Mesenchymal stem cell-based HSP70 promoter-driven VEGFA induction by resveratrol promotes angiogenesis in a mouse model

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Received: 21 January 2015 / Revised: 17 March 2015 / Accepted: 24 March 2015 / Published online: 10 April 2015
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Abstract Several studies of stem cell-based gene therapy have indicated that long-lasting regeneration following vessel ischemia may be stimulated through VEGFA gene therapy and/or MSC transplantation for reduction of ischemic injury in limb ischemia and heart failure. The therapeutic potential of MSC transplantation can be further improved by genetically modifying MSCs with genes which enhance angiogenesis following ischemic injury. In the present study, we aimed to develop an approach in MSC-based therapy for repair and mitigation of ischemic injury and regeneration of damaged tissues in ischemic disease. HSP70 promoter-driven VEGFA expression was induced by resveratrol (RSV) in MSCs, and in combination with known RSV biological functions, the protective effects of our approach were investigated by using ex vivo aortic ring coculture system and a 3D scaffolds in vivo model. Results of this investigation demonstrated that HSP promoter-driven VEGFA expression in MSC increased approximately 2-fold over the background VEGFA levels up-

on HSP70 promoter induction by RSV. Exposure of HUVEC cells to medium containing MSC in which VEGFA had been induced by *cis*-RSV enhanced tube formation in the treated HUVEC cells. RSV-treated MSC cells differentiated into endothelial-like phenotypes, exhibiting markedly elevated expression of endothelial cell markers. These MSCs also induced aortic ring sprouting, characteristic of neovascular formation from pre-existing vessels, and additionally promoted neovascularization at the MSC transplantation site in a mouse model. These observations support a hypothesis that VEGFA expression induced by *cis*-RSV acting on the HSP70 promoter in transplanted MSC augments the angiogenic effects of stem cell gene therapy. The use of an inducible system also vastly reduces possible clinical risks associated with constitutive VEGFA expression.

Keywords Stem cell-based gene therapy · Resveratrol · Hsp70 promoter · VEGFA · Angiogenesis · Mouse model

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Introduction

Ischemia-reperfusion-associated damage to cardiovascular tissue as a result of disease, injury, or surgery is the major cause of morbidity and mortality (Pagidipati and Gaziano 2013). Such damages may be the consequence of vascular redistribution in multiple tissues as a result of angiogenesis (Risau 1997). Tissue ischemia reduces the oxygen level and nutrient supplies to the cells, leading to increased secretion of angiogenic growth factors, which promote angiogenesis and prevent or delay cell damage (Leeper et al. 2010; Reinders et al. 2006). Vascular endothelial growth factor A (VEGFA) is upstream angiogenic growth factor that drives angiogenic sprouting from pre-existing vessels to increase oxygen supply (Gerhardt 2008).

Reports have indicated that hypoxia-inducible factor 1 binds the hypoxia-induced transcriptional element of the VEGFA promoter to induce VEGFA expression to stimulate endothelial cell proliferation, migration, and survival and vascular permeability (Kimura et al. 2000). Low VEGFA expression may increase the risk for cardiac and symptomatic peripheral vascular disease (Lambrechts et al. 2005; Porcu et al. 2002). Several approaches are available for the delivery of exogenous VEGFA transgenes in mesenchymal stem cells (Moon et al. 2014) to reduce ischemic injury in limb ischemia and heart failure. In expression of an endogenous transgene, an inducible transgene system is preferred for controlled transgene expression. The heat shock protein 70 (HSP70)-inducible gene expression system is based on heat treatment and chemical induction (Ait-Aissa et al. 2000) and has previously been used in gastric cancer inhibition (Isomoto et al. 2006) and RNA interference expression (Liao et al. 2013). Resveratrol (RSV) is a phytochemical inducer of heat shock factor protein 1 (HSF-1) that activates HSP70 promoter activity (Putics et al. 2008). *Trans*-resveratrol (*t*-RSV) was shown to possess anti-inflammatory, anti-oxidant, anti-carcinogenic, anti-tumor, and immunomodulatory properties (Shakibaei et al. 2009). Rats pretreated with *t*-RSV were boosted in the myocardium transplantation environment by overexpressing the nuclear factor erythroid-2-related factor (Nrf2) and redox-factor 1 (Ref1) antioxidant genes (Iizuka et al. 2005). *t*-RSV induced tube formation by the human coronary artery endothelial cells and mediated neovascularization in a rat model (Kaga et al. 2005). In contrast, only a few of studies have been reported on the biological effects of *cis*-resveratrol (*c*-RSV) on cells. We showed in a recent study that *c*-RSV reduced IL-1 β secretion, in contrast to increased IL-1 β secretion by *t*-RSV (Huang et al. 2014). Moreover, pretreatment with *c*-RSV reduced ATP-induced reactive oxygen species (ROS) production. These results indicate that the anti-inflammatory actions of *c*-RSV in human acute monocytic leukemia cell (THP-1)-differentiated macrophages may be associated with the inhibition of noncanonical inflammasome pathway (Huang et al. 2014).

In recent years, autologous MSC-based therapy has attracted much attention in angiogenesis in cardiovascular disease because MSCs release proangiogenic factors in response to paracrine angiogenic signaling to allow the growing vasculature to grow new blood vessels (Chun et al. 2013; Lee et al. 2013). Despite reported successes, it was demonstrated that the amount of engrafted MSCs decreased dramatically after 24 h of transplantation due to exposure to toxic and oxidative microenvironments (Leblond et al. 2009). In this work, we aimed to test, in a mouse angiogenesis model, that VEGFA expression increased by *c*-RSV acting on the HSP70 promoter in transplanted MSC augments the angiogenic effects of stem cell gene therapy.

Material and methods

Cell lines

Mesenchymal stem cells (MSCs) were isolated from bone marrow of C57BL/6 female mice at ≤ 8 weeks after gestation. MSCs were purchased from Life Technologies (Carlsbad, CA). Cells were maintained in Dulbecco's Modified Eagle's Medium/Ham's Nutrient Mixture F-12 (DME/F12; Life Technologies) supplemented with 10 % fetal bovine serum (FBS; Life Technologies), 2 mM L-glutamine (Life Technologies), and 1 % penicillin/streptomycin (Life Technologies). Human umbilical vein endothelial cell line (HUVEC) was purchased from the Bioresource Collection and Research Center, Hsinchu, Taiwan. Cells were cultured in M199 medium (M199; Life Technologies) supplemented with 5 % heat-inactivated FBS, 50 $\mu\text{g}/\text{mL}$ penicillin/streptomycin, endothelial cell growth supplement (EGM-2 BulletKit; Lonza Inc, Allendale, NJ). Human acute monocytic leukemia THP-1 cells (TIB-202; American Type Culture Collection, Manassas, VA) were cultured in RPMI 1640 medium supplemented with 10 % (*v/v*) heat-inactivated FBS, 100 units/mL of penicillin, and 100 $\mu\text{g}/\text{mL}$ of streptomycin. Cell lines were cultured at 37 °C in a humidified incubator containing 5 % CO₂.

Preparation of conditioned medium

THP-1 cells were plated in 6-well plates at 2×10^6 cells per well. The cells were differentiated to adherent macrophages by overnight culture in complete medium supplemented with 500 ng/mL of phorbol 12-myristate 13-acetate (PMA), and then replaced with fresh completed medium for an additional 24 h, the THP-1-conditioned media (THP-CM) was harvested. Stable MSCs harboring the HSP promoter-driven VEGFA gene (abbreviated as HSP-VEGFA-MSC) were plated in 6-well plates at 5×10^5 cells per well and were treated with 5 μM *c*-RSV or *t*-RSV for 12 h. Subsequently, the RSV

treated-HSP-VEGFA-MSC-conditioned media was harvested.

Construction of lentiviral vectors and transduction into MSCs

The human HSP70 promoter was excised from pHBCAT, a gift from Dr. R. Morimoto (Northwestern University, Chicago, IL). The rat vascular endothelial growth factor A cDNA (VEGFA; Genbank ID: AY033506) was amplified by RT-PCR using specific primers (Table 1). The PCR fragment and the HSP70 promoter were subcloned into the pLenti-LT-hyg vector to generate HSP-VEGFA (Fig. 1a). Virus stocks were prepared by cotransfecting the HSP-VEGFA construct with three packaging plasmids, pMDLg/pRRE, CMV-VSVG, and RSV-Rev, into 293 T cells following previous method (Chan et al. 2001). The viral supernatants were harvested 36–48 h later, filtered, and centrifuged at $20,000\times g$ for 90 min. The viral titer was determined by the method of end point dilution through counting the number of infected red cells at $\times 100$ magnification under a fluorescence microscope 96 h after infection to 293 T cells. Titer in the transducing units was computed as follows: (TU)/mL=(the numbers of red fluorescent cells) $\times$ (dilution factor) / (volume of virus solution). Titers of the viral particles were quantified by HIV quantification ELISA kit. MSCs were seeded in 12-well plate, and the cells were transduced with an equal ratio of viral particle of HSP-VEGFA virus particle, and the stably transduced cells were designated as HSP-VEGFA-MSC.

Cell proliferation test

Serum-starved HSP-VEGFA-MSCs were plated at a density of 1×10^4 cells/well in a 24-well culture plate and cultured for 24 h. The cells were treated with the indicated concentrations of *c*-RSV or *t*-RSV in serum-reduced Opti-MEM (2 % FBS) for 12 h. After changing to fresh culture medium, cell number was counted by the Countess® Automated Cell Counter (Invitrogen) every 12 h.

Cell viability MTT assay

MSC cells were plated at a density of 1×10^5 cells/well in a 12-well culture plate (BD Biosciences) and were cultured to confluency. The cells were treated with *c*-RSV or *t*-RSV for 48 h. Then, the cell viability was detected using the MTT method at 540 nm by VERSAMax spectrophotometry (Molecular Devices).

Luciferase activity assay

Luciferase activity was assayed using a Promega assay kit according to the manufacturer's instructions. Briefly, cells were plated at a density of 1×10^5 in a 24-well plate, lysed with Passive Lysis Buffer, and assayed by the Dual-Luciferase Reporter Assay System (Hung et al. 2014a). The luminescence intensity was measured by a GLOMAX 20/20 Luminometer. The luciferase units were expressed as relative luciferase units.

Quantitative real-time RT-PCR

Total RNA was prepared from the cell lines and was treated with DNase I (New England BioLabs, Ipswich, MA). RNAs were reverse transcribed into cDNAs at 42 °C for 60 min using Moloney Murine Leukemia Virus Reverse Transcriptase (Life Technologies). After the oligo(dT)-primed reverse transcription reaction, quantitative real-time RT-PCR was performed using LightCycler 480 SyberGreen I Master Mix and LightCycler® 480 Instrument (Roche, Mannheim, Germany) as previously described (Hung et al. 2014b). Sequences of the mouse and human gene-specific primers used are listed in Table 1. For normalization, the β -actin (for human genes) and *Gapdh* (for mouse genes) mRNA levels of each RNA preparation were determined. Relative gene expression was determined by the $\Delta\Delta C_t$ method, where C_t is threshold cycle. The relative mRNA levels were normalized to the mRNA level of the reference β -actin gene for human samples and *Gapdh* gene for mouse samples. The melting

Table 1 PCR primers

Gene name	Forward primers 5'→3'	Reverse primers 5'→3'
Rat VEGFA	ATGAACTTTCTGCTCTCTTGG	TCACCGCCTTGGCTTGTC
Human VEGFA	CTCCACCATGCCAAGTGGTC	AGGAACATTTACACGTCTGCG
Human VEGFR2	GCAGGGGACAGAGGGACTTG	GAGGCCATCGCTGCACTCA
Human β -actin	GCGAGAAGATGACCCAGATC	CCAGTGGTACGGCCAGAGG
Mouse CD31	ACGCTGGTGCTCTATGCAAG	TCAGTTGCTGCCCATTCATCA
Mouse VEGFR2	CTGGAGCCTACAAGTGCTCG	GAGGTTTGAATCGACCCTCG
Mouse VEGFA	CTGTGCAGGCTGCTGTAAC	ACAGTGATTTCTGGCTTTGTTT
Mouse HO-1	AAGCCGAGATAGCTGAGTTCA	GCCGTGTAGATATGGTACAAGGA
Mouse GAPDH	TCCCACCCAGAGACAGCCGC	CCGTTACACCCGACCTTCAC

Fig. 1 Cytotoxicity effects of RSV on cell viability and transcriptional activities of the heat shock promoter responses to challenges in MSCs. **a** Schematic representation of the lentiviral vectors, the 2A peptides sequence (T2A) from *Thossea asigna* virus was used as a linker connecting the red fluorescence protein (DsRed), hygromycin and luciferase (LUC) gene to generate the lentiviral multi-cistronic expression vector, pLenti-LT-hyg. pLenti-HSP70p-VEGFA-Luciferase-DsRed-hygromycin (HSP-VEGFA), pLenti-CAGp-VEGFA-Luciferase-DsRed-hygromycin (CAG-VEGFA). **b** MSCs and **c** HUVECs were treated with different concentrations of *c*-RSV or *t*-RSV for 48 h and

subjected to the MTT assay. The viability of untreated cells was considered to be 100 %. **d** Stable HSP-VEGFA-MSC was treated with 5 or 10 μ M *c*-RSV or *t*-RSV for 24 h. Stable HSP-VEGFA-MSC was simultaneously treated with 5 μ M *c*-RSV or *t*-RSV and **e** the indicated temperatures or **f** conditioned medium collected from cultured human monocyte (THP-1 CM) for 24 h. At the end of the treatment, the HSP70 promoter activity was examined by luciferase assay. Mean $\pm$ S.D. of three independent experiments are shown. * p <0.05 and ** p <0.01 indicate differences between the treated cells and the control

curve of the amplification product was always checked to ensure a single clean peak that represented good quality quantitative real-time RT-PCR data.

Western blot analysis

Total cellular proteins were isolated from cell lines by the PRO-PREP™ Protein Extraction Solution (Intron Biotechnology, Kyonggi-do, Korea), and Western blot analysis was performed as described previously (Chong et al. 2015). In brief, an amount of 25 or 50 μ g of total proteins from cell lysates or conditioned media was loaded onto each lane, and the proteins were

separated in sodium dodecyl sulfate polyacrylamide electrophoresis (SDS-PAGE; Bio-Rad Laboratories, Hercules, CA). After electrophoresis, the resolved proteins were transferred to PVDF membrane (EMD Millipore, Billerica, MA). The membranes were blocked with 5 % skimmed milk powder (Anchor, Kowloon, Hong Kong) in phosphate-buffered saline-Tween (PBS-T): phosphate-buffered saline (PBS, Sigma-Aldrich) containing 0.1 % Tween-20 in Sigma-Aldrich for 1 h and probed overnight with the following antisera at appropriate dilutions: 1:1000 dilution of the anti-HO-1 (MBL International, Woburn, MA), a 1:1000 dilution of the anti-VEGF (Santa Cruz Biotechnology Inc., Dallas, TX), and a 1:10,000 dilution of

the anti β -actin (EMD Millipore) antisera in PBS-T. Identification of each protein was achieved with the Western Lighting Plus Reagent (Perkin Elmer, Waltham, MA) using an appropriate horseradish peroxidase (HRP)-conjugated secondary antibodies (Jackson Immuno Research Laboratories, West Grove, PA). Protein levels in the Western blot analysis were detected and quantified by the LAS-3000 chemiluminescence detection device (Fujifilm, Valhalla, NY). To adjust for loading differences, the optical density of each protein was normalized to that of the β -actin band.

Heat shock challenges

Before heat shock stress, fresh medium was added to the cells for 120 min at 37 °C. Culture plates were sealed with parafilm and immersed into a shaking bath maintained at various temperatures for 5 min each. After heat shock challenges, cells were refed with fresh medium and returned to the CO₂ incubator at 37 °C for 24 h (Chong et al. 2013).

Tube formation ability assay

The tube formation assay was carried out with the μ -slide angiogenesis system from Ibidi (Integrated BioDiagnostics, Germany). The μ -slides were coated with growth factor-reduced BD Matrigel (BD Biosciences) and placed at 37 °C for 1 h to polymerize. HUVEC cells were harvested and resuspended in conditioned medium from mock, *c*-RSV-, or *t*-RSV-treated HSP-VEGFA-MSC at a density of 2×10^5 cells per mL. From this solution, 50 μ L was applied in each μ -slide well and incubated for 6 h. Tube formation was measured using microscopic images of five different areas. Tubular length and the total number of tubes were quantified.

Aortic ring coculture MSCs

The protocol for the preparation of the mouse aortic ring was as described by Baker et al (2012). Briefly, the mouse aortic ring was seeded into a matrigel-coated wells. HSP-VEGFA-MSC seeded into transwell and was then treated with *c*-RSV or *t*-RSV. After 10 days of coculture, confocal laser scanning was performed to assess vessel outgrowth from the aortic ring.

Formation of 3D scaffolds in vivo

Eight-week-old male C57BL/6JNarl mice were purchased from the National Laboratory Animal Center (Taipei, Taiwan). The mice were maintained in an air-conditioned animal facility under constant temperature and humidity conditions with a 12:12 light-dark cycle and were allowed ad libitum diet and drinking water. Mice were randomly picked

to different groups, and there were at least six or more mice in each group. All experimental procedures were approved by the Institutional Animal Care and Use Committee of the Chang Gung University. The stable HSP-VEGFA-MSCs were seeded into a three-dimensional laminar silk scaffold (Wray et al. 2011), and then, the scaffolds were subcutaneously implanted into right lateral subcutaneous pockets of the mice. The mice with or without the stem cell transplantation were intraperitoneally injected 20 mg/kg *c*-RSV every other days. Four weeks after implantation, the animals were sacrificed, and the number of new blood vessels covering the diffusion scaffolds was observed under a bright field microscope.

Endothelial induction of MSCs

MSCs were used for the endothelial induction protocol (Janeczek Portalska et al. 2012). Briefly, the 6-well plates were covered with 1 mL of growth factor-reduced Matrigel (BD Bioscience) diluted 1:1 in EGM-2 without growth factors. The stable HSP-VEGFA-MSCs were plated in 6-well plates at 5×10^5 cells per well and were treated with 5 μ M *c*-RSV in EGM-2 BulletKit without VEGFA. The cultured medium was replaced every second day; at the end of the 10-day incubation period, RNA was harvested for quantitative real-time RT-PCR.

Statistical analysis

The surviving fractions and the relative luminescence units were measured in triplicate and expressed as the mean $\pm$ S.D. Comparisons between two groups were analyzed using a two-tailed Student's *t*-test. The results were analyzed by one-way ANOVA followed by Tukey's post *hoc* test for analyzing parametric data. All statistical analyses were performed using Graph Pad Prism (GraphPad, San Diego, CA). *p < 0.05 was considered statistically significant.

Results

Low-dosage RSV treatment does not cause acute cytotoxicity in MSCs and HUVECs

To examine whether *c*-RSV and *t*-RSV were toxic to MSCs or HUVECs, 5 to 100 μ M concentrations of the drugs were tested. Treatment of MSCs with 5 μ M *c*-RSV did not induce significant changes in cell viability compared with the no-treatment control. A slight decrease in cell survival was observed with *t*-RSV at 5 μ M (Fig. 1b). A low 20 μ M *t*-RSV dosage moderately damaged the HUVECs, but the same *c*-RSV dosage did not affect cell viability (Fig. 1c). Thus, only the *t*-RSV at concentrations >10 μ M caused massive cell death in both cell types.

HSP70 promoter-driven transgene expression is effectively activated by RSV and/or heat shock in HSP-VEGFA-MSC stable cells

To examine whether the HSP70 promoter-driven transgene is activated by RSV, inflammation, and oxidative/ischemic stress, HSP-VEGFA-MSC stable cells were generated by using a lentiviral expression system with the VEGFA gene under the regulation of the HSP70 promoter. After treatment of the infected cells with 5 μ M of either isoform of RSV, HSP70 promoter activities increased up to 2-fold after *c*-RSV treatment and 3-fold after *t*-RSV treatment (Fig. 1d).

Studies have shown that modulators of the heat shock response can have combinatorial synergy with heat stress (Jurivich et al. 1994). Possible heat shock and RSV synergistic effects on activating HSP70 transcriptional activity were next investigated. Simultaneous exposure to *c*-RSV or *t*-RSV and heat shock resulted in further activation of HSP70 promoter activity (Fig. 1e). Both RSV isoforms combined with 44 °C heat shock increased HSP70 promoter activity ~1.5-fold. Moreover, simultaneous exposure at 46 °C fully activated the promoter activity.

Furthermore, studies have shown that elevated levels of proinflammatory cytokines, particularly IFN- γ and TNF- α , selectively inhibit HSP70 production. The HSP-VEGFA-MSCs were simultaneously treated with *c*-RSV or *t*-RSV and a conditioned medium from THP-1 cells (THP-CM). The results showed that THP-CM suppressed 20 % of the HSP70 promoter activity (Fig. 1f). Interestingly, simultaneous exposure of cells to *c*-RSV and THP-CM showed no change in promoter activity. However, a discernible decrease in activity resulted from simultaneous treatment with *t*-RSV. Taken together, the results indicate that 5 μ M of both isoforms of RSV had low cytotoxicity and strongly induced HSP70 activity.

RSV induces endothelial differentiation in MSCs and promotes tube formation in HUVECs

To determine whether the HSP70 promoter can conditionally regulate gene expression, VEGFA expression was examined under RSV treatment in HSP-VEGFA-MSC. Western blot results showed that both RSV forms upregulated VEGFA and hemeoxygenase 1 (HO-1) protein expression, but *t*-RSV was more potent than *c*-RSV (Fig. 2a). Furthermore, exposure to *t*-RSV enhanced VEGFA secretion in HSP-VEGFA-MSC compared with the control and *c*-RSV treatment. We next investigated whether VEGFA secreted by HSP-VEGFA-MSC could stimulate angiogenesis in endogenous endothelial cells. HUVECs developed tubules when grown in conditional medium derived from *c*-RSV pretreated HSP-VEGFA-MSC. The total tube length increased 2-fold compared with that grown in conditioned medium from mock pretreated HSP-VEGFA-MSC. However, *t*-RSV-pretreated HSP-VEGFA-

MSC inhibited tube formation of HUVECs (Fig. 2b, c). *VEGFA* mRNA levels were significantly upregulated in HUVECs grown in conditioned medium from RSV-pretreated HSP-VEGFA-MSC. Conversely, the vascular endothelial growth factor receptor (*VEGFR*) mRNA expression levels were significantly upregulated in HUVECs grown in the MSC or HSP-VEGFA-MSC conditioned medium. The *c*-RSV group showed a ~2-fold increase, but the *t*-RSV group showed no significant change, compared with the mock group (Fig. 2d). Lower *VEGFR2* induction in the *t*-RSV group in HUVECs may explain the low tube formation ability.

To characterize the phenotype of HSP-VEGFA-MSC cultured in EGM-2 medium (without VEGFA) with or without RSV after 10 days, the expression of endothelial genes *CD31*, *HO-1*, *VEGFA*, and *VEGFR2* was assessed. The results demonstrated that RSV had a synergistic effect on the endothelial differentiation of MSCs when combined with endothelial cell culture conditions (Fig. 3). *CD31* expression increased 5-fold with *c*-RSV and 3-fold with *t*-RSV treatment (Fig. 3a). *HO-1* acts as an upstream signal of *VEGFA*, and its expression level increased 2- and 3-fold with *c*-RSV and *t*-RSV treatment, respectively (Fig. 3b). *VEGFA* expression increased 1.5-fold with both RSV isoforms (Fig. 3c). *VEGFR2* expression increased 2.5- and 1.5-fold with *c*-RSV and *t*-RSV treatment (Fig. 3d).

RSV conditionally regulates HSP-VEGFA-MSC-dependent angiogenesis in aortic rings ex vivo and in 3D scaffolds in vivo

An ex vivo coculture system was established to determine the angiogenic effects for the MSC and the aortic ring (Fig. 4a). After 10 days of coculture, there was an obvious expansion of tubules, which are presumably a combination of endothelial cells, mesenchymal cells, and perivascular cells, in the placebo- and RSV-treated HSP-VEGFA-MSC groups compared with the aortic ring-only group (Fig. 4a). The field of view occupied by tubules in Fig. 4a indicated that exposure of HSP-VEGFA-MSC to *c*-RSV or *t*-RSV promoted angiogenesis. To confirm these findings, we analyzed the sprouting using a higher magnification and found that there was additional vessel development from aortic intimal/subintimal layers. Thus, the angiogenesis observed in our analysis was due to formation of new vessels and did not sprout from the initial vessels (Fig. 4b).

Three-dimensional (3D) laminar silk scaffolds were seeded with HSP-VEGFA-MSC and implanted subcutaneously into mice. Four weeks later, the subcutaneous blood vessels surrounding the site of the HSP-VEGFA-MSC scaffold were examined. This area was thin and semi-transparent, allowing for the visualization of blood vessels (Fig. 4c). Vessels and their associated branches began to spread and extended toward the HSP-VEGFA-MSC scaffold.

Fig. 2 RSV induced gene expression in HSP-VEGFA-MSC and c-RSV-pretreated HSP-VEGFA-MSC-conditioned medium enhances HUVEC tube formation. **a** Stable HSP-VEGFA-MSC was treated with 5 μ M of c-RSV or t-RSV for 24 h. At the end of treatment, total cellular VEGFA, exogenous VEGFA, and HO-1 protein expression levels were detected in whole cell lysates by Western blot analysis. **b** HUVECs were cultured with conditioned medium (CM) from 5 μ M c-RSV or t-RSV pretreated HSP-VEGFA-MSC or untreated HSP-VEGFA-MSC CM (Mock) or

unconditioned medium (Control) for 8 h. **c** Tube formation was quantified by the angiogenesis analyzer plug-in for ImageJ. **d** Real-time PCR was performed to examine the *VEGFR2* and *VEGFA* mRNA levels in HUVECs. Values were normalized to that of β -actin and were expressed relative to the control (Ctrl) group. Data represent mean $\pm$ S.D. of three independent experiments. * p <0.05 and ** p <0.01 indicate differences between the treated cells and the control

The scaffold-implanted mice treated with c-RSV presented neovascularization/angiogenesis effects surrounding the scaffold-folds, which showed similar results compared with the HSP-

VEGFA-MSC group (Fig. 4c). In contrast, fewer blood vessels and branches were observed surrounding the scaffolds without c-RSV treatment (Fig. 4d).

Fig. 3 Endothelial marker expression in c-RSV- or t-RSV-treated HSP-VEGFA-MSC. Stable HSP-VEGFA-MSC treated with 5 μ M c-RSV or t-RSV was cultured in EGM-2 (without VEGFA) and matrigel for 10 days. At the end of incubation, gene expression of **a** *CD31*, **b** *HO-1*, **c** *VEGFA*, and **d** *VEGFR2* was assessed by real-time PCR. Values were normalized to *Gapdh* and were expressed relative to the respective control group. Data represent mean $\pm$ S.D. of three independent experiments. * p <0.05 and ** p <0.01 indicate differences between the treated cells and the control

Fig. 4 *c*-RSV-treated HSP-VEGFA-MSC promotes new vessel branching in an ex vivo aortic ring coculture system. **a** Mouse aortic rings were seeded into a 9-well dish with Matrigel. HSP-VEGFA-MSC was seeded into transwells and then treated with *c*-RSV or *t*-RSV. The culture medium was replaced every second day. After 10 days of coculture, confocal laser scanning was performed to assess outgrowth of vessels from the aortic rings and **b** quantification of the total sprouts area was performed by Wimasys. **c** Stable HSP-VEGFA-MSC was seeded

into a three-dimensional laminar silk scaffold, which was subcutaneously implanted into the right lateral subcutaneous pockets in mouse. Four weeks after implantation, the animals were sacrificed and the number of new blood vessels covering the diffusion scaffolds was observed under a bright field microscope, and **d** quantification of the total vessels network length by Wimasys. Data represent means $\pm$ S.D. of three independent experiments. * $p < 0.05$ and ** $p < 0.01$ indicate differences between the treated cells and the mock control

Discussion

The main challenge in stem cell-based treatment of cardiovascular diseases (Fierro et al. 2011; Leblond et al. 2009) is safety and effectiveness of the delivery systems used. We present in this study a novel MSC-based gene therapy system by using an RSV-inducible HSP70 promoter to regulate VEGFA expression, adding a further level of regulation on the sole application of MSC.

VEGFA is a key pleiotropic growth factor that affects the generation of new blood vessel networks, proliferation and differentiation of endothelial cells, and the formation of mature blood vessel via angiogenesis (Kimura et al. 2000). VEGFA-based gene therapy has indeed been successfully used to treat ischemia in animal models and in clinical trials (Kalil et al. 2010). Studies have demonstrated that the MSC effects on proliferation and differentiation through signals arising from microenvironmental niches are controlled by growth factors such as VEGFA and basic fibroblast growth factor, bFGF (Xiao et al. 2007). In particular, VEGFA also induces neural stem cell proliferation via one of its receptors, VEGFR2, in a dose-dependent manner (Xiao et al. 2007). VEGFA expressed in adenoviral-transduced MSCs was shown to act through paracrine and/or autocrine signaling to affect cell growth (Lin et al. 2008). Heat shock induces HSP70 expression in monocytes and endothelial cells (Gawad et al. 2009). The synergistic cooperation RSV with heat shock treatment used in this work caused a more powerful triggering of HSF1 regulation of the HSP70 promoter (Fig. 1d). Other

mechanisms of induction of HSF1 expression may also cooperate synergistically with the action of to achieve an enhanced effect on HSP70 promoter expression.

Ischemic injury is associated with inflammatory responses, filtration of many types of inflammatory cells, secretion of inflammatory mediators such as IFN- γ , TNF- α , and IL-12, and blocking of activators of angiogenic processes in ischemic tissues (Liu et al. 1999). In this study, the effects of inflammatory cytokines on the expression of HSP70 promoter-driven luciferase reporter genes were examined (Fig. 1f), and the results demonstrated that THP-1 CM caused a 20 % downregulation of gene expression in HSP-VEGFA-MSC under RSV induction.

The *t*-RSV isomer, but not *c*-RSV, exhibits antiangiogenic activity (Belleri et al. 2008). RSV dosage is critical in angiogenesis not only in inducing HSP70 promoter expression but also because RSV in both forms is extensively metabolized and exerts differential effects on angiogenesis in vitro (Fig. 2b) and in vivo (Fig. 4a). *t*-RSV enhanced angiogenesis at low doses (100 nM to 5 μ M) but inhibited angiogenesis at higher doses (10 to 100 μ M), depending on the cell type (Baron-Menguy et al. 2007; Chen and Easton 2011). Studies have shown that low-dose *t*-RSV stimulated angiogenesis by increasing the eNOS-stimulated VEGFA. On the other hand, higher doses may affect antioxidants on oxidative stress as a result of angiogenesis-related processes (Chen and Easton 2011).

Protective genes in the cell act as a defense against ROS and oxidative stress. HO-1 plays a key role in oxidant

responses (Zhang et al. 2002). Overexpression of HO-1 protects cells against oxidative injury, inhibits cellular proliferation, increases cell death upon exposure to sublethal or lethal doses of H₂O₂ (Zhang et al. 2002), and plays a role in other diverse biological processes (Galbraith 1999). The increased antioxidant activity of *t*-RSV is likely due to high-level HO-1 overexpression, which causes oxidative stress-induced cellular apoptosis. Low levels of reactive oxygen species as a cell signaling molecule to mediate endothelial cell proliferation and generally stimulate angiogenesis and vasculogenesis (Luczak et al. 2004; Ushio-Fukai and Urao 2009). The potential angiogenesis and antiangiogenesis effects of *c*-RSV or *t*-RSV remain to be investigated.

Despite reported successes, possible side effects that may arise from clinical applications of VEGFA should be closely monitored. It has been reported that VEGFA overexpression may lead to the development of neovascularization in the eye in a rat model (Spilsbury et al. 2000), blood-brain barrier leakage after stroke (Wang et al. 2005), rapid microvessel enlargement, and increased vascular permeability in the heart (Rissanen et al. 2004). Possible carcinogenic transformation of MSC and tissue cells as a result of high-level VEGFA expression constitutes another source of risks which should also be addressed (Moon et al. 2003). Moreover, it is known that increased mammalian target of rapamycin (mTOR) activities in cancer cells can lead to secretion of VEGFA and platelet-derived growth factor β , promoting angiogenesis by increasing mTOR activities in vascular cells (Guertin and Sabatini 2007; Advani 2010). The genetically modified MSCs that conditionally expressed VEGFA under the control of a *c*-RSV-inducible HSP70 promoter described in this work should alleviate, or vastly reduce, possible clinical risks associated with constitutive VEGFA expression as described. In conclusion, we demonstrated that *c*-RSV spatial and temporal regulation of VEGFA expression enhanced the angiogenic capacity of stem cell-based gene therapy for treatment of ischemic diseases and lower clinical risks.

Acknowledgments This work was supported in part by the Ministry of Science and Technology (Taiwan), NSC 99-2632-B-182-001-MY3 and MOST 103-2320-B-182-021, Chang Gung Memorial Hospital Grant (CMRPD 34012, CMRPD 180133, CMRPD1B0472, CMRPD1B0473), and the Ministry of Education, Taiwan (EMRPD1C0121).

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