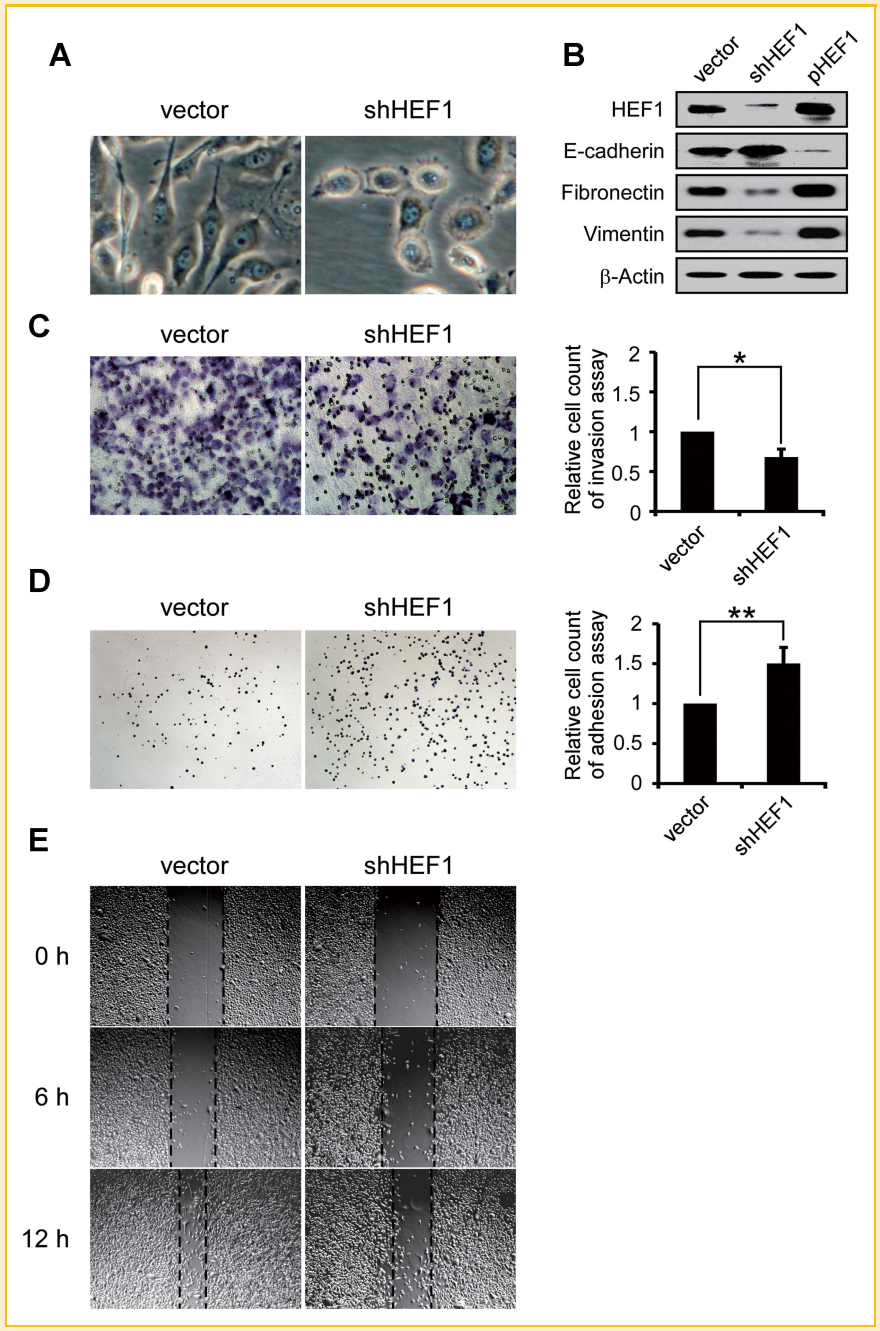

Fig. 2. HEF1 is a positive regulator of aggressiveness and EMT of PC-3 cells. A: PC-3 cells transfected with shRNA to silence HEF1 (shHEF1) appear morphologically different compared with those treated with vehicle control (vector). B: Total cell lysates from PC-3 cells treated as in (A) were subjected to Western blotting, which showed the effect of silencing HEF1 in PC-3 cells on expression of EMT markers (E-cadherin, fibronectin, and vimentin). β-Actin was detected as a loading control. Different treatments applied imposed different impacts on invasion (C), adhesion (D), and migration (E) of PC-3 cells. In invasion and adhesion assays, relative cell counts were calculated and normalized to those of vector control treatment. Data are presented as the mean ± SEM of each group (C and D, right panels. $^{*}P < 0.05$; $^{**}P < 0.01$).

co-transfected into PC-3 with the miR-145 mimic, or elevated when HEF1-targeting shRNA was co-transfected into PC-3 with the miR-145 inhibitor. The results indicated that HEF1 may influence the expression of miR-145 through a feedback mechanism. As shown in Figure 3B, the ectopic miR-145 increased expression of E-cadherin and decreased expression of fibronectin and vimentin in PC-3 cells, indicating that EMT was partially blocked by overexpressed HEF1. Inhibition of miR-145 reduced expression of E-cadherin and promoted expression of fibronectin and vimentin in PC-3 cells, but this promotion of EMT was partially blocked by the HEF1-specific shRNA (quantified in Fig. S2). Furthermore, knockdown of miR-145 increased the cell invasiveness and migration rate and reduced the adhesion of PC-3 cells, while those effects were partially blocked by shHEF1 (Fig. 3C,D). These findings highlighted HEF1 as an

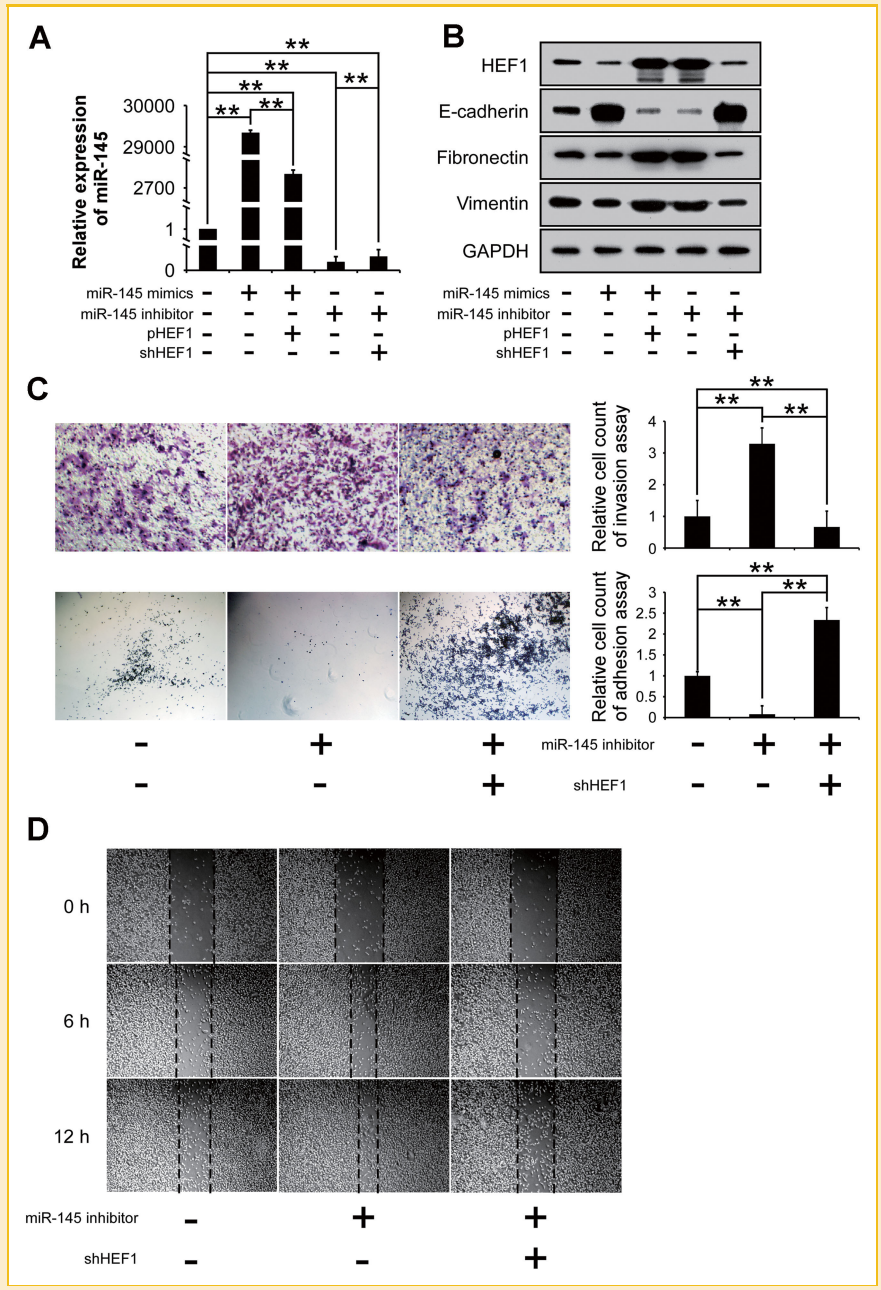

Fig. 3. HEF1 mediates miR-145-regulating EMT and aggressiveness in PC-3 cells. A: RNA from PC-3 cells treated with different factors were subjected to real-time PCR assay. Data are presented as means ± SEM. B: Total cell lysates prepared from PC-3 cells cultured as in (A) were analyzed by Western blotting. Results show that different treatments differentially influenced expression levels of EMT markers (E-cadherin, fibronectin, and vimentin). GAPDH was detected as loading control. C: Different treatments on PC-3 cells imposed different impacts on their capacities for invasion (the upper panel) and adhesion (lower panel). Relative cell counts were calculated and normalized to those of vector control treatment. Data are means ± SEM of each group (right panels, $^{**}P < 0.01$). D: Different treatments imposed different impacts on the migration ability of PC-3 cells.

important factor in the miR-145-mediated regulation of EMT and aggressiveness in PC-3 cells.

INHIBITION OF HEF1 REPRESSES BONE INVASION OF PC-3 CELLS IN VIVO

To further confirm the significant influence of HEF1 on the development and progression of PCa metastasis in vivo, an intra-tibial injection mouse model was established. Four weeks after intra-tibial inoculation, skeletal lesions of all animals in the left tibias were remarkably larger than those in the right tibias (Fig. 4A), indicating that PC-3/shHEF cells had lower bone invasive ability compared with PC-3/vector cells. These observations were confirmed by histological analysis and H&E staining (Fig. 4B). The extent and areas of skeletal lesions shown in the X-rays were also scored (Fig. 4C), revealing significantly smaller tumors and less bone invasion in PC-3/shHEF1 cells compared with PC-3/vector cells. The

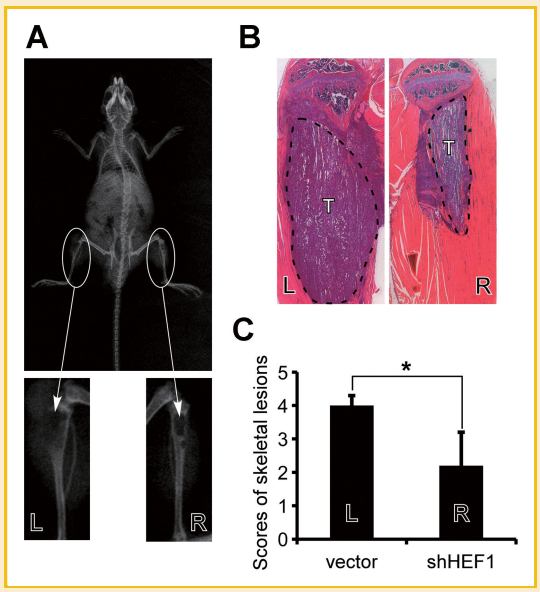

Fig. 4. Inhibition of HEF1 represses bone invasion and metastasis of PC-3 cells in vivo. Male SCID mice were inoculated with PC-3 cells through the intra-tibial route as described in Methods and Materials Section. A: PC-3 cells transfected with vehicle control were inoculated in left tibias, and those transfected with shHEF1 in the right tibias. Skeletal lesions in radiographs are indicated by arrows. B: Histological analysis of skeletal samples in (A) was carried by H&E staining (magnification, 25×). Tumors are indicated by dotted outlines and marked with a "T." C: Lesion scores of vector and shHEF1 treated specimens were evaluated as described in Methods and Materials Section and presented as the mean ± SEM of each group. $^{*}P < 0.05$.

results indicated that inhibition of HEF1 inhibited the development and aggressiveness of PCa in bone.

HEF1 IS NEGATIVELY CORRELATED WITH MIR-145 EXPRESSION IN PCA SPECIMENS AND POSITIVELY CORRELATED WITH CLINICOPATHOLOGY OF PCA PATIENTS

To determine whether HEF1 expression is correlated with the level of miR-145 in PCa specimens, we collected a group of clinical resected samples (Table I). After IHC staining for expression of HEF1 in primary and bone metastatic PCa specimens, miR-145 was detected by qRT-PCR. As shown in Figure 5A, the level of HEF1 was most visibly elevated in bone metastatic specimens and moderately increased in primary cancer samples, while it was lowly expressed in normal prostate tissues. HEF1 expression in primary or bone metastatic specimens was inversely correlated with the corresponding miR-145 expression (Spearman correlation = −0.800, $P < 0.001$, Fig. 5B; Spearman correlation = −0.603, $P = 0.022$, Fig. 5C).

To further identify whether HEF1 expression is correlated with clinicopathology in PCa patients, the HEF1 level of the primary PCa specimens with bone metastasis (n = 10) had an average IHC score of 9.2, compared to those (n = 12) without bone metastasis with an average IHC score of 5.2 ($P < 0.01$, Fig. 5D). Furthermore, we assessed whether the expression of HEF1 was related to total serum prostate-specific antigen (PSA) level, free PSA (fPSA) level and Gleason score in primary PCa patients. A significant positive correlation was found between the expression of HEF1 and serum fPSA level (Spearman correlation = 0.656, $P = 0.0010$, Fig. 5E), as well as between the expression of HEF1 and Gleason score (Spearman correlation = 0.426, $P = 0.048$, Fig. 5F). These results indicated that overexpression of HEF1 was associated with tumor progression and bone metastasis.

TABLE I. Clinical Characteristics of Prostate Cancer Patients

Characteristics	n (%) or median (IQR)
All patients	22
Age, years	72.5 (68–78)
Bone metastasis	
+, positive	10 (45.5)
−, negative	12 (54.5)
fPSA, ng/ml	
0–2	5 (23)
2–10	5 (23)
10–30	6 (27)
≥30	6 (27)
Gleason score	
2–4	1 (4.5)
5–7	10 (45.5)
8–10	11 (50)

PSA, prostate specific antigen; IQR, interquartile range.

DISCUSSION

We previously implicated the function of miR-145 in suppressing bone metastasis of PCa and its involvement in regulating EMT [Peng et al., 2011]. In the present study, we further identified the link between miR-145 and HEF1 in the regulation of EMT programs and metastasis of PCa. Our results identified HEF1 as a direct target of miR-145 mediating its regulation of EMT and invasion in PCa bone metastasis (Fig. 6).

Some studies have found that several miRNAs play a critical role in regulating EMT in cancer progression through targeting EMT regulators, Snail1, ZEB1, and ZEB2 [Kim et al., 2011; Bullock et al., 2012]. Up-regulation of miR-34a has been shown to result in Snail-dependent EMT activation in colon, breast and lung carcinoma cells by directly targeting Snail [Kim et al., 2011]. The miR-200 family is also known to induce EMT by altering ZEB1 and ZEB2 expression [Bullock et al., 2012]. In this study, HEF1 obviously decreased E-cadherin and promoted EMT of PC-3 cells, and regulatory functions of miR-145 on aggressiveness and EMT were found to be partially mediated by HEF1. Specifically, we revealed the metastasis-promoting protein HEF1 as a direct target of miR-145. Interestingly, a recent study simultaneously verified that HEF1, directly targeted by miR-145, increases invasiveness of glioblastomas [Speranza et al., 2012]. This result strongly supports our discovery that HEF1 may promote EMT and bone invasion in PCa under the negative regulation of miR-145. In addition, many studies identified that pri-miR-145 levels are significantly increased following TGF-β1 treatment, which was found to induce rapid HEF1 degradation in a proteasome-dependent fashion [Feng et al., 2004; Davis-Dusenbery et al., 2011; Mayorga and Penn, 2012]. But controversially, it was also reported that TGF-β1 promoted HEF1 expression in a dose-dependent manner [Zheng and McKeown-Longo, 2002]. It

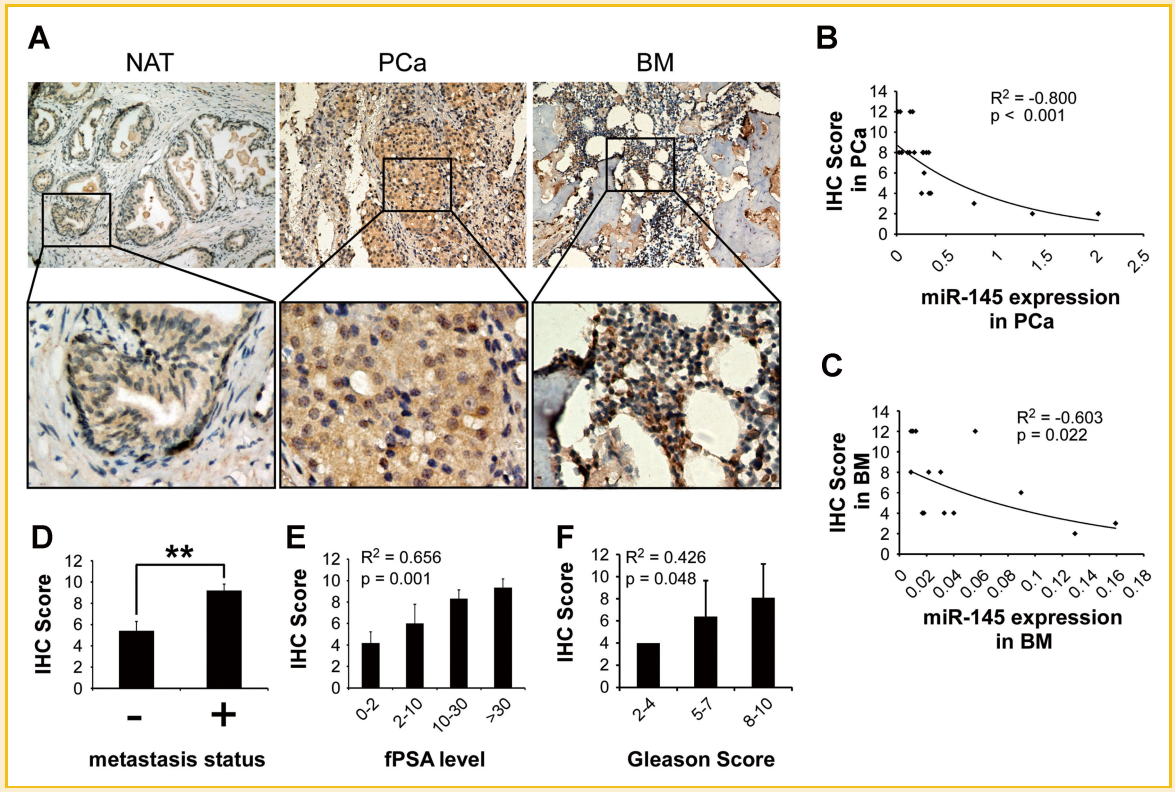

Fig. 5. HEF1 is negatively correlated with miR-145 expression in PCa specimens and positively correlated with clinicopathology of PCa patients. A: Sections show expression of HEF1 by immunochemistry in non-tumor adjacent tissue (NAT), primary prostate cancer (PCa) and bone metastasis (BM). Stainings of HEF1 are in yellow and brown, while nuclei counterstained with hematoxylin are in blue. Pictures were taken under magnifications of 100× (upper panel) and 200× (lower panel). B,C: Expression of miR-145 and IHC scores of HEF1 in clinical specimens were calculated, and correlation analysis was performed in primary PCa samples (B) and bone metastasis samples (C). D: IHC scores of HEF1 in samples from patients with bone metastases was significantly higher than from those without metastases (means ± SEM. $^{**}P < 0.01$). E,F: Levels of free PSA (E) and Gleason scores (F) in patients with primary tumors are presented on the *x* axis. The *y* axis reflects the mean IHC score of HEF1 within each group. Bars represent standard errors.

is interesting that TGF-β1 decreases HEF1 while upregulating miR-145, but also promotes it in a different circumstance. Since there is no researches about the relationships between TGF-β1, miR-145 and HEF1 in PCa, further study has to be conducted to illustrate this issue.

One of the hallmarks of EMT is the down-regulation of the cell–cell adhesion protein E-cadherin, resulting in destabilization of the adherens junctions that connect cells [Huber et al., 2005]. Some studies have found that HEF1 is able to activate the ERK cascade and subsequently induce Snail and Slug up-regulation, which bind to the E-cadherin promoter to inhibit its expression [Kong et al., 2011]. Moreover, HEF1-dependent signaling through the SRC kinase promotes E-cadherin removal from cell junctions and its lysosomal degradation in mammalian cells [Tikhmyanova and Golemis, 2011]. Therefore, HEF1 may regulate EMT by indirectly decreasing expression of E-cadherin. Furthermore, in PC-3 cells, we have

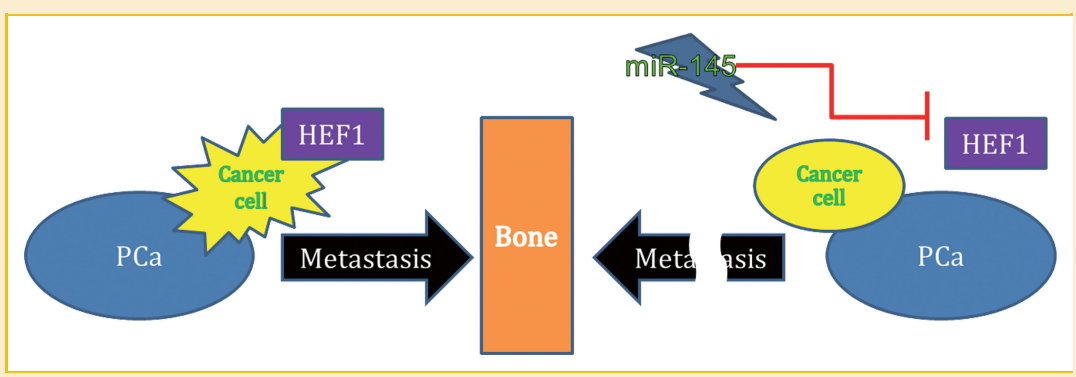

Fig. 6. HEF1 mediated the function of miR-145 in inhibiting PCa bone metastasis. A brief and pithy diagram about the mechanism.

also found that miR-145 can negatively regulate the level of c-Myc and expression of miR-9, which is directly induced by Myc [Ma et al., 2010]. Because miR-9 can directly down-regulate E-cadherin and induce mesenchymal traits and the formation of micrometastases [Ma et al., 2010], miR-145 may modulate EMT through the c-Myc/miR-9/E-cadherin axis. Importantly, we also found that ZEB2, which a putative target of miR-145 [Boominathan, 2010], was decreased by miR-145. Therefore, miR-145 may regulate EMT via targeting ZEB2. Recently, Gao et al. [2012] found N-cadherin which is an important marker of the mesenchymal phenotype to be directly and negatively targeted by miR-145 in a luciferase-based assay. Taken together, those findings suggest that since HEF1 is not a transcription factor, miR-145 may regulate EMT by other mechanisms and HEF1 plays, at least in part, a role in this process.

Besides regulating EMT, miR-145 can regulate invasion and metastasis of cancers through directly targeting oncogenes such as MUC1 and FSCN1 [Kano et al., 2010; Sachdeva and Mo, 2010], or indirectly suppressing metastasis-related factors such as MMP9 which is downstream effector of N-cadherin [Gao et al., 2012]. In this study, our results showed that miR-145 regulated metastasis-promoting protein HEF1, which can directly enhance migration and invasion of cancer cells [Croft and Olson, 2008; Bui et al., 2009]. In addition, miR-145 can directly regulate stem cell markers, such as c-Myc, Oct4, Sox2, and KLF4 [Sachdeva et al., 2009; Xu et al., 2009]. All these findings suggest that miR-145 may also regulate cancer invasion and metastasis by modulating metastasis-promoting proteins and cancer stem cells or stem cell properties. Therefore, the notion that cancer cells routinely pass through a complete EMT program is likely to be simplistic in cancer metastasis [Sanz-Moreno et al., 2008]. The migration and invasion events of cancer cells controlled by miRNAs do not necessarily require a complete EMT phenotype.

Several studies have shown that elevated expression of HEF1 promotes cancer progression and invasion in many cancer types, especially in melanoma, breast cancer and colorectal cancer [Kim et al., 2006; Izumchenko et al., 2009; Li et al., 2011]. However, Seo et al. [2011] reported that HEF1 seems to regulate the progression of chronic myelogenous leukemia (CML) in a negative manner, presumably by attenuating extramedullary hyperplasia. In this study, expression of HEF1 was found to be low in normal prostate cells, modest in primary PCa and high in bone metastasis, so the result showed HEF1 may be an indicator for the malignancy of PCa. Moreover, in primary PCa samples, the higher expression of HEF1 strongly indicated a higher degree of metastatic properties and was positively significantly associated with the fPSA level and Gleason Score, which are powerful indicators of poor prognosis. These results suggest that HEF1 plays a significant role in invasion and bone metastasis of PCa. In addition, HEF1 is a cytoskeletal protein that can directly enhance migration and invasion of cancer cells [Croft and Olson, 2008; Xu et al., 2009], beyond just regulating E-cadherin [Kong et al., 2011; Tikhmyanova and Golemis, 2011]. Moreover, overexpression of HEF1 also can promote cancer cell growth, migration and invasion by activating various signal transduction pathways, such as extracellular signal-regulated kinase, p38, RhoA, and Aurora A [Pugacheva and Golemis, 2005; Dadke et al., 2006; Singh et al., 2007; Xia et al., 2010]. These observations suggest that HEF1 may promote bone invasion of PCa through multiple pathways.

CONCLUSION

In summary, this study demonstrates that HEF1, a direct target of miR-145, promotes EMT and bone invasion in PCa and that miR-145 suppresses EMT and invasion, at least in part, through targeting HEF1. These findings imply that miR-145 and HEF1 may be used as novel therapeutic targets in bone metastasis of PCa. However, taking miRNAs into clinical practice remains a challenging issue, and our following studies will be aimed towards addressing those hurdles.

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