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Research paper

Epidermal growth factor/heat shock protein 27 pathway regulates vasculogenic mimicry activity of breast cancer stem/progenitor cells

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ABSTRACT

Tumor vascularization, which is mainly contributed by angiogenesis and vascularization, is necessary for tumor maintenance and progression. Vasculogenic mimicry (VM), vascular-like channels which are lack of the involvement of endothelial cells, has been observed in aggressive cancers and also involves in tumor vascularization. Breast cancer stem/progenitor cells (BCSCs) have been identified as a subpopulation of breast cancer cells with markers of $CD24^{-}CD44^{+}$, high aldehyde dehydrogenase activity ($ALDH^{+}$) or could be enriched by mammosphere cultivation. These cells have been proven to be associated with tumor vascularization. Here we investigated the molecular mechanisms in VM activity of BCSCs. By periodic acid-Schiff or hematoxylin–eosin stain, we found that there were VM structures in two xenografted human breast cancer tissues established from $CD24^{-}CD44^{+}$ or $ALDH^{+}$ cells. Only $ALDH^{+}$ or mammosphere-forming BCSCs could form tube structures on matrigel-coated surface as similar as microvascular endothelial cells. Inhibition of the phosphorylation of epidermal growth factor receptor (EGFR) by gefitinib or knockdown of EGFR by lentiviral shRNA abolished the *in vitro* VM activity of BCSCs. By quercetin treatment, a plant flavonoid compound which is known to suppress heat shock proteins, or siRNA-mediated gene silencing, both Hsp27 expression and VM capability of BCSCs were suppressed. Forced expression of phosphor-mimic form of Hsp27 in $ALDH^{+}$ BCSCs could overcome the inhibitory effect of gefitinib. In conclusion, our data demonstrate that VM activity of BCSCs is mediated by EGF/Hsp27 signaling and targeting this pathway may benefit to breast cancer therapy.

1. Introduction

Tumor vascularization is required for cancer progression when tumors expand more than 1–2 mm^3, a size which the delivery of nutrients and oxygen could not rely on diffusion [1] and is usually explained mainly by angiogenesis, a process involves the proliferation and migration of endothelial cells from preexisting blood vessels [2]. In addition to angiogenesis, the mechanisms of tumor vascularization also include angioblasts recruitment, cooption, mosaic vessels and vasculogenic mimicry [1,2]. Vasculogenic mimicry (VM) is defined by the formation of perfusable, matrix-rich and vasculogenic-like networks by tumor cells themselves without endothelial cells being detected [3]. VM was first discovered in aggressive uveal melanoma by a periodic acid-Schiff (PAS) stain which revealed the matrix within tissues [4]. The red blood cells could be found in PAS-positive VM networks and they seem to be connected with peripheral endothelial-lined vasculatures [4]. VM is not restricted to melanoma but also being discovered in other cancers such as breast, prostate and ovarian carcinomas [5]. The

Abbreviations: EGF, epidermal growth factor; Hsp27, heat shock protein 27; VM, vasculogenic mimicry; BCSCs, breast cancer stem/progenitor cells; ALDH, aldehyde dehydrogenase; PAS, periodic acid-Schiff; VEGF, vascular endothelial growth factor; EMT, epithelial–mesenchymal transition.

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invasive breast cancer cells were found to display VM activity when cultured on matrigel and it could be inhibited by celecoxib, an inhibitor of cyclooxygenase-2 [6]. Overexpression of Her2 in MCF7 cells, which is a non-invasive breast cancer cell line, induced VE-cadherin expression and VM activity [7]. Recently, VM in triple negative breast cancer has been found to be associated with CD133 expression and CD133+ cells in triple negative cells was considered as cancer stem cells [8].

Cancer stem/progenitor cells (CSCs) are a subpopulation of cancer cells with tumor-initiating, differentiation and drug resistance capabilities [9,10]. Targeting CSCs is considered as the key for successful cancer therapy [11,12]. In breast cancer, CSCs could be identified with surface markers $CD24^{-}CD44^{+}$ [13], intracellular aldehyde dehydrogenase (ALDH) activity [14] or mammospheres that proliferate as spheroids in a non-adherence and serum-free condition [15,16]. From the characteristics of differentiation capacity of CSCs and the importance of tumor vascularization of cancer progression, it is possible that CSCs may contribute to tumor vascularization by transdifferentiation into endothelial progenitor cells (EPCs). In ovarian cancer study, when the type I epithelial ovarian cancer cells (EOCs), which are $CD44^{+}$ cells and with characteristics of CSCs, xenografted in nude mice, the vascular structures within tumors were mainly consisted of cells with positivity of human CD34 marker [17]. These type I EOCs formed vascular structures when cultured in matrigel condition *in vitro* and transdifferentiated into $CD34^{+}$ cells [17]. In addition to transdifferentiation into EPCs, CSCs may secret angiogenic factors to stimulate tumor angiogenesis. In glioma study, the tumors of spheroid cells, which were enriched with CSCs, exhibited higher microvessel density and induced increased EPC recruitment [18]. The secretion of angiogenic factors, vascular endothelial growth factor (VEGF) and stromal-derived factor 1, was responsible for the tumor vascularization stimulation ability of glioma CSCs [18]. Other reports demonstrated that glioma CSCs could transdifferentiate into endothelial cells and support tumor vascularization. 20–90% of tumor endothelium carried the same genetic alteration as tumor cells [19]. When glioma CSCs cultured in endothelial condition, their progeny displayed features of functional endothelial cells [19]. Blocking VEGF signaling suppressed the maturation of tumor endothelial progenitors into endothelium and inhibition of Notch signaling abolished the transition of glioma CSCs into endothelial progenitors [20]. These reports suggest CSCs may support tumor vascularization through direct transdifferentiation into endothelial cells or progenitors. In breast cancer study, the vascular-associated markers expressed in aggressive MDA-MB-231 breast cancer cell line, which was described as an embryonic-like cell type, but not nonaggressive MCF7 cells [21]. Also using spheroid culture, the sphere-forming cells of MCF7 arranged into VM structures in response to nutrient limitation and expressed endothelial markers CD31, angiopoietin-1 and endoglin [22].

Although VM in breast cancer seems to be associated with breast CSCs (BCSCs), the mechanisms are not fully understood. Spheroid forming MCF7 cells could form VM structures *in vitro* through the up-regulation of osteopontin, which was an oncoprotein and a target of miR-299-5p [22]. In this present study, we investigate the regulating molecules in VM activity of BCSCs and discover that epidermal growth factor (EGF) could stimulate VM activity of BCSCs. It has been reported that the activation of heat shock protein 27 (Hsp27) is required for EGF-induced angiogenesis in endothelial cells [23]. Forced expression of Hsp27 in non-angiogenic human breast cancer cells could induce expansive tumor growth and increase vascular proliferation *in vivo* [24], but if Hsp27 participates in EGF-induced VM activity of BCSCs remains unknown. Here we provide experimental evidences to demonstrate that Hsp27, as well as its phosphorylation, is critical for EGF-induced VM activity of BCSCs.

2. Materials and methods

2.1. Ethics statement

All the studies involving human participates were fully encoded to protect patient confidentiality and were utilized under a protocol approved by the Institutional Review Board of Human Subjects Research Ethics Committees of Chung Shan Medical University Hospital, Taichung, Taiwan. All the patients enrolled in this study have signed an Informed Consent Form to agree to participate in this study and to publish the results.

All the animal studies were operated following a protocol approved by Institutional Animal Care & Use Committee of Chung Shan Medical University, Taichung, Taiwan (Approval No. 1103).

2.2. Reagents

Gefitinib, the EGFR inhibitor, was purchased from Tocris Bioscience (Bristol, UK). Quercetin was purchased from Calbiochem (Darmstadt, Germany). These chemicals were dissolved in dimethyl sulfoxide (DMSO, Sigma–Aldrich, St. Louis, MO, USA).

2.3. Fluorescence activated cell sorting (FACS)

Anti-CD24-PE, anti-CD44-APC, antibodies were purchased form BD Biosciences (San Jose, CA, USA). ALDEFLUOR assay, which was originally used for isolation of human hematopoietic progenitors [25], was used for isolation of stem/progenitor cells from AS-B244 and assay kit was purchased from StemCell Technologies Inc. (Vancouver, BC, Canada). Cell labeling with fluorescent-conjugated antibodies or ALDEFLUOR assay was performed according to the manufacturer's recommendations. Sorting of antibody-labeled cells was carried out on a FACSAria™ cell sorter (BD Biosciences).

2.4. Xenotransplantation of human breast cancer cells

BC0145 or BC0244 human breast cancer cells were established from primary human breast cancer specimens as described previously [26,27]. Before inoculation of tumor cells, 8-week-old female NOD/SCID mice (Tzu Chi University, Hualien, Taiwan) received a sub-lethal dose of gamma irradiation. 10^5 of $CD24^{-}CD44^{+}$ BC0145 or $ALDH^{+}$ BC0244 tumor cells were mixed with 5×10^5 normal human breast fibroblasts/site in 2 mg/ml Matrigel and were subcutaneously injected into mammary fat pads of mice. Mice were monitored for tumor growth weekly.

2.5. PAS and hematoxylin–eosin (H&E) stain

Xenografted tumor tissues were embedded with paraffin and 1 μm of sections were used for PAS or H&E stain after deparaffinization process. PAS or H&E stain was performed with commercial reagent (Sigma–Aldrich) according to the manufacturer's recommendations.

2.6. Cell culture

AS-B145 and AS-B244 breast cancer cells which derived from BC0145 or BC0244 xenograft human breast cancer cells were established as our previous reports [26–28] and maintained in MEMα medium (Life Technologies, Grand Island, NY, USA) supplemented with 10% fetal bovine serum (Hyclone Thermo Fisher Scientific Inc., Waltham, MA, USA), bovine insulin (0.1 mg/ml,

Sigma–Aldrich), sodium pyruvate (1 mM, Life Technologies), and Glutamax (2 mM, Life Technologies) in a 5% CO_2 air humidified atmosphere at 37 °C. HMEC-1, a human microvascular endothelial cell line, was maintained in M200 medium supplemented with 1× low serum growth supplement (LSGS, Life Technologies).

2.7. Mammosphere cultivation

Mammosphere cultivation was performed as our previous reports [27–29]. Briefly, cells were harvested from a monolayer culture and prepared as density of 1×10^4 cells/ml in DMEM/F12 medium (Life Technologies) contain 0.5% methylcellulose (Sigma–Aldrich), 0.4% bovine serum albumin (Life Technologies), 10 ng/ml EGF (PeproTech, Rocky Hill, NJ, USA), 10 ng/ml bFGF (PeproTech), 5 μg/ml insulin (Sigma–Aldrich), 1 μg/ml hydrocortisone (Sigma–Aldrich) and 4 μg/ml heparin (Sigma–Aldrich). 2 ml of cell solution were seeded into each well of ultralow attachment 6-well-plate (Corning Life Sciences, Tewksbury, MA, USA) and incubated for 7 days. Mammospheres were collected with a 100 μm cell strainer (BD Biosciences) and dissociated with HyQTase (Merck Millipore, Billerica, MA, USA).

2.8. Matrigel-based in vitro VM activity assay

Wells of μ-microslide (ibidi GmbH, Martinsried, Germany) were coated with 10 μl of matrigel (BD Biosciences) at 8 mg/ml, 37 °C for overnight. $ALDH^+$ or mammosphere cells were suspended at 2×10^4 cells/50 μl M200 medium containing 1× LSGS and loaded into one matrigel-coated well. The slide was then incubated at 37 °C, 5% CO_2 incubator and the VM structures were recorded by inverted microscopy every 2 h. Images of wells were analyzed with Tubeness function in Image J software (NIH, Bethesda, MA, USA) and VM scores were calculated according to a formula described by Aranda et al [30].

2.9. shRNA lentivirus transduction

Lentiviral shRNA vectors (TRCN0000231722 for sh-LacZ, TRCN0000039634 for sh-EGFR#1, TRCN0000121067 for sh-EGFR#2) were obtained from National RNAi Core Facility at the Institute of Molecular Biology (Academia Sinica, Taipei, Taiwan). VSV-G-pseudotyped lentiviruses were produced by RNAi Core Facility at the Institute of Clinical Medicine (Nation Cheng Kung University, Tainan, Taiwan). For transduction of lentivirus, cells were plated at 2×10^5 cells per well in six-well plates and transiently transduced with lentivirus in the presence of 8 μg/ml polybrene (Sigma–Aldrich) for 24 h. Successfully transduced cells were further selected by 2 μg/ml puromycin for 3 days.

2.10. Western blot

Cells were harvested, lysed in M-PER Mammalian Protein Extraction Reagent (Pierce Thermo Fisher Scientific Inc., Waltham, MA, USA) and determined the protein concentration by BCA reagent (Pierce Thermo Fisher Scientific Inc.). 25 μg of extracted protein were separated by using 10% SDS-PAGE and transferred to the PVDF membrane (Immobilon-P, Merck Millipore). The membrane were then blocked with 5% skimmed milk/TBS/0.05% Tween-20 at room temperature for 1 h followed by incubation with primary antibodies (mouse anti-EGFR and rabbit anti-phosphor $EGFR^{Tyr1173}$ antibodies were purchased from Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA; rabbit anti-Hsp27 antibody was purchased from Enzo Life Sciences, Inc.; rabbit anti-GAPDH antibody was purchased from MDBio Inc., Taipei, Taiwan) at 4 °C overnight. Peroxidase-conjugated anti-rabbit or anti-mouse IgG (Cell Signaling Technology, Inc., Danvers, MA, USA) were used as secondary antibodies. Developed chemiluminescence signals from catalyzed ECL substrate (PerkinElmer Inc., Waltham, MA, USA) were detected by Luminescence-Image Analyzer (LAS-4000mini, GE Healthcare Biosciences, Pittsburgh, PA, USA).

2.11. RNA interference

Negative control siRNAs or Hsp27 specific siRNAs were purchased from Santa Cruz Biotechnology Inc., prepared by serum-free medium as concentration of 50 nM and complexed with Turbofect™ transfection reagent (Fermentas Inc. MA, USA). After incubation at room temperature for 15 min, RNA-transfection reagent mixtures were seeded in wells of 6-well-plate and then 1 ml of 2×10^5 cells/ml cell suspension were then added into wells. Transfected cells were harvested after 48 h and used for further application.

2.12. Plasmid construction and transfection

Phosphor-dead (Hsp27A) or phosphor-mimic (Hsp27D) form of Hsp27 was constructed from pDsRed-Hsp27 plasmid which was constructed previously [27] by QuikChange™ Lightning Multi Site-Directed Mutagenesis Kit (Agilent Technologies, Santa Clara, CA, USA) with primers as follow:

S15A-F: 5′-ctgcggggccccgcctgggacccctt-3′
S15A-R: 5′-aaggggtcccaggcggggccccgcag-3′
S78,82A-F: 5′-ccgcgcgctcgcccggcaactcgccagcggggtc-3′
S78-82A-R: 5′-gaccccgctggcgagttgccgggcgagcgcgcgg-3′
S15D-F: 5′-cctgcggggccccgactgggacccctttc-3′
S15D-R: 5′-gaaggggtcccagtcggggccccgcagg-3′
S78-82D-F: 5′-gccgcgcgctcgaccggcaactcgacagcggggtct-3′
S78-82D-R: 5′-agaccccgctgtcgagttgccggtcgagcgcgcggc-3′

For overexpression of Hsp27 mutants, plasmids were transfected into cells by Turbofect transfection reagent. Briefly, 1×10^6 cells were seeded in a 10 cm culture dish at one day before transfection. 5 μg of plasmids were dissolved in 500 μl of serum-free RPMI-1640 medium (Gibco Life Technologies) and 10 μl of Turbofect transfection reagent were added for reagent–DNA complexing at room temperature for 15 min. Reagent–DNA complexes were then added into dish with 5 ml of antibiotics-free culture medium, cultured for 48 h and harvested for further experimentations.

3. Results

3.1. Vasculogenic mimicry activity of breast cancer stem/progenitor cells

We have previously established two breast cancer xenografts (BC0145 and BC0244) from human breast cancer specimens and demonstrated the existence of breast cancer stem/progenitor cells (BCSCs) [26,27]. To investigate the contribution of BCSCs in tumor vasculogenic mimicry (VM), we first used PAS stain to observe the VM structures within xenografted tumor tissues derived from $CD24^-CD44^+$ BC0145 cells or $ALDH^+$ BC0244 cells. Both xenografted BC0145 and BC0244 tumors contained PAS-positive VM structures and some of them contained erythrocytes (Fig. 1A). The H&E stain is commonly used for the demonstration of the presence of erythrocytes VM structures in several reports [31–33]. In H&E stain results, erythrocytes were clearly found in VM channels which were lined by tumor cells with basement membrane in both

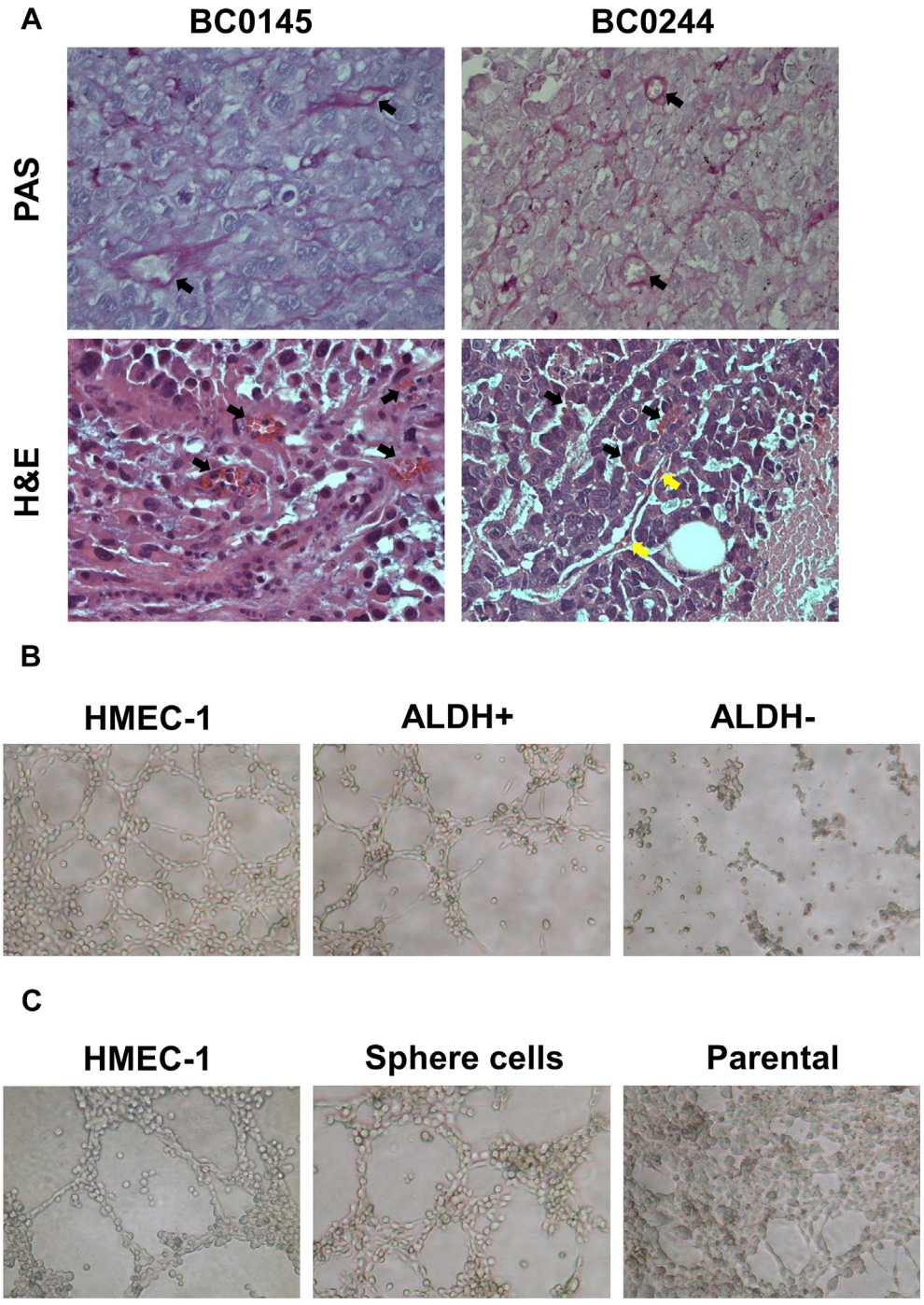

Fig. 1. *In vivo* and *in vitro* observations of VM activity of BCSCs. (A) 10^5 of $CD24^-CD44^+$ BC0145 or $ALDH^+$ BC0244 xenografted breast cancer cells were sorted and injected into mammary fat pads of NOD/SCID mice for 4 weeks. VM in tumor tissues were determined by PAS (upper panel) and H&E (lower panel) stain. Black arrows indicated erythrocytes containing VM structures and yellow arrows indicated erythrocytes containing endothelial vessels. (B) $ALDH^-$ or $ALDH^+$ cells were sorted from AS-B244 cells and seeded into matrigel-coated wells to observe the formation of vessel-like structures. HEMC-1 endothelial cells were used as positive control. Data were presented from one of three independent experiments. (C) Parental or mammosphere-forming AS-B145 cells were collected and seeded into matrigel-coated wells to observe the formation of vessel-like structures. HMEC-1 cells were used as positive control. Data were presented from one of three independent experiments.

BC0145 and BC0244 tumors (Fig. 1A). Some of erythrocytes containing VM structures in BC0244 tumors were observed to connect with endothelial vessels (Fig. 1A). Both PAS and H&E stain demonstrated the presence of VM structures in BCSCs derived human breast cancer xenografts. We next separated $ALDH^-$ (non-BCSCs) or $ALDH^+$ (BCSCs) cells from AS-B244 cells by FACS and performed *in vitro* VM assay by culturing on matrigel-coated wells with medium for cultivation of endothelial cells. $ALDH^+$ AS-B244 cells, but not $ALDH^-$, could form vessel-like structures as similar as HMEC-1, an immortalized human microvascular endothelial cell line (Fig. 1B). In addition to sorting of $ALDH^+$ cells, we also used mammosphere cultivation to enrich BCSCs from AS-B145 cells. In comparison with normal cultured AS-B145 cells which were maintained in two dimensional cultures, mammosphere cells

displayed obvious VM activity (Fig. 1C). From these results, it suggests that there is a strong VM activity of BCSCs.

3.2. EGF mediates VM activity of BCSCs

We next wanted to search for factors which could regulate VM activity of BCSCs. We first compared the VM activity of ALDH$^+$ AS-B244 cells in medium for cultivation of endothelial cells (M200 in supplemented with LSGS) or breast cancer cells (MEMα). ALDH$^+$ AS-B244 cells lost their VM activity in MEMα medium (Fig. 2Aii) while there was a strong VM activity of these cells in M200/LSGS medium (Fig. 2Ai). After comparison of medium used for cultivation of endothelial cells and breast cancer cells (Table 1), we hypothesized that EGF or basic fibroblast growth factor (bFGF) might involve in regulating VM activity of BCSCs. By supplementation of EGF or bFGF into MEMα medium, EGF was found to stimulate VM activity of ALDH$^+$ AS-B244 cells in MEMα medium (Fig. 2Aiii) and there was no significant difference between EGF and M200/LSGS groups ($P = 0.239$, Fig. 2B). The effect of bFGF in stimulating VM activity of ALDH$^+$ AS-B244 cells was minor (Fig. 2Aiv) but still significant ($P = 0.03$, Fig. 2B). Supplementation with EGF plus bFGF also stimulated VM activity of ALDH+ AS-B244 cells in MEMα medium (Fig. 2Av) but there was no significant difference when compared with supplementation of EGF alone ($P = 0.245$, Fig. 2B). To further confirm that EGF signaling is the key factor in VM activity of BCSCs, gefitinib was used to block the phosphorylation and activation of EGF receptor (EGFR). Treatment of ALDH$^+$ AS-B244 cells with gefitinib inhibited the phosphorylation of EGFR^{Tyr1173} in a dose-dependent manner (Fig. 3A). 5 μM of gefitinib significantly abolished VM activity of ALDH$^+$ AS-B244 cells ($P < 0.01$, Fig. 3B). In addition to gefitinib

Table 1
A comparison of components in medium for cultivation of endothelial or cancer cells.

Cell type	Endothelial cells	Breast cancer cells
Medium name	M200	MEMα
Supplements	LSGS[a] contains:	10% fetal bovine serum
	2% fetal bovine serum	Insulin (5 μg/ml)
	EGF (10 ng/ml)	Penicillin (10 units/ml)
	bFGF (3 ng/ml)	Streptomycin (10 μg/ml)
	Heparin (10 μg/ml)	Glutamate (2 mM)
	Hydrocortisone (1 μg/ml)	Sodium pyruvate (1 mM)

[a] Low serum growth supplement, the information of contents was obtained from product data sheet.

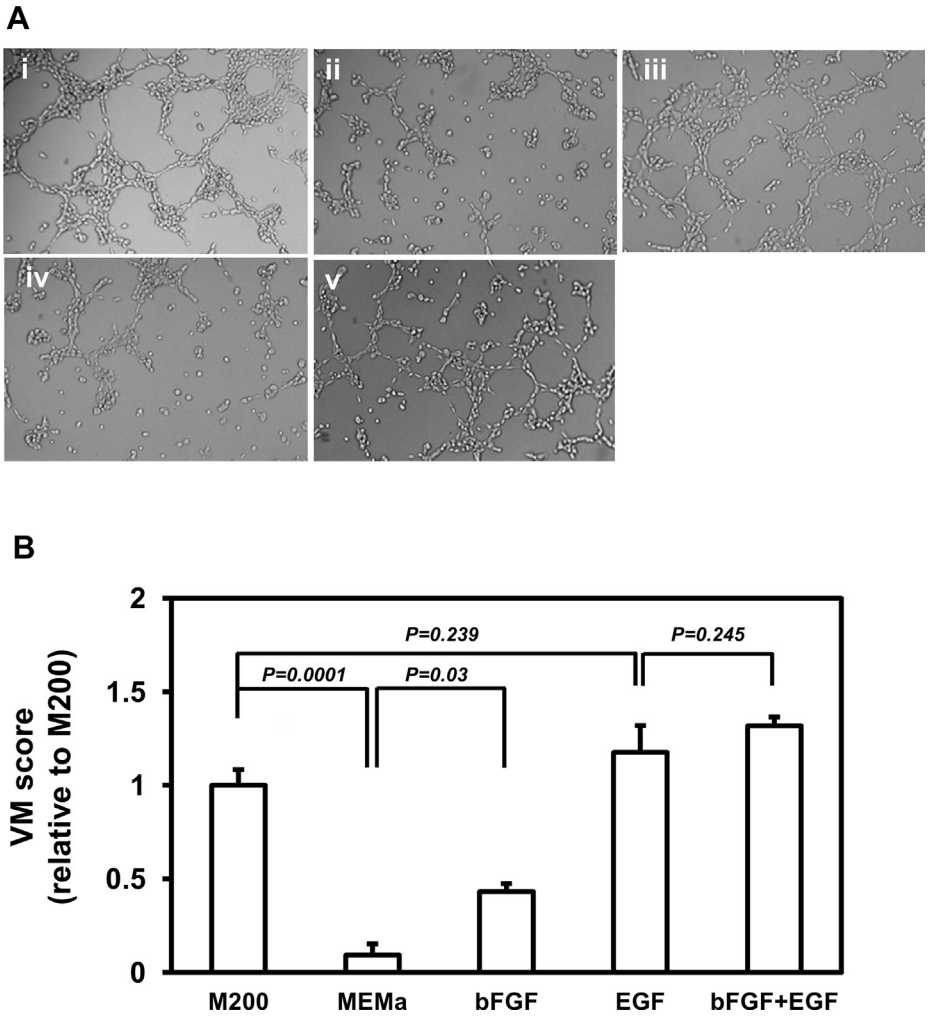

Fig. 2. EGF stimulates *in vitro* VM activity of ALDH$^+$ BCSCs. (A) ALDH$^+$ AS-B244 cells were sorted and seeded into matrigel-coated wells to observe the formation of vessel-like structures in different conditions. i, M200 + LSGS; ii, MEMα; iii, MEMα + erythrocytes EGF (10 ng/ml); iv, MEMα + erythrocytes bFGF (3 ng/ml); v, MEMα + EGF (10 ng/ml) + bFGF (3 ng/ml). Data were presented from one of three independent experiments. (B) Images from three wells of each condition in (A) were used for analysis of VM scores and data were presented from one of three independent experiments as the relative value to M200 group.

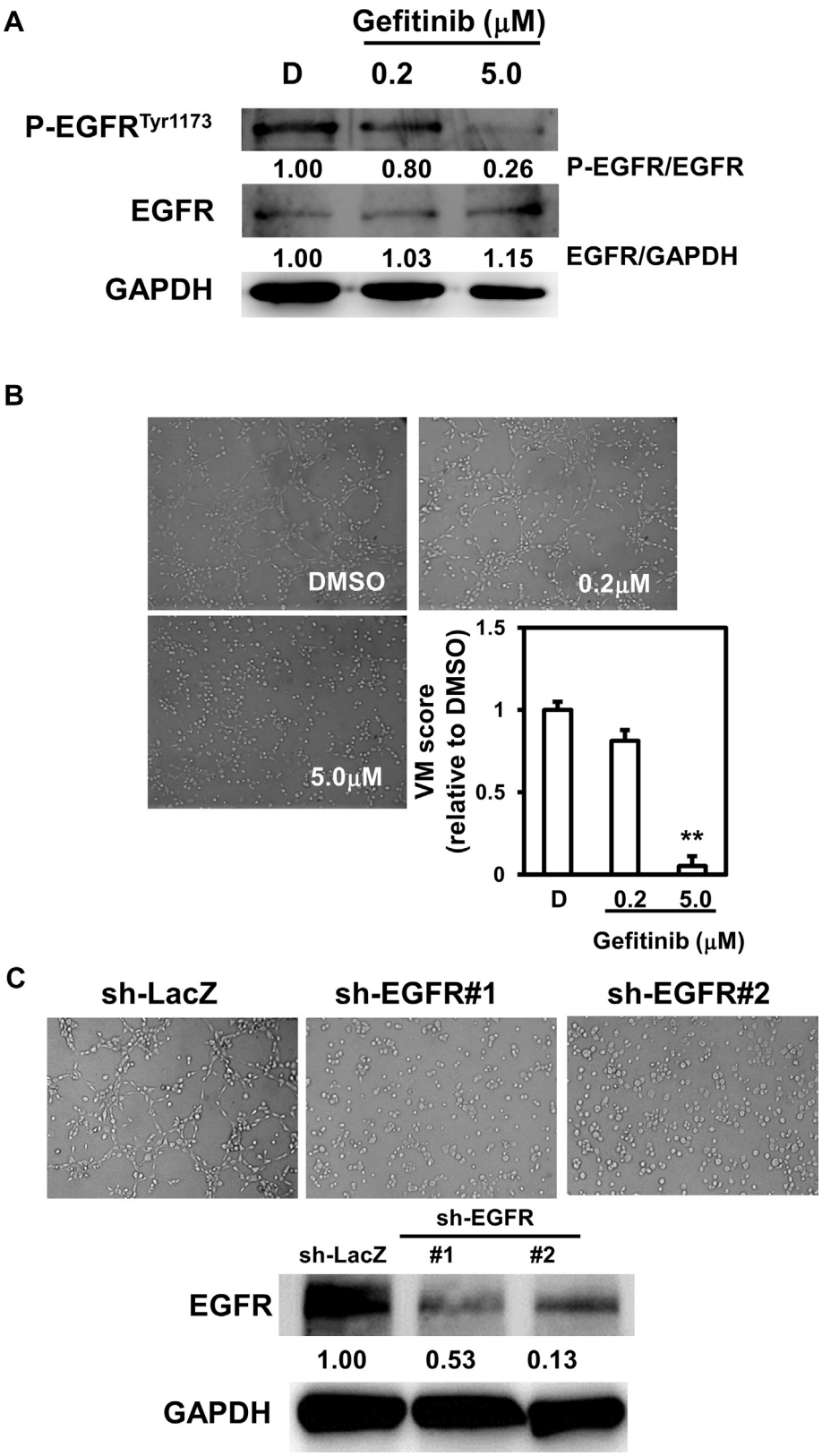

Fig. 3. Disruption of EGFR activation or expression suppresses VM activity of ALDH$^+$ BCSCs. (A) ALDH$^+$ AS-B244 cells were suspended in M200 medium containing LSGS and treated with 0.1% DMSO or indicated concentration of gefitinib for 1 h. The activation of EGFR was determined by western blot of tyrosine 1173 phosphorylation. The inserted numbers indicated the relative protein expression in comparison with 0.1% DMSO. Data were presented from one of three independent experiments. (B) DMSO or gefitinib treated cells were further seeded into matrigel-coated wells to observe the formation of vessel-like structures. Images from three wells of each condition were used for analysis of VM scores and data were presented one of three independent experiments as the relative value to 0.1% DMSO group. D, 0.1% DMSO; **, $P < 0.01$. (C) ALDH$^+$ AS-B244 cells were transduced with lentivirus carrying control shRNA (sh-LacZ) or EGFR specific shRNA (sh-EGFR#1 or sh-EGFR#2) for 24 h and selected for successfully transduced cells by 2 μg/ml puromycin for further 3 days. Selected cells were plated on matrigel-coated microwells for *in vitro* VM determination. Knockdown efficiency was determined by western blot analysis of EGFR expression. The inserted numbers indicated the relative protein expression in comparison with sh-LacZ transduced cells.

treatment, we also applied lentiviral transduction of EGFR specific short hairpin RNA (shRNA) to knockdown EGFR in ALDH$^+$ AS-B244 cells to examine the role of EGFR signaling in the VM activity of BCSCs. In comparison with control sh-LacZ transduced cells, transduction with EGFR specific shRNA lentivirus (sh-EGFR#1 or sh-EGFR#2) in ALDH$^+$ AS-B244 cells completely abolished their *in vitro* VM activity (Fig. 3C). These results demonstrate that EGFR signaling is the key regulator in VM activity of BCSCs.

3.3. Hsp27 is the downstream molecule in EGF-mediated VM activity of BCSCs

Recently, it has been reported that knockdown of Hsp27 suppressed EGF-induced endothelial cord formation within tumors [23]. We have also demonstrated Hsp27 controls the maintenance of BCSCs [27]. We next wanted to investigate the role of Hsp27 in VM activity of breast cancer stem/progenitor cells. Quercetin, an Hsp27 inhibitor, decreased Hsp27 expression in ALDH$^+$ AS-B244 cells in a dose-dependent manner (Fig. 4A). VM activity of ALDH$^+$ AS-B244 cells was significantly inhibited by quercetin at 25 μM or 50 μM ($P < 0.01$, Fig. 4B). In addition to quercetin, we also applied siRNA-mediated gene silencing to decrease the Hsp27 expression. Specific Hsp27 siRNA could effectively decrease Hsp27 expression in both AS-B145 sphere cells and ALDH$^+$ AS-B244 cells (Fig. 4C). Knockdown of Hsp27 in AS-B145 sphere cells or ALDH$^+$ AS-B244 cells significantly suppressed their VM activity in M200+ LSGS medium ($P < 0.01$, Fig. 4D). We further examined if Hsp27 could be a downstream molecule of EGF-mediated VM activity of BCSCs. Supplementation of EGF in MEMα medium significantly stimulated VM activity of ALDH$^+$ AS-B244 cells transfected with negative control siRNA ($P < 0.05$, Fig. 5Aiii and B) while knockdown of Hsp27 completely blocked EGF-induced VM activity of ALDH$^+$ AS-B244 cells ($P < 0.01$, Fig. 5Aiv and B). In order to investigate the involvement of Hsp27 activation in EGF-induced VM activity of BCSCs, we constructed phosphor-dead (Hsp27A) and phosphor-mimic (Hsp27D) form of Hsp27 in tagged with DsRed protein and overexpressed them in ALDH$^+$ AS-B244 cells. Overexpression of Hsp27A significantly suppressed the VM activity of BCSCs in presence of EGF ($P = 0.048$, Fig. 6A and B). Forced expression of Hsp27D in BCSCs did not enhance VM activity under EGF stimulation ($P = 0.103$, Fig. 6A and B) but it could overcome the inhibitory effect of gefitinib in VM structures ($P = 0.005$, Fig. 6A and B). These results suggest that Hsp27 is the key downstream molecule in EGF-mediated VM activity of BCSCs.

4. Discussion

There are several molecular pathways to regulate VM in tumors including vascular, embryonic/stem cell, and hypoxia related signaling pathways. In vascular signaling pathways, VE-cadherin could regulate VM activity in aggressive melanoma cells through activating erythropoietin-producing hepatocellular

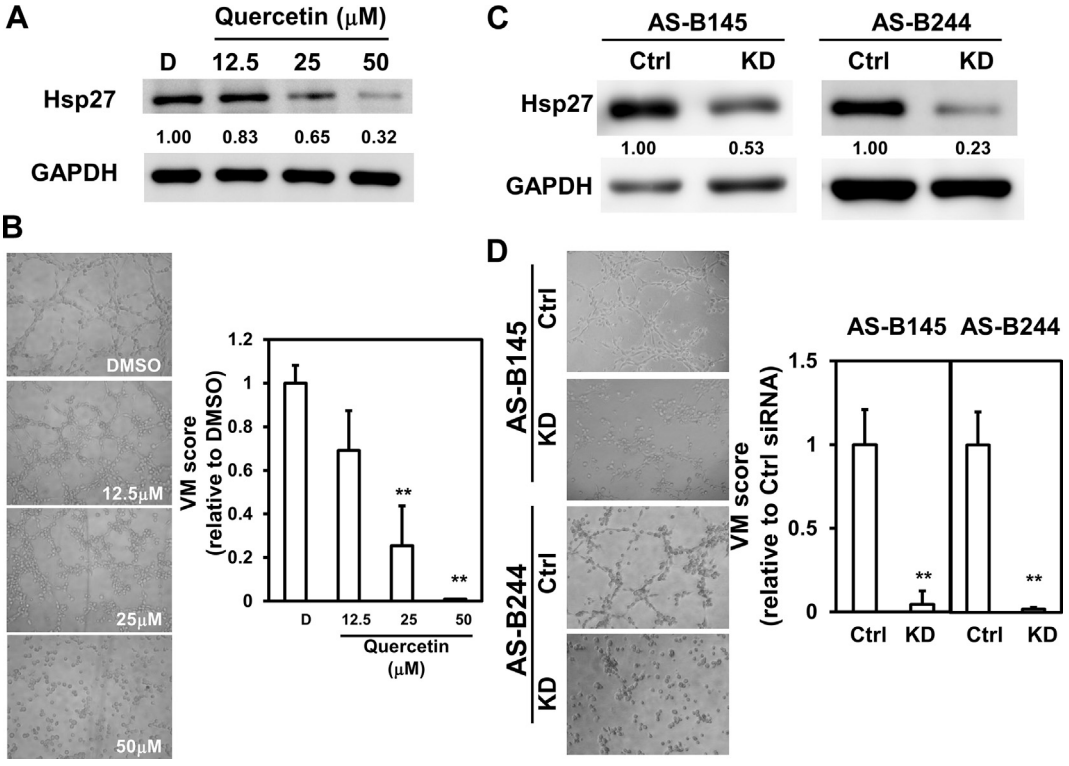

Fig. 4. Disruption of Hsp27 expression suppresses EGF-induced VM activity of ALDH$^+$ BCSCs. (A) ALDH$^+$ AS-B244 cells were sorted and treated with 0.1% DMSO (D) or indicated concentration of quercetin for 1 h. Expression of Hsp27 was determined by western blot. The inserted numbers indicated the relative Hsp27 expression in comparison with 0.1% DMSO. Data were presented from one of three independent experiments. (B) DMSO or quercetin treated cells were further seeded into matrigel-coated wells to observe the formation of vessel-like structures. Images from three wells of each condition were used for analysis of VM scores and data were presented as the relative value to 0.1% DMSO group. D, 0.1% DMSO; **, $P < 0.01$. (C) Mammosphere-forming AS-B145 or ALDH$^+$ AS-B244 cells were transfected with negative control (ctrl) or Hsp27 specific (KD) siRNA oligos for 48 h. Hsp27 expression as determined by western blot. The inserted numbers indicated the relative Hsp27 expression in comparison with negative control siRNA transfected cells. Data were presented from one of two independent experiments. (D) Negative control (ctrl) or Hsp27 specific (KD) oligos transfected cells were further seeded into matrigel-coated wells to observe the formation of vessel-like structures. Images from three wells of each condition were used for analysis of VM scores and data were presented from one of two independent experiments as the relative value to negative control siRNA transfected group. **, $P< 0.01$.

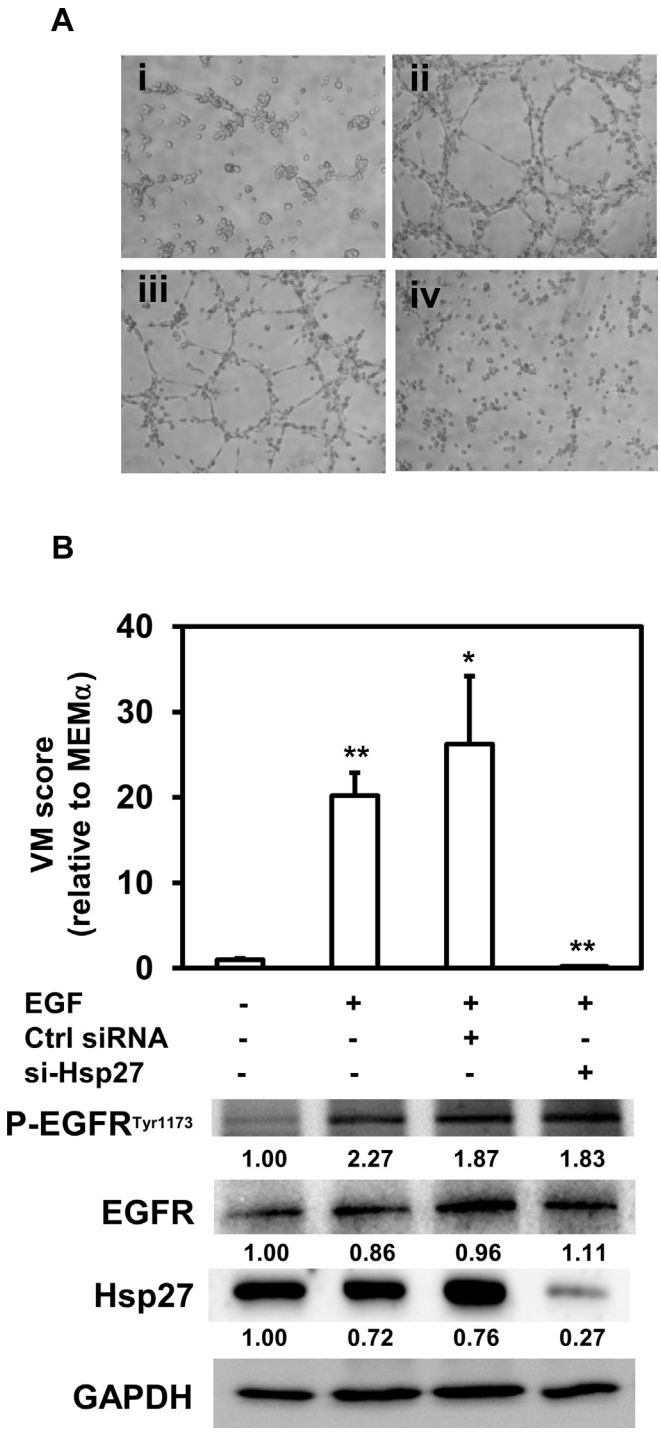

Fig. 5. Knockdown of Hsp27 inhibits EGF-induced VM activity of ALDH$^+$ BCSCs. (A) ALDH$^+$ AS-B244 cells were transfected with negative control (ctrl siRNA) or Hsp27 specific (si-Hsp27) siRNA oligos for 48 h, suspended in MEMα medium and seeded into matrigel-coated wells for observation of the formation of vessel-like structures. i, non-transfected cells; ii, non-transfected cells with EGF; iii, ctrl-siRNA transfected cells with EGF; iv, si-Hsp27 transfected cells with EGF. (B) Images from three wells of each condition in (A) were used for analysis of VM scores and data were presented from one of two independent experiments as the relative value to non-transfected cells and without EGF group. *, $P < 0.05$; **, $P < 0.01$. The expression of p-EGFR^{tyr1173}, EGFR or Hsp27 was determined by western blot. Data were presented from one of two independent experiments.

carcinoma-A2 (EphA2) [34]. The activation of EphA2 in melanoma cells further activates phosphoinositide 3-kinase (PI3K) [35], which is a downstream molecule in receptor tyrosine kinases signaling pathways [36]. In present study, we discovered that the VM activity of BCSCs was regulated by EGF/EGFR signaling pathway, which has been widely demonstrated to turn on the activation of PI3K/Akt signaling cascade [37]. In addition to PI3K/Akt, the activated EGFR could be internalized into nucleus and become a co-transcriptional activator to turn on target gene expression such as COX2 and cyclin D1 [38]. In MDA-MB-231 metastatic breast cancer cells, suppression of COX2 activity by celecoxib or specific siRNA inhibited their VM activity [6]. Together with our findings, it will be interesting to investigate if the nuclear EGFR pathway occurs in BCSCs to upregulate the expression of COX2.

It has been reported that vascular endothelial growth factor (VEGF) signal activates p38 MAPK and leads to the phosphorylation of Hsp27 [39]. In endothelial cells, H_2O_2 or VEGF-induced actin polymerization is Hsp27 phosphorylation dependent [39]. SB203580, a small molecule inhibitor of p38 MAPK, inhibited the phosphorylation of Hsp27 as well as actin reorganization and cell migration in human umbilical vein endothelial cells (HUVEC) [40]. When endothelial cells were exposed to angiogenesis inhibitors, such as endostatin, fumagillin or TNP-470, the expression of Hsp27 was decreased, although TNP-470 treatment in HUVEC cells caused up-regulation of Hsp27 phosphorylation and nuclear localization of Hsp27 [41]. These demonstrate that Hsp27 phosphorylation is a critical regulator in VEGF-induced angiogenesis of endothelial cells. In tumor angiogenesis, Hsp27 expression has been reported to be significantly up-regulated in angiogenic MDA-MB-436 breast cancer xenografts [24]. Knockdown of Hsp27 in angiogenic MDA-MB-436 cells resulted in long-term dormancy when xenotransplanted into SCID mice [24]. The phenomena might be due to the down-regulation of angiogenic factors, such as VEGF-A or bFGF secreted from Hsp27 knockdown MDA-MB-436 cells [24]. In present study, suppression of Hsp27 through quercetin or specific siRNA abolished EGF-induced VM activity of BCSCs. This is the first report to demonstrate that Hsp27 regulates VM activity of tumor cells. We have previously demonstrated that Hsp27 regulates epithelial–mesenchymal transition (EMT) in BCSCs and decreasing the expression of Hsp27 could inhibit cell migration capability of BCSCs [27]. The loss of VM activity in Hsp27 downregulated BCSCs could be a result of inhibition cell migration. In hepatocellular carcinoma cells, it has been reported that the expression of VE-cadherin is up-regulated by Twist1 [42], an important transcriptional factor in EMT process [43]. Recently, Hsp27 has been demonstrated to regulate IL-6 induced EMT in prostate cancer [44]. Silencing of Hsp27 decreased the binding of STAT3, which is a main downstream molecule in IL-6 induced signaling pathway, on Twist1 promoter and led to its down-regulation [44]. It will be interesting to investigate if Twist1 involves in EGF/Hsp27 mediated VM activity of BCSCs.

There are disappointing results in the clinical trials of anti-angiogenesis agents in cancer therapy. The trial of monoclonal anti-VEGF antibody, bevacizumab, has been failed in Phase III which results indicated that there was no benefit when administered with capecitabine [45,46]. One of the mechanisms of tumor resistance to bevacizumab may be due to the VM activity of cancer cells and there is no effect of bevacizumab to VM [5]. Gefitinib is one of the anti-angiogenesis agents approved by Food and Drug Administration of USA without any information about its effect to VM [3]. Here we are the first to provide evidences that gefitinib could suppress VM activity of BCSCs. We further demonstrated that Hsp27 was the downstream molecule in EGF-induced VM activity of BCSCs. These results provide a new insight to develop therapeutic strategies in against tumor VM by combination of anti-angiogenesis and anti-Hsp27 agents for treatment of breast cancer.

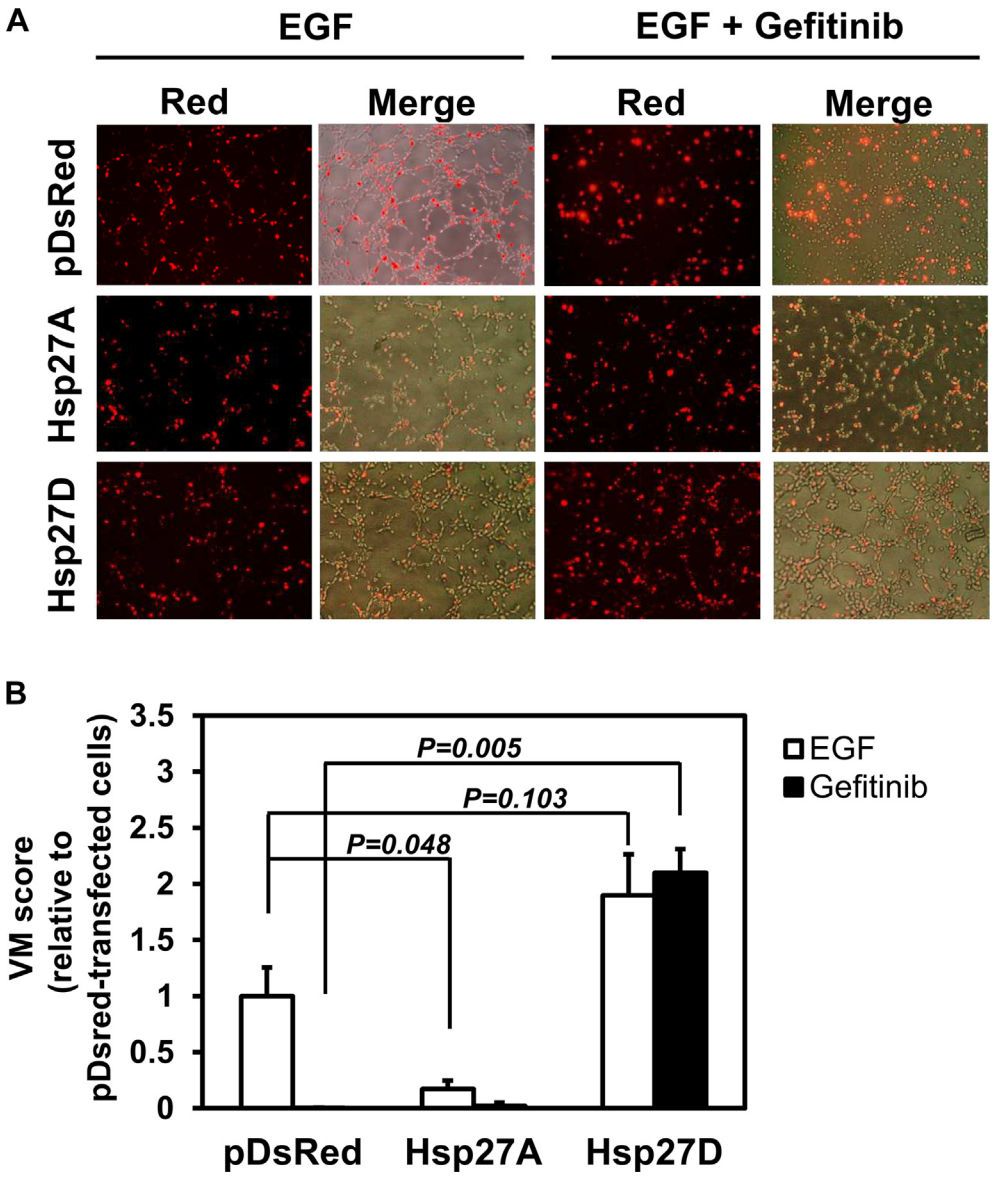

Fig. 6. Phosphorylation status of Hsp27 determines EGF-stimulated VM activity of ALDH$^+$ BCSCs. (A) ALDH$^+$ AS-B244 cells were transfected with indicated plasmids for 48 h and harvested for *in vitro* VM analysis. The concentration of EGF or gefitinib was 10 ng/ml or 5 μM, respectively. (B) Images from three wells of each condition in (A) were used for analysis of VM scores and data were presented from one of two independent experiments as the relative value to pDsRed-transfected group. A *P* value less than 0.05 was considered as significant difference.

5. Conclusions

Our data demonstrate that EGF/Hsp27 pathway participates in the VM activity of BCSCs for the first time and also discover that gefitinib, the EGFR inhibitor, could interfere with VM for the first time. These results provide a new insight to develop therapeutic strategies in against tumor VM by combination of anti-angiogenesis and anti-Hsp27 agents for treatment of breast cancer.

Disclosure statement

The authors have no conflicts of interest.

Authors' contributions

CHL participated in data analysis and manuscript preparation; YTW, HCH and YY performed experiments and contributed to data analysis; ALY participated in providing the human breast cancer cell lines established from primary human breast cancer specimens and research design; WWC participated in research design, analyzing data and manuscript preparation. All authors read and approved the final manuscript.

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