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Rosuvastatin elicits KDR-dependent vasculogenic response of human placental stem cells through PI3K/AKT pathway

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ABSTRACT

The growth and plasticity of engrafted human mesenchymal stem cells is regulated by external stimuli. Rosuvastatin (RSV) promotes myocardial neovascularization and limits myocardial remodeling in patients with chronic heart failure (CHF). While these non-lipid benefits may in part depend on the activation of stem cells, experimental evidence that RSV directly elicits vasculogenic differentiation of human mesenchymal stem cells is still lacking.

We assessed whether RSV may drive a gene program of vascular commitment and the secretion of trophic mediators with antiapoptotic, angiogenic and antifibrotic activities in human mesenchymal stem cells from full-term placentas (FMhMSCs).

With real-time RT-PCR, immunofluorescence, chemiluminescence, Western blot analysis, and *in vitro* vasculogenesis assays, we show that RSV enhanced expression of vascular endothelial growth factor (VEGF), kinase insert domain receptor (KDR), encoding a major VEGF receptor, hepatocyte growth factor (HGF), and platelet-derived growth factor-BB (PDGF-BB) in a time- and dose-dependent manner. GATA-4 and Nkx-2.5 transcription was not affected. RSV enhanced capillary-like formation *in vitro*, but capillary-embedded FMhMSCs lacked endothelial marker expression, suggesting a role of pericyte-like elements in tube formation. In HUVEC/FMhMSC cocultures, RSV increases PDGFRβ expression in FMhMSCs, and enhanced capillary density and organizational efficiency, promoting a long-lasting survival of tubular networks. RSV also activated PI3K–Akt pathway; the vasculogenic effects of the statin were abrogated following PI3K inhibition by LY294002.

In conclusion, RSV-induced increase in capillary formation was dependent on VEGF and KDR. RSV promotes the activation of paracrine signals for vascular commitment of FMhMSCs through PI3K–Akt pathway. This observation may pave the way to the use of RSV as a pharmacological enhancer of stem cell potential for cardiovascular cell therapy.

1. Introduction

Statins have been shown to elicit a host of non-cholesterol-lowering responses, including reduced systemic inflammation [1], enhanced angiogenesis [2], and bone marrow cell (BMC) mobilization [3]. Rosuvastatin (RSV), a relatively new molecule, prevented progressive left ventricular remodeling and promoted neoangiogenesis in failing heart of patients [4,5] and animal models [6]. This outcome was partially ascribed to normalization of cardiac cytokine expression, including TNF-α, and increase in circulating BMCs [6] and endothelial progenitor cells [7], an event that

Abbreviations: RSV, rosuvastatin; CHF, chronic heart failure; FMhMSCs, human mesenchymal stem cells from fetal membranes of term placenta; VEGF, vascular endothelial growth factor; KDR, kinase insert domain receptor; PDGF-BB, platelet-derived growth factor-BB; vWF, von Willebrand factor.

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may potentially account for myocardial neovascularization and rescue.

Whether RSV may directly commit human adult stem cells toward a cardiovascular fate and/or enhance the paracrine secretion of trophic mediators with angiogenic, antiapoptotic and antifibrotic properties remains to be established. Addressing this issue may be of particular biomedical relevance. In fact, only poor or negligible myocardial rescue has been obtained so far in randomized clinical trials with simple stem cell transplantation in failing heart [8]. Conversely, Strauer et al. [9] demonstrated that cell therapy improves ventricular performance and prognosis in patients with chronic heart failure when stem cells were administered in addition to standard therapeutic regimes. It is conceivable that pharmacological agents modulate the magnitude of stem cell potential. *Ex vivo* preconditioning with synthetic molecules harboring differentiating and paracrine "logics" remarkably enhanced the cardiovascular commitment in both mouse embryonic [10] and human stem cells [11,12], improving their rescuing properties *in vivo* in animal models of heart failure [11,13,14]. These findings prompt further studies to assess whether drugs already available for conventional cardiovascular therapy (*i.e.* RSV) may also be used for stem cell preconditioning to enhance the potential for a cell therapy.

Here, we investigated whether exposure to RSV of human mesenchymal stem cells from fetal membranes of term placenta (FMhMSCs) may activate the transcription of vascular lineage-promoting genes, and whether RSV may drive the secretion of growth factors enhancing vasculogenesis *in vitro*.

2. Methods

2.1. Rosuvastatin preparation

Rosuvastatin (RSV) Ca^{2+} – salt was kindly provided by AstraZeneca and it was dissolved in DMSO at the final concentration of 50 mM.

2.2. Cell isolation

Term placenta obtained from caesarian sections were rapidly rinsed in PBS containing penicillin and streptomycin and used immediately. Pieces from fetal membranes were minced and digested for 10 min in DMEM with 0.25% trypsin–EDTA, 10 U/ml DNaseI and 0.1% collagenase. Tissues were pipetted vigorously up and down avoiding foam for 5 min; larger pieces of tissue were allowed to settle under gravity for 5 min. Each supernatant was transferred to a fresh tube, neutralized with FBS, then spun at $1000 \times g$ for 10 min. Each pellet was resuspended in 5 ml of DMEM containing 20% FBS, 10 U/ml penicillin and 100 μg/ml streptomycin. FMhMSCs were seeded into culture flasks and grown at 37 °C in 5% CO_2. Non-adherent cells were removed after 1 week and medium (with 10% FBS) was changed subsequently every 4 days.

Human umbilical vascular endothelial cells (HUVECs) were isolated in our laboratory with the method described by Davis [15] from human umbilical cord obtained during elective caesarian sections. The cells collected were grown in EGM-2 medium (Lonza) at 37 °C in 5% CO_2.

2.3. Flow cytometry analysis (FACS) and cellular phenotyping

To assess the mesenchymal origin of fibroblast-like cells isolated from fetal membranes we performed FACS analysis. Cells were harvested by treatment with 0.08% trypsin–EDTA and incubated with 1 μg/10^6 cells conjugated antibodies for 40 min at 4 °C in the dark. The antibodies used were: anti-CD105-Alexa Fluor 488 (Chemicon), anti-CD73-PE (BD), anti-CD29-PE-Cy5 (BD), anti-CD90-R-PE (BD), anti-CD166-R-PE (BD), anti-CD14-APC (BD), anti-CD34-FITC (BD), anti-CD44-FITC (BD), and anti-CD45-Per-CP (BD). After washing, cells were analyzed on a flow cytometer (FACSCalibur, Becton Dickinson, San Jose, CA, USA) by collecting 10,000 events and the data analyzed using the Cell Quest Software (Becton Dickinson).

Adipogenic (Chemicon), osteogenic (Chemicon) and chondrogenic (Lonza) differentiations were performed to test the multipotency of FMhMSCs, following the manufacturer's instructions.

For HUVEC characterization, anti-CD31 (Santa Cruz) was used as primary antibody and fluorescent conjugated goat anti-mouse (MP Biomedicals) as secondary antibody. Cells were analyzed on a flow cytometer.

2.4. Cell treatment and viability

Drug sensitivity was estimated by the MTT method, essentially as described by the manufacturer's instructions (SIGMA). FMhMSCs (7500 cells/cm^2) were seeded into 24-well plates and then treated with different concentrations of RSV (0.1–75 μM) for 1, 2, 3 and 6 days. Untreated cells and DMSO treated cells were used as control. Data were collected by reading at 570 nm with a multi-well plate reader (Dynex Technology).

Cell viability was determined by the trypan blue dye exclusion test. The cells were treated with RSV (0.1–75 μM). After 1, 2, 3 and 6 days, both attached and floating cells were harvested and they were counted by using a hemocytometer.

To assess whether RSV, in range between 0.1 and 10 μM, below the toxic concentration, may coax cells into a "cardiovascular decision", we performed comparative analyses of the expression of targeted cardiogenic and angiogenic genes and immunocytochemical assessment of lineage-restricted markers, as detailed below.

2.5. Gene expression

Real-time RT-PCR was used to assess the time- and dose-dependent effect of RSV on the gene expression of vascular endothelial growth factor (VEGF), KDR, hepatocyte growth factor (HGF), GATA-4, Nkx-2.5 and Akt. Primer sequences were: human GAPDH reverse: 5′ TGTGGTCATGAGTCCTTCCA 3′, forward: 5′ CAGCCTCAAGATCATCAGCA 3′; human VEGF reverse: 5′ ACACAGGATGGCTTGAAGATG 3′, forward: 5′ AGAAGGAGGAGGGCAGAATC 3′; human HGF reverse: 5′ ACTCCAGGGCTGACATTTGAT 3′, forward: 5′ ATTTGGCCATGAATTTGACCT 3′; human KDR reverse: 5′ GAGCTCTGGCTACTGGTGATG 3′, forward: 5′ CTGCAAATTTGGAAACCTGTC 3′; human GATA-4 reverse: 5′ TAGCCTTGTGGGGAGAGCTT 3′, forward: 5′ TGGCCTGTCATCTCACTACG 3′; human Nkx-2.5 reverse: 5′ GCGCACAGCTCTTTCTTTTC 3′, forward: 5′ CAAGTGTGCGTCTGCCTTT 3′; human Akt: reverse: 5′ CTTAATGTGCCCGTCCTTGT 3′, forward: 5′ TCTATGGCGCTGAGATTGTG 3′.

Data were normalized using GAPDH as an index of cDNA content after reverse transcription. Amplification included initial denaturation at 95 °C for 10 min, 50 cycles of denaturation at 95 °C for 10 s, annealing at 59–63 °C for 6–10 s, and extension at 72 °C for 10 s, performed at a temperature transition rate of 20 °C/s. Fluorescence was measured at the end of each extension step. Specificity of the product was determined by a melting curve analysis, conducted after completion of the cycling process with the aid of a temperature ramp (from 55 to 95 °C at 0.1 °C/s) and continuous fluorescence monitoring.

Samples were run in duplicate, and the average threshold cycle (C_t) value was used for calculations. Relative quantification of mRNA expression was calculated with the comparative C_t method using the "Delta–delta method" for comparing relative expression results between treatments in Real-time RT-PCR [16].

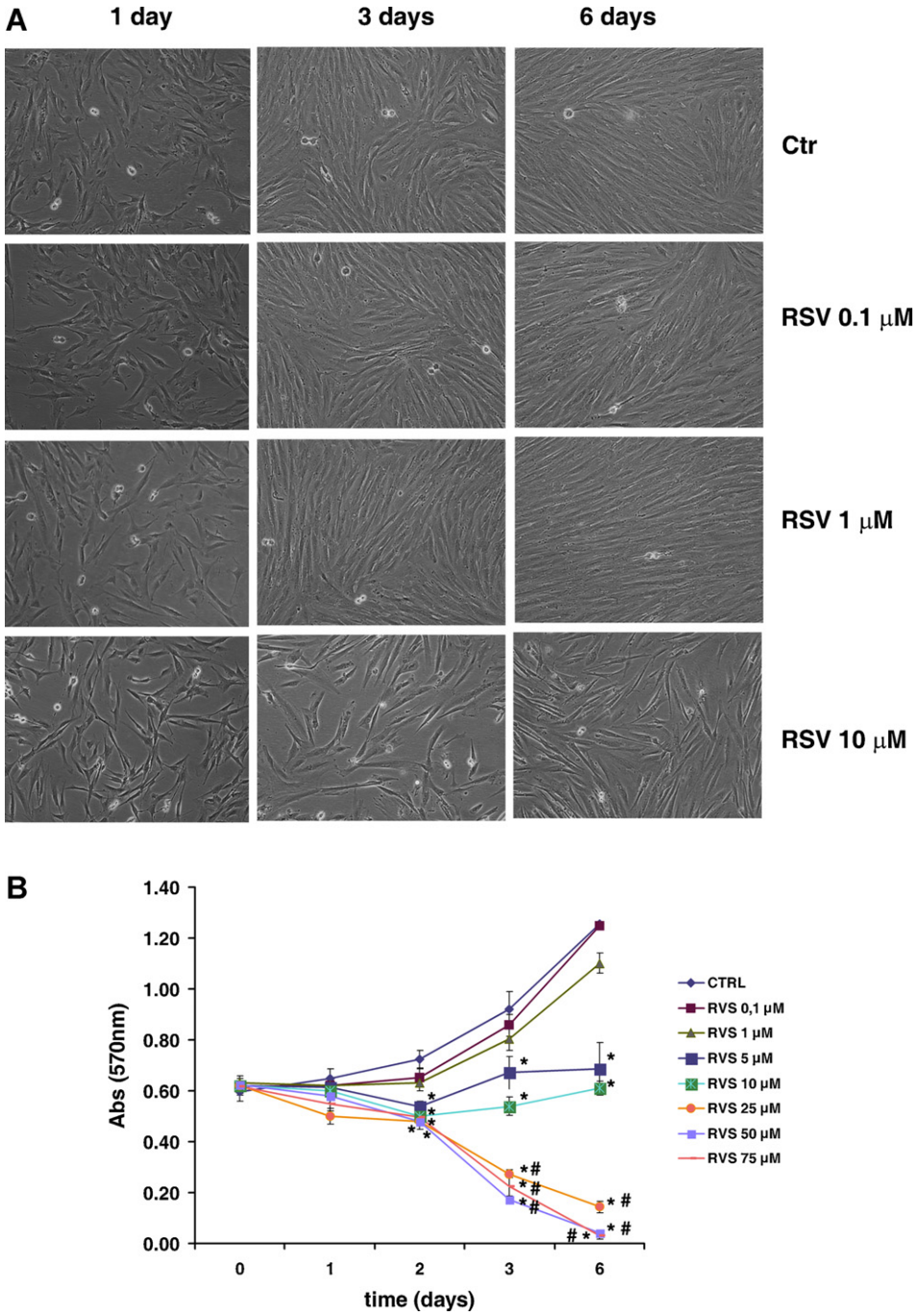

Fig. 1. Treatment of FMhMSCs with RSV and MTT proliferation assay. (A) FMhMSCs (7500 cells/cm^2) were seeded into 75 cm^2 flasks and treated after 24 h with different concentrations of RSV (0.1–1–10 μM) for 1, 3 and 6 days (100×, original magnification). (B) FMhMSCs (7500 cells/cm^2) were seeded into 24-well plates and then treated with different concentrations of RSV (0.1–75 μM) for 1, 2, 3 and 6 days. Data were collected by reading at 570 nm with a multi-well plate reader ($n = 4$). Statistical analysis: ANOVA Dunnett's Multiple Comparison Test, *significantly different from untreated cells (Ctrl); unpaired t-test, #significantly different from 5 to 10 μM treated cells; $P < 0.05$.

2.6. Effect of rosuvastatin on growth factor production

We assessed whether RSV (0.1–10 μM) acts on the secretion of VEGF, HGF and platelet-derived growth factor-BB (PDGF-BB) using Bio-Plex Pro Human Cytokine 3-Plex assay from Bio-Rad, following the manufacturer's instructions. Cell culture supernatants and total protein content were collected at different times of treatment (16 h, 1 day, 3 and 6 days).

PDGF-BB production in HUVECs cocultured with FMhMSCs has been evaluated using Bio-Plex Pro Human Cytokine Plex assay from Bio-Rad, following the manufacturer's instructions. FMhMSCs and HUVECs have been cocultured using cell culture insert with 0.4 μm pore diameter (BD). Total proteins were extracted 8 and 24 h after the treatment.

All the samples were assayed at least in duplicate and normalized with total protein content.

2.7. In vitro vasculogenesis

Analysis of capillary-like tube formation was performed using Cultrex® Basement Membrane Extract (BME, Thema). Cells were seeded in gel-precoated wells and cultured in the absence or

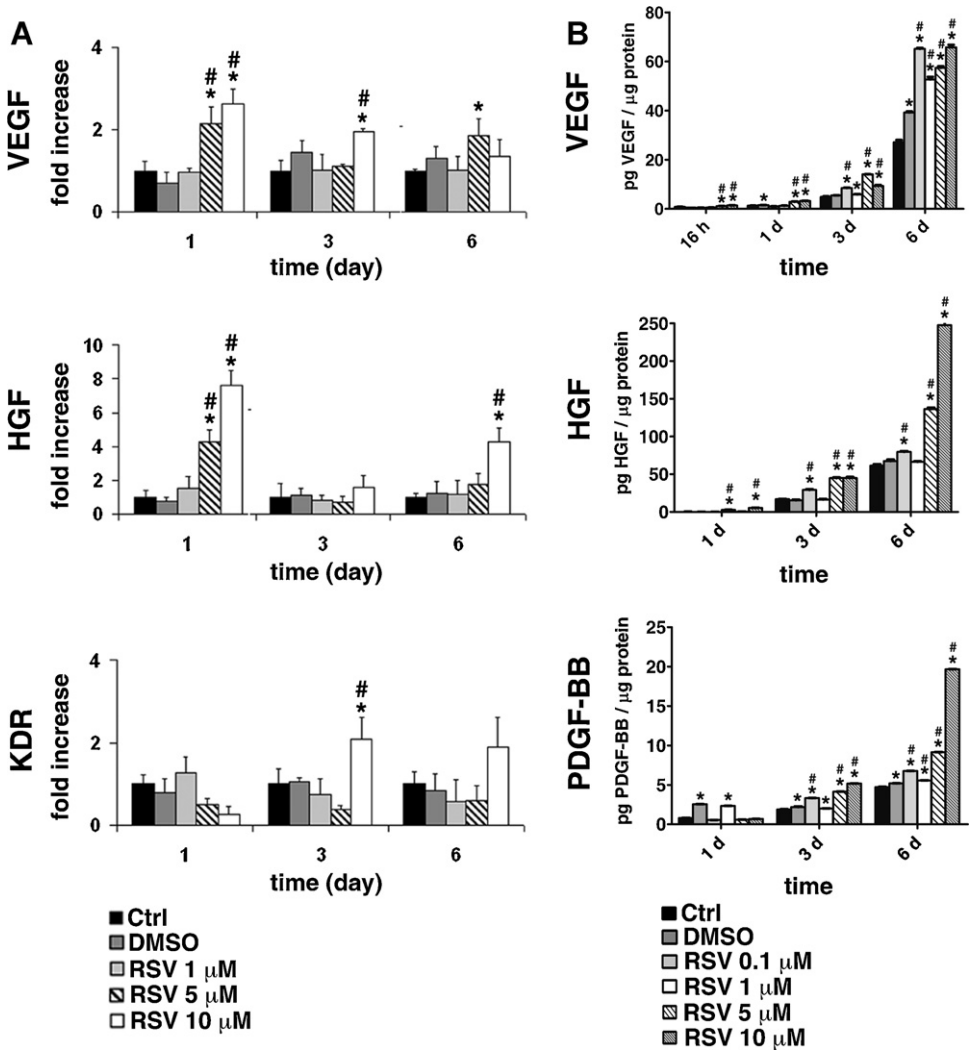

Fig. 2. RSV effects on gene expression and cytokine secretion. (A) VEGF, HGF, and KDR gene expression was assessed by real-time RT-PCR. FMhMSCs were cultured in the absence or presence of 0.02% DMSO, and RSV concentrations of 1, 5, or 10 μM. The mRNA abundance in untreated cells was defined as 1, and fold increase of VEGF, HGF, and KDR mRNA from RSV-treated cells was plotted relative to that value (mean ± SEM; $n = 3$). (B) Time-course analysis of VEGF, HGF, PDGF-BB, secreted by FMhMSCs cultured in the absence or presence of RSV (0.1–1–5–10 μM) (mean ± S.E.M.; $n = 3$). Statistical analysis: ANOVA Dunnett's Multiple Comparison Test, *significantly different from untreated cells (Ctrl), #significantly different from solvent-treated cells (DMSO); $P < 0.05$.

presence of RSV at different concentrations, with or without 10 μM LY294002 (Alexis), or neutralizing antibodies anti-VEGF (final dilution 1:500, AbCam), and anti-KDR (final dilution 1:1000, AbCam).

For capillary-like tube formation assay in cocultured cells, HUVECs and FMhMSCs were stained with two fluorescent viable dyes, PKH2 (green) and PKH26 (red), respectively, following the manufacturer's instructions (Sigma). Then cells were seeded in a ratio of 3:1 in gel-precoated wells and cultured in the absence or presence of RSV 10 μM.

Capillary-like structures were observed at regular time intervals, and photographed using an inverted optical microscope equipped with a digital sight camera (Nikon).

2.8. Immunostaining of tube-like structures in BME

After tube-like structures formation, performed in chamber slides (BD), supernatant was removed and BME was dissolved using cell recovery solution (BD), then the cells were fixed with 2% paraformaldehyde at room temperature for 5 min. After fixation, cells were washed thrice with PBS/TWEEN20 and, only for intracellular antigens, permeabilized with 0.2% Triton X-100 at room temperature for 10 min. Aspecific antibody binding sites were blocked by incubating with 5% bovine serum albumin for 1 h at 37 °C. Then, cells were labeled with specific primary antibodies for 1 h at room temperature. Excess primary antibody was removed by three washes with PBS/TWEEN20 and the cells were stained at 37 °C for 1 h with fluorescein-conjugated goat IgG. The monoclonal antibodies used were: anti-von Willebrand factor (vWF) antibody (DAKO), anti-VEGF (BD), anti-CD31 (Santa Cruz), and anti-CD34 (BD). For negative control, samples were processed omitting the primary antibody.

2.9. Western blotting

Fifty micrograms of total cell lysate were subjected to SDS-PAGE. Phospho-Akt rabbit monoclonal antibody (1:2000 final dilution, Cell Signaling), total Akt rabbit polyclonal antibody (1:1000 final dilution, Cell Signaling), followed by incubation with horseradish peroxidase-conjugated antibody to rabbit IgG (1:2000 final dilution, Cell Signaling) were used to evaluate Akt expression and

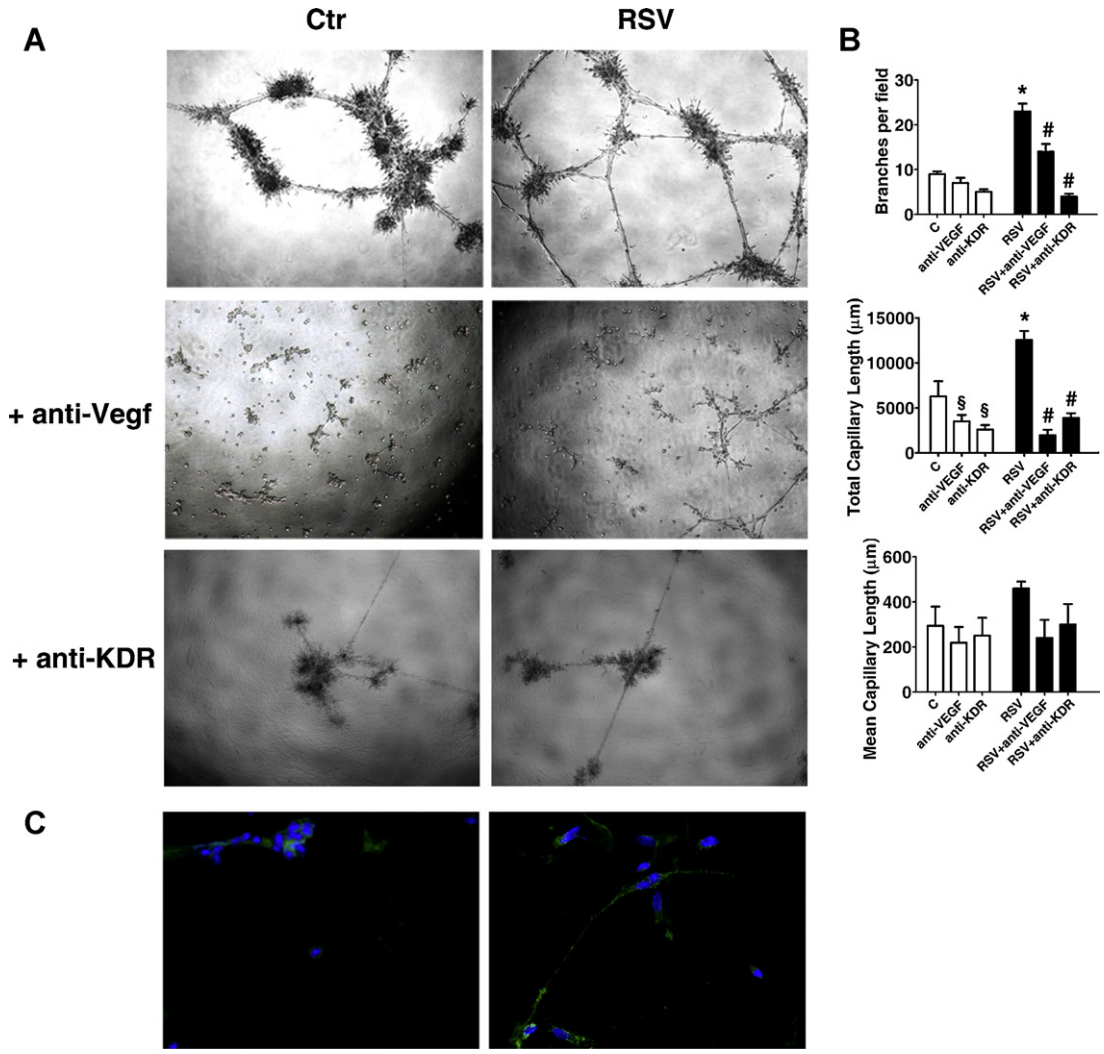

Fig. 3. RSV promotes FMhMSC *in vitro* vasculogenesis through a VEGF-dependent mechanism. (A) Tube-like formation was assessed in semisolid medium in FMhMSCs cultured for 24 h in the absence or presence of 10 μM RSV, and in cells exposed to anti-VEGF or anti-KDR (final dilution 1:500 and 1:1000, respectively) neutralizing antibodies in the absence or presence of 10 μM RSV. All images are representative of three experiments with similar results (40×, original magnification). (B) Quantification and morphological assessment of tube-like structures in semisolid medium (A) were performed by NIS-Elements D Nikon software (version 3.06). The parameters branches per field, the total capillary length, and the average capillary length were expressed as mean ± S.E.M. ($n = 3$). *Significantly different (increase) from untreated cells (Ctr); #significantly different (reduction) from RSV treatment; §significantly different (reduction) from untreated cells (Ctr); $P < 0.05$. (C) After tube-like structures formation, untreated and RSV 10 μM treated cells were stained with anti-VEGF specific antibody. RSV treatment increases VEGF production during vasculogenesis (100×, original magnification).

activation. The expression PDGFRβ in FMhMSCs cocultured with HUVECs has been evaluated using Western blotting. FMhMSCs and HUVECs have been cocultured using cell culture inserts with 0.4 μm pore diameter (BD). Total proteins were extracted 8 and 24 h after the treatment.

PDGFRβ (1:1000 final dilution, AbCam) and tubulin (1:2000 final dilution, Cell Signaling) rabbit polyclonal antibodies were used, followed by incubation with horseradish peroxidase-conjugated antibody to rabbit IgG (Cell Signaling).

Antigen–antibody complexes were visualized by ECL Western blotting detection reagents (GE Healthcare), according to the manufacturer's instructions.

2.10. Statistical analysis

The statistical analysis was performed using GraphPad Prism ver. 5. Data were evaluated by using a two-tailed, unpaired Student's *t*-test, and ANOVA Dunnett's Multiple Comparison Test, assuming a *P* value less than 0.05 as the limit of significance.

3. Results

3.1. Cell treatment

Nontoxic effect was observed following FMhMSC exposure to RSV ranging from 0.1 to 10 μM throughout 6 days of treatment (Fig. 1).

3.2. Effect of rosuvastatin on gene activation and growth factor secretion

RSV increased the gene expression of VEGF, HGF, and KDR, encoding a major VEGF receptor in a time- and dose-dependent fashion (Fig. 2A). The stimulatory effect peaked at a concentration of 10 μM, reaching a maximum after 1-day exposure for both VEGF and HGF transcription, and after 3-day treatment in the case of KDR. RSV did not affect the gene expression of GATA-4 and Nkx-2.5, two cardiac lineage promoting genes, nor did it change Akt transcription (data not shown). RSV time- and dose-dependently increased PDGF-BB and HGF release in the culture medium (Fig. 2B).

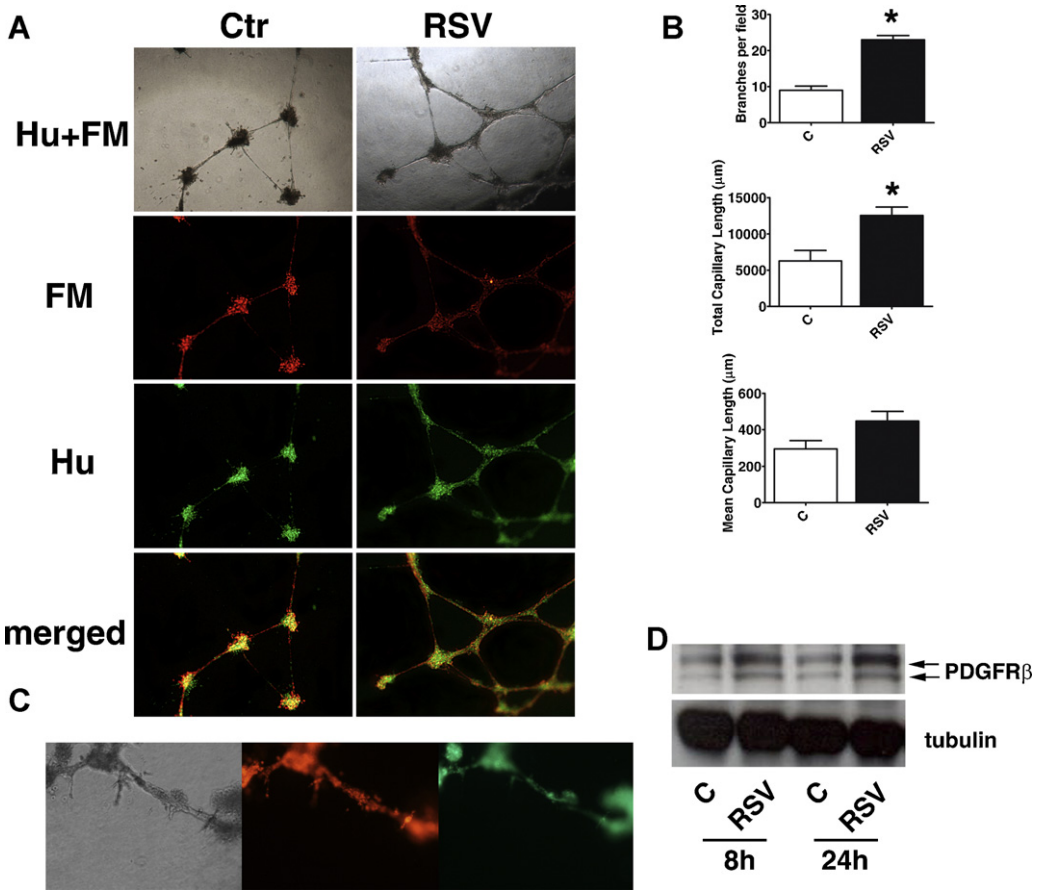

Fig. 4. RSV increases the ability of cocultured HUVECs and FMhMSCs to form capillary-like structures and promotes tubular network survival. (A) Cocultures were performed with HUVECs and FMhMSCs in a ratio of 3:1, seeded in gel-precoated wells. Before seeding, cells were labeled with fluorescent viable dyes, green PKH2 (HUVECs, Hu) and red PKH26 (FMhMSCs, FM). Then, cells were seeded and treated with medium alone or RSV 10 μM. All images are collected at 24 h and are representative of three separate experiments with similar results (40×, original magnification). (B) Quantification and morphological assessment of tube-like structures in semisolid medium (A) were performed by NIS-Elements D Nikon software (version 3.06). The parameters branches per field, the total capillary length, and the average capillary length were expressed as mean ± S.E.M. ($n = 3$). *Significantly different from untreated cells. $P < 0.05$. C, tubule-like structure in cocultures of HUVECs and FMhMSCs treated with RSV 10 μM (100×, original magnification). (D) PDGFRβ expression in FMhMSCs cocultured with HUVECs in presence or absence of RSV 10 μM for 8 and 24 h. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

The RSV action became evident after 24 h, progressively increasing after 3-days, resulting in a remarkable and long-lasting increment for up to 6 days of treatment. RSV also time-dependently increased release of VEGF throughout 6 days. Surprisingly, 0.1 μM concentration induces VEGF secretion as well as RSV 10 μM after 6 days of treatment (Fig. 2B).

3.3. Effect of rosuvastatin on vasculogenesis in vitro

In FMhMSC cultures grown in semisolid medium (BME), RSV increased both the number of capillary-like tubes and their branching assembly as compared with untreated controls (Fig. 3A and B). The density of the RSV-induced network peaked at 24 h, persisting for up to 48 h. The ability of FMhMSCs to form capillary-like structures *in vitro* was strongly reduced, although not completely abrogated, by blocking VEGF and its major receptor, KDR, using specific antibodies (Fig. 3A and B), even in the presence of RSV. vWF-, CD31-, or CD34-positive cells were not detected within the capillary-like tubes after 24 h of treatment with RSV (data not shown). Nevertheless, VEGF staining was consistently enhanced in capillary-like structures induced by 10 μM RSV (Fig. 3C), as compared with untreated controls.

We also investigated whether RSV-treated FMhMSCs may aid HUVECs to form capillary-like structures in semisolid medium. In FMhMSC and HUVEC cocultures, RSV increased both the number of capillary-like tubes and their organizational efficiency (Fig. 4A–C). Within the tubular network, most of the HUVECs were closely associated with and surrounded by FMhMSCs (Fig. 4C), a feature resembling that described for hMSCs behaving as pericyte/nurse-like elements [11]. While the observed ability of FMhMSCs (Figs. 3 and 7) or HUVECs (Fig. 5) to form capillary-like structures *in vitro*, in the absence or presence of RSV, occurred in as early as within 2 h, and persisted for up to 2 days (not shown), the overall capillary network formed by FMhMSCs and HUVECs cocultured with RSV persisted for up to 1 week (Fig. 6). It is well known that PDGF-BB/PDGFRβ axis is commonly involved in recruitment of pericytes by endothelial cells promoting the stability of the vasculature [17]. While RSV did not affect the PDGF-BB production in HUVECs cocultured with FMhMSCs separated by a membrane insert (data not shown), after 8 h of treatment the molecule induced an increase in PDGFRβ expression in stem cells persistent for up to 24 h (Fig. 4D).

3.4. Effect of phosphatydilinositol-3 kinase inhibitor on rosuvastatin-mediated vasculogenesis

The spontaneous process of capillary-like tube formation was completely abrogated in the presence of LY294002, a phosphoinositide-3 kinase (PI3K) inhibitor (Fig. 7A and B). The RSV-mediated increase in capillary-like tubes was also suppressed by

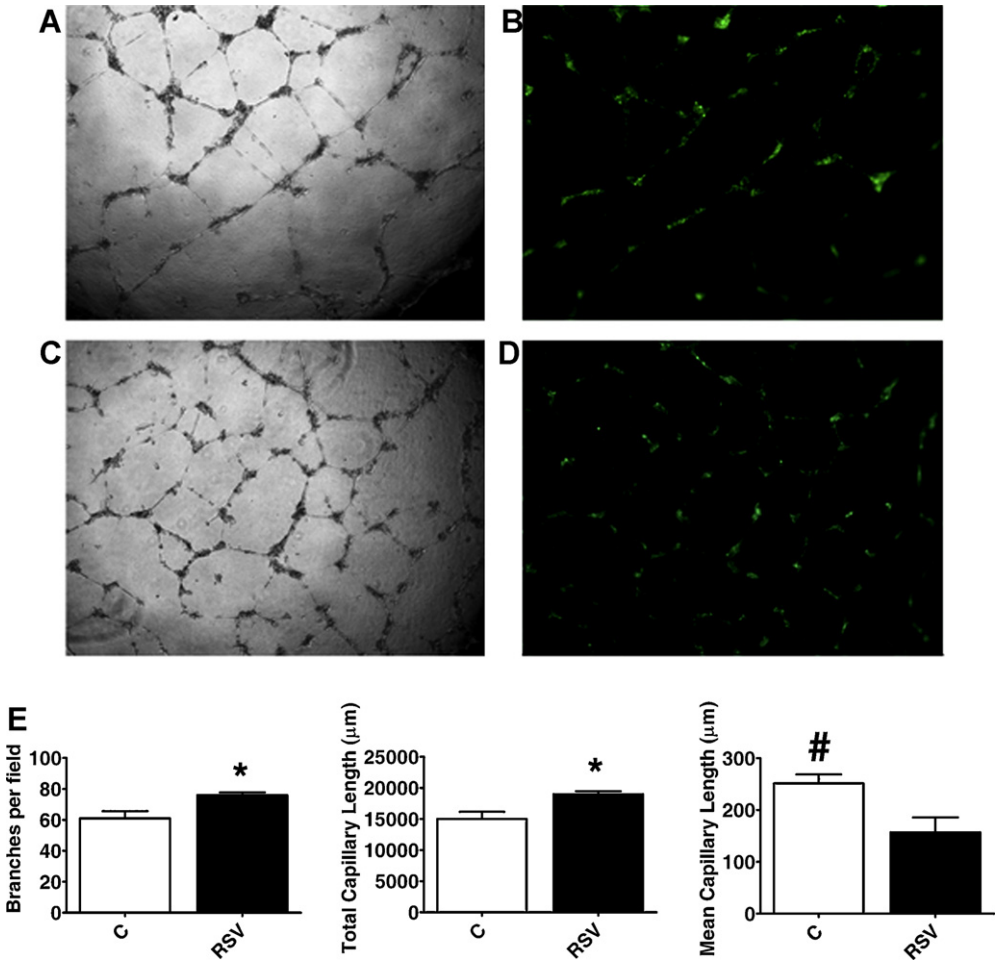

Fig. 5. RSV increases the ability of HUVECs to form capillary-like structures. (A–D) Before seeding, HUVECs were labeled with fluorescent viable dye green PKH2. Then, cells were seeded and treated with medium alone (A and B) or RSV 10 μM (C and D). Phase contrast (A and C) and fluorescent images (B and D) are representative of three experiments with similar results at 24 h (40×, original magnification). (E) Quantification and morphological assessment of tube-like structures in semisolid medium were performed by NIS-Elements D Nikon software (version 3.06). The parameters branches per field, the total capillary length, and the average capillary length were expressed as mean ± S.E.M. ($n = 3$). *Significantly different from untreated cells (C), #significantly different from treated cells (RSV); $P < 0.05$. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

the same inhibitor (Fig. 7A and B). RSV rapidly primed Akt phosphorylation within 30 min of treatment. Inhibition of PI3K, which is placed upstream to Akt signaling, suppressed RSV-mediated Akt phosphorylation (Fig. 7C).

4. Discussion

We provide evidence that RSV may behave as an effective activator of stem cell-mediated angiogenic potential. The RSV-induced increase in VEGF and HGF gene expression and release is noteworthy, since both factors modulate pro-angiogenic, pro-survival and antifibrotic responses *in vitro* and *in vivo* [11,18–21]. These soluble factors are involved in limiting the ischemic remodeling in infarcted hearts in a paracrine manner [22]. However, it is still debated whether common drugs may strictly modulate the paracrine angiogenic response in a receptor-dependent manner. VEGF binds with high affinity to tyrosine kinase receptors Flt-1 and KDR, activating distinct signal transduction pathways [23]. Among them, KDR plays a major role in the VEGF mediated angiogenesis [24]. Thus, a defect within the VEGF–KDR system could result in impaired physiologic coronary angiogenesis in ischemic myocardium [25]. Previous studies suggested that the activation of autocrine VEGF loop in selected microenvironment contributes to optimal differentiation of stem cells in a receptor-dependent manner [26] and rescues stem cell survival [27]. Therefore, it is conceivable that the RSV exposure of FMhMSCs may change the microenvironment and increase the KDR transcription, which primes a paracrine/autocrine circuitry enhancing capillary-like formation during stem cell differentiation. Thus, KDR activation should be the main target of statin-elicited angiogenic response. This intriguing hypothesis is supported by our findings: (i) under RSV treatment, cells embedded within capillary tubes continued to express VEGF, and (ii) a neutralizing VEGF antibody significantly reduced RSV-induced capillarogenesis. However, VEGF antagonism failed to completely abrogate the paracrine angiogenic response by stem cells, suggesting the involvement of other factors. Our findings in part support previous studies showing that KDR is activated in a VEGF-independent fashion by other soluble factors (*e.g.* cytokines) [28,29] and strengthen the needs of further dissecting the complexity of signals forming functionally mature vessels from stem cells. In fact, it is well known that VEGF mainly primes endothelial differentiation, but it is still not well defined which mechanisms underline the stem cell-derived mature vasculogenesis. In our study, persistence of VEGF expression within stem cells in the absence of endothelial markers highlights the notion that VEGF is an early signal and FMhMSCs may have been committed only in part along an endothelial fate by VEGF, rather acting as pericyte-like elements supporting tube formation, growth and functional maturity. This is further inferred by the observation that: (i) RSV also increased capillary-like density in cocultures of HUVECs and FMhMSCs, (ii) stem cells closely

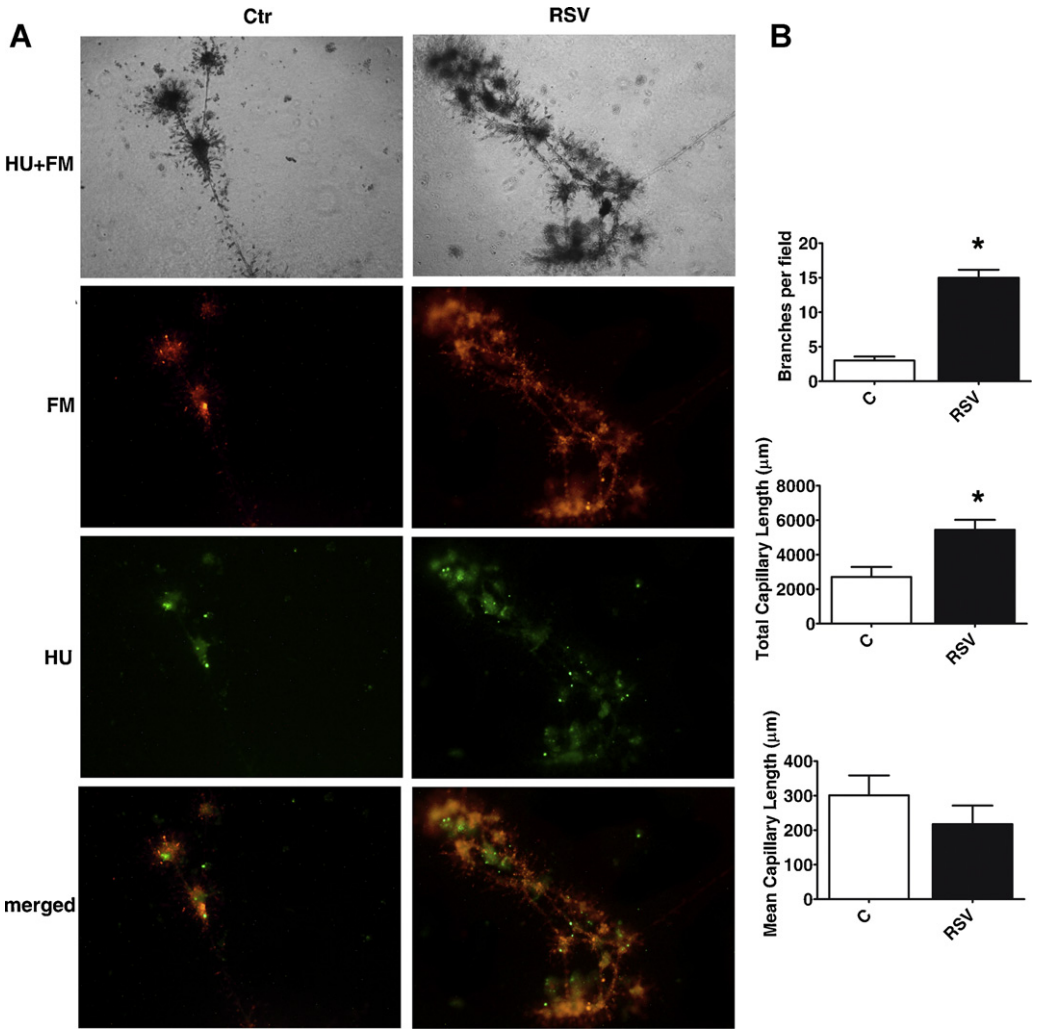

Fig. 6. The capillary network formed by FMhMSCs and HUVECs cocultured with RSV persisted up to one week. (A) Cocultures were performed with HUVECs and FMhMSCs in a ratio of 3:1, seeded in gel-precoated wells and treated with medium alone or RSV 10 μM. Before seeding, cells were labeled with fluorescent viable dyes, green PKH2 (HUVECs, HU) and red PKH26 (FMhMSCs, FM). All images are representative of three experiments with similar results at 7 days (40×, original magnification). (B) Quantification and morphological assessment of tube-like structures in semisolid medium were performed by NIS-Elements D Nikon software (version 3.06). The parameters branches per field, the total capillary length, and the average capillary length were expressed as mean ± S.E.M. ($n = 3$). *Significantly different from untreated cells (Ctr); $P < 0.05$. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

associated with and gathered around HUVECs along the assembled tubes, and (iii) the tubular network formed by RSV-exposed cocultures survived significantly longer and with higher organizational efficiency than that elicited by statin-treated HUVECs. To this end, the ability of RSV to enhance stem cell expression of PDGFRβ in HUVEC/FMhMSC cocultures may be worthy of consideration, due to the major role of PDGFBB/PDGFRβ axis in supporting pericyte/endothelial cell interplay and vasculature stability [17].

Recent study showed that activation of the PI3K/Akt-dependent signal pathway is involved in vasculogenesis of mouse embryonic stem cells [30]. Moreover, statins promote the activity of endothelial nitric oxide synthase, which is a hallmark of mature vasculature [31], through a PI3K/Akt-dependent pathway in endothelial cells in a dose-dependent manner [32].

We showed *in vitro* that the RSV-dependent angiogenesis was directly accompanied by the activation of a PI3K/Akt-dependent signal pathway in FMhMSCs in a time-dependent fashion.

FMhMSC exposure to RSV resulted in Akt phosphorylation, an event associated with signaling networks ensuring proper vessel growth and assembly [2]. Recent studies showed that Akt phosphorylation is augmented during the endothelial differentiation [33] and its inhibition significantly impairs the endothelial differentiation of mesenchymal stem cells [34]. It is well established that PI3K is the critical activator of Akt during angiogenesis in both physiological and pathological conditions [35], and this pathway is well preserved in some progenitor cells [36]. Previous study demonstrated that HMG-CoA reductase inhibitors increase the number of differentiated endothelial progenitor cells (EPCs), which is dose-dependently limited by PI3K-inhibition [37]. This study assessed that statins can increase the number of EPCs and improve angiogenic response through modulation of PI3K/Akt pathway, yet it was not clear whether the PI3K/Akt activation following statin exposure was critical for angiogenic response also in the absence of VEGF mediation. Moreover, the previous studies did not investigate the role of PI3K/Akt pathway during statin exposure of more undifferentiated cells such as FMhMSCs.

In our investigation, the block of spontaneous FMhMSC-derived vasculogenesis by a PI3K inhibitor demonstrates that Akt is a conserved player in the vasculogenic potential of human mesenchymal stem cells. The finding that inhibition of PI3K, an

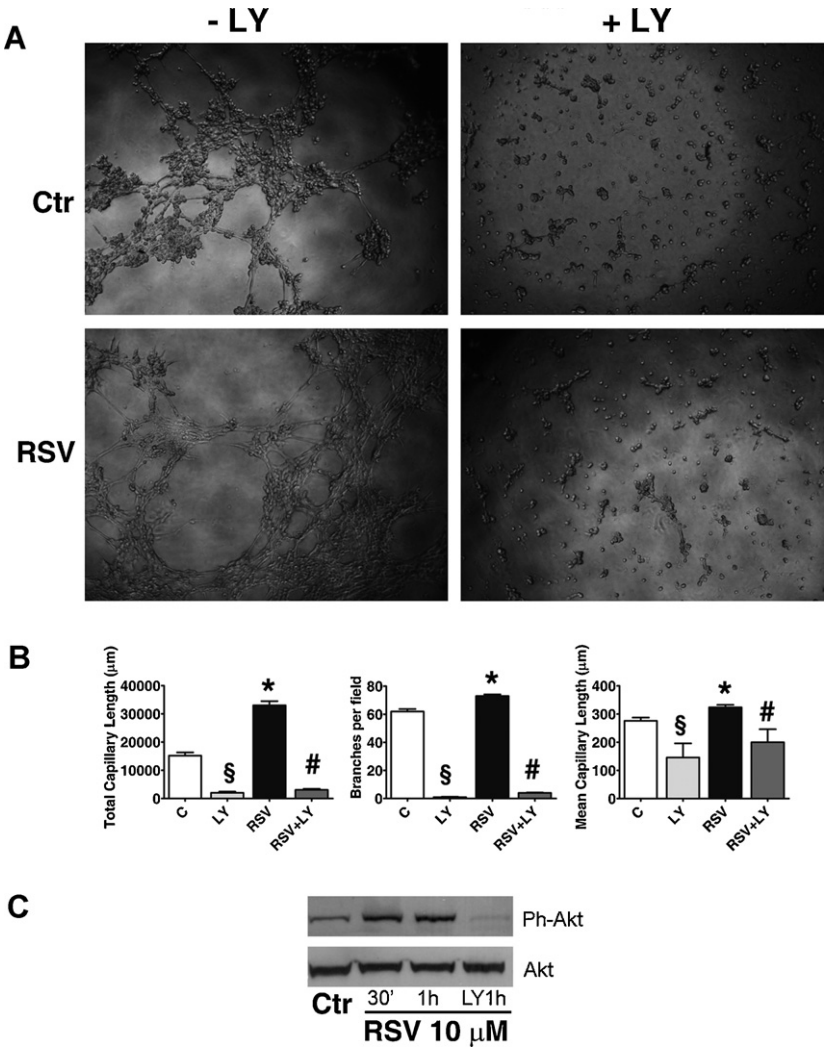

Fig. 7. RSV stimulates Akt phosphorylation in FMhMSCs. (A) Tube-like formation was assessed in FMhMSCs cultured in semisolid medium after 2 h of treatment with medium alone, 10 μM LY294002 (LY), 10 μM RSV, 10 μM LY294002 (LY) + 10 μM RSV (40×, original magnification). (B) Quantification and morphological assessment of tube-like structures in semisolid medium were performed by NIS-Elements D Nikon software (version 3.06). The parameters branches per field, the total capillary length, and the average capillary length were expressed as mean ± S.E.M. ($n = 3$). *Significantly different from untreated cells (C); #significantly different (reduction) from RSV; §significantly different (reduction) from untreated cells (C); $P < 0.05$. (C) Akt phosphorylation was assessed by immunoblot analysis in cell extracts from FMhMSCs cultured in the absence or presence of 10 μM RSV or 10 μM LY294002 (LY), for the indicated times. All images are representative of three experiments with similar results.

upstream Akt activator, abrogated both rosuvastatin-induced Akt patterning and *in vitro* capillarogenesis, indicates that the ability of statin to enhance vascular commitment in FMhMSCs is also related to PI3K-dependent activation of Akt. Further studies are on the way to deeply dissect the mechanistic bases of RSV action.

In conclusion, the results of the present study demonstrate that rosuvastatin can modulate *in vitro* the vasculogenesis by human mesenchymal stem cells also in a VEGF-independent manner. In this way, the PI3K/Akt pathway appears to play a major role for KDR-induced increase in angiogenic response following exposure to statin. The augmentation of vasculogenic response by pharmacological modulation of the signaling pathways, which regulate KDR-activation during FMhMSCs differentiation, may be a novel therapeutic concept to improve neovascularization in a VEGF-independent manner.

Conflict of interest

The authors declare no potential conflicts of interest.

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