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Silencing stromal interaction molecule 1 by RNA interference inhibits the proliferation and migration of endothelial progenitor cells

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ABSTRACT

Knockdown of stromal interaction molecule 1 (STIM1) significantly suppresses neointima hyperplasia after vascular injury. Endothelial progenitor cells (EPCs) are the major source of cells that respond to endothelium repair and contribute to re-endothelialization by reducing neointima formation after vascular injury. We hypothesized that the effect of STIM1 on neointima hyperplasia inhibition is mediated through its effect on the biological properties of EPCs. In this study, we investigated the effects of STIM1 on the proliferation and migration of EPCs and examined the effect of STIM1 knockdown using cultured rat bone marrow-derived EPCs. STIM1 was expressed in EPCs, and knockdown of STIM1 by adenoviral delivery of small interfering RNA (siRNA) significantly suppressed the proliferation and migration of EPCs. Furthermore, STIM1 knockdown decreased store-operated channel entry 48 h after transfection. Replenishment with recombinant human STIM1 reversed the effects of STIM1 knockdown. Our data suggest that the store-operated transient receptor potential canonical 1 channel is involved in regulating the biological properties of EPCs through STIM1. STIM1 is a potent regulator of cell proliferation and migration in rat EPCs and may play an important role in the biological properties of EPCs.

1. Introduction

Endothelial cell damage is an important pathophysiological step occurring during atherosclerosis and restenosis, and it follows percutaneous coronary interventions such as angioplasty and stenting [1,2]. Accelerated re-endothelialization effectively inhibits smooth muscle cell (SMC) migration, proliferation, and the resulting neointima formation; therefore, the study of re-endothelialization may be important in the prevention of the early stages of atherosclerosis and restenosis. The traditional paradigm of vascular repair is based on the proliferation and migration of pre-existing mature endothelial cells from the adjacent vasculature [3]. Recently, endothelial progenitor cells (EPCs), which can be mobilized and recruited into injured vessels, have become increasingly recognized to play a key role in the maintenance of vascular integrity and to act as "repair" cells in response to vascular injury [4,5]. However, the regulatory mechanisms of the biological properties of EPCs remain unclear, especially with respect to ion channels [6,7].

Calcium (Ca^{2+}) signaling is essential for a variety of functions, such as the regulation of proliferation, differentiation, and gene transcription. A major Ca^{2+} entry pathway component in non-excitable cells, including vascular cells, are store-operated Ca^{2+} channels (SOCs) [8]. SOCs are activated in response to receptor-mediated or passive depletion of intracellular Ca^{2+} stores to regulate many Ca^{2+}-dependent cellular functions. Major components of SOCs are stromal interaction molecule 1 (STIM1), ORAI and transient receptor potential (TRP) protein families [9]. SOCs are present in the salivary gland and endothelial and smooth muscle cells; however, the characteristics of SOCs in different cell types are quite distinct [8]. In non-excitable EPCs, the characteristics and components of SOCs are yet to be elucidated.

STIM1 is a sensor of SOCs [9] and plays an important role in the proliferation of endothelial cells and vascular SMCs (VSMCs) [10,11]. In a previous study, we showed that knockdown of STIM1 significantly suppressed neointimal hyperplasia after vascular injury. By reducing neointima formation after vascular injury, STIM1 may represent a novel therapeutic target in the prevention of restenosis after vascular intervention due to the central role of EPCs in the process of re-endothelialization [11,12]. Therefore, we hypothesized that STIM1 affects the biological properties of EPCs. Here, we present evidence that STIM1 has an essential role in the proliferation and migration of EPCs.

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2. Materials and methods

Materials and methods are described in detail in the Supplemental materials.

2.1. Adenoviral transduction

All animal procedures were approved by the Care of Experimental Animals Committee of the Third Military Medical University. Adenovirus (Ad) constructs expressing a non-silencing control (NSC) or rat STIM1 small interfering RNA (si/rSTIM1) or the human STIM1 (hSTIM1) cDNA were previously created in our laboratory [11]. EPCs were transduced with Ad-si/rSTIM1, Ad-si/rSTIM1 + Ad-hSTIM1, or NSC for 48 h and used in the experiments.

2.2. [^{3}H]-Thymidine incorporation and cell migration assay

DNA synthesis in EPCs was measured by [^{3}H]-thymidine incorporation. EPCs were seeded onto 24-well plates, and 1 μCi of [methyl-^{3}H]-thymidine was added to each well. Incorporated [^{3}H]-thymidine was precipitated with 10% trichloroacetic acid and counted with a liquid scintillation counter. EPC migration was determined using a modified Boyden chamber assay.

2.3. Intracellular free Ca^{2+} measurements

The EPCs placed in a special chamber were loaded with the Ca^{2+} indicator Fluo-3/AM (5 μM; Beyotime, Jiangsu, China). After loading, changes in intracellular Ca^{2+} ($[Ca^{2+}]_i$) levels in individual cells were measured using a digital imaging system equipped with a laser confocal scanning microscope (LSCM). The SOC-mediated influx of Ca^{2+} following stimulation with 1 μM thapsigargin (Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA) was estimated as described previously [11].

2.4. Statistical analysis

Data from at least three independent experiments are expressed as mean ± SD SPSS 11.0 software was used for statistical analysis. Data were analyzed in pairs (test and control) using *t*-tests, and *P*-values less than 0.05 were considered statistically significant.

3. Results

3.1. STIM1 is expressed in EPCs

After 4–7 days of culture, adherents EPCs were characterized by LSCM and flow cytometry. The majority of cells (91.3 ± 1.6%) stained positive for Dil-AcLDL and lectin (Supplementary Fig. 1) and expressed endothelial/stem cell markers including CD34 (77.32%), VEGFR-2 (91.24%) and CD133 (90.58%), but not CD45 (4.86%) (Fig. 1A).

To determine if STIM1 mRNA and protein are expressed in EPCs, semi-quantitative RT-PCR and western blotting were performed with total RNA and cytosolic protein extracts from cultured bone-marrow EPCs. STIM1 mRNA was present at fairly high levels as deduced by semi-quantitative RT-PCR in quiescent EPCs (Fig. 1B, left). Western blots revealed that the expected 84-kDa STIM1 protein was present in EPCs (Fig. 1B, right). Immunocytochemistry was used to further investigate the subcellular localization of STIM1 in EPCs, and STIM1 was found to be localized in the cytoplasm of EPCs (Fig. 1C). In addition, control cells that were not incubated with anti-STIM1 antibody exhibited no green fluorescence (data not shown). We conclude from these observations that STIM1 was expressed in the primary EPCs.

3.2. siRNA-induced STIM1 silencing inhibits the proliferation and migration of EPCs

Rat bone marrow-derived EPCs were cultured for *in vitro* experiments. An adenovirus construct expressing NSC, hSTIM1, or si/rSTIM1 was transfected into the EPCs. In cultured EPCs, the transfection efficiency of the adenovirus as calculated by GFP expression was 90.4 ± 2.1% (Supplementary Fig. 2). The STIM1 levels were detected by semi-quantitative RT-PCR and western blotting 48 h post-transduction. Transduction of EPCs with Ad-si/rSTIM1 at multiplicities of infection (MOI) of 10 and 20 plaque-forming units (pfu)/cell effectively decreased STIM1 mRNA and protein expression at 48 h post-transduction compared with the NSC cells. STIM1 mRNA decreased to 24.6 ± 1% at 10 pfu/cell MOI and 23.3 ± 1% at 20 pfu/cell MOI, whereas the protein levels decreased to 23.2 ± 1% and 21.6 ± 1%, respectively (Fig. 2A). However, the co-transfection of Ad-hSTIM1 (MOI 10 pfu/cell) with Ad-si/rSTIM1 (MOI 10 pfu/cell) restored the expression of STIM1 at both the mRNA level (99.5 ± 4%) and the protein level (93.9 ± 1.2%; Fig. 2A) compared with NSC-transfected cells. Interestingly, transfection of EPCs with Ad-si/rSTIM1 decreased the uptake of [^{3}H]-thymidine at 48 h after infection when compared with NSC (MOI 10 pfu/cell, 6990 ± 265 vs. 2373 ± 116; MOI 20 pfu/cell, 5701 ± 399 vs. 1194 ± 126; $n = 6$, $P < 0.05$; Fig. 2B). The co-transfection of Ad-hSTIM1 (MOI 10 pfu/cell) reversed the effects of STIM1 knockdown on [^{3}H]-thymidine uptake (2292 ± 227 vs. 6580 ± 232, $n = 6$, $P < 0.05$; Fig. 2B). These results demonstrate that knockdown of STIM1 suppressed the proliferation of EPCs *in vitro*.

Next, we used the modified Boyden chambers to assess the effects of STIM1 on EPC migration. As shown in Fig. 2C, transfection of EPCs with Ad-si/rSTIM1 decreased the number of migrating cells significantly 48 h after infection compared with NSC-transfected cells (MOI 10 pfu/cell, 11.66 ± 0.88 vs. 32.00 ± 1.15; MOI 20 pfu/cell, 9.66 ± 0.88 vs. 30.33 ± 1.76; $n = 5$, $P < 0.05$). On the si/rSTIM1 (MOI 10 pfu/cell) background, Ad-hSTIM1 (MOI 10 pfu/cell) re-expression reversed the effects of STIM1 knockdown in the number of migrating cells (14.00 ± 0.57 vs. 34.00 ± 1.15, $n = 5$, $P < 0.05$). These results demonstrate that knockdown of STIM1 inhibited the migration of EPCs *in vitro*.

Additionally, we evaluated the effect of si/rSTIM1 on SOCE, which was activated by the depletion of intracellular Ca^{2+} stores using 1 μM thapsigargin in the absence of extracellular Ca^{2+}, followed by the addition of extracellular Ca^{2+} to a final concentration of 2 mM. The thapsigargin-mediated SOCE may be attributed to the release of Ca^{2+} from the sarcoplasmic reticulum, and the infection of Ad-si/rSTIM1 at 48 h resulted in a marked decrease in SOCE. However, on the si/rSTIM1 background, the co-transfection of cells with Ad-hSTIM1 reversed the effects of STIM1 knockdown on intracellular Ca^{2+} in EPCs (Fig. 2D). These results demonstrate that SOCE may have a key role in the proliferation and migration of EPCs.

3.3. TRPC1–SOC cooperates with STIM1 to mediate the SOCE of EPCs

By semi-quantitative PCR and western blotting, we found that TRPC1 was expressed in EPCs (Fig. 3A). We also found that TRPC1 localized to the plasma membrane and at intracellular sites in EPCs by immunocytochemistry (Fig. 3B). Additionally, semi-quantitative RT-PCR and western blotting were used to address whether the knockdown or re-expression of STIM1 affected the expression of TRPC1. Semi-quantitative RT-PCR demonstrated that the transcript levels greatly decreased 48 h after si/rSTIM1 transfection (Fig. 3C). The TRPC1 protein level also significantly decreased 48 h after treatment with si/rSTIM1 (Fig. 3C). However, Ad-hSTIM1 re-expression reversed the effects of STIM1 knockdown on TRPC1. To determine if STIM1 associated with the TRPC1 channel in rat EPCs, a co-immunoprecipitation study was performed (Fig. 3D).

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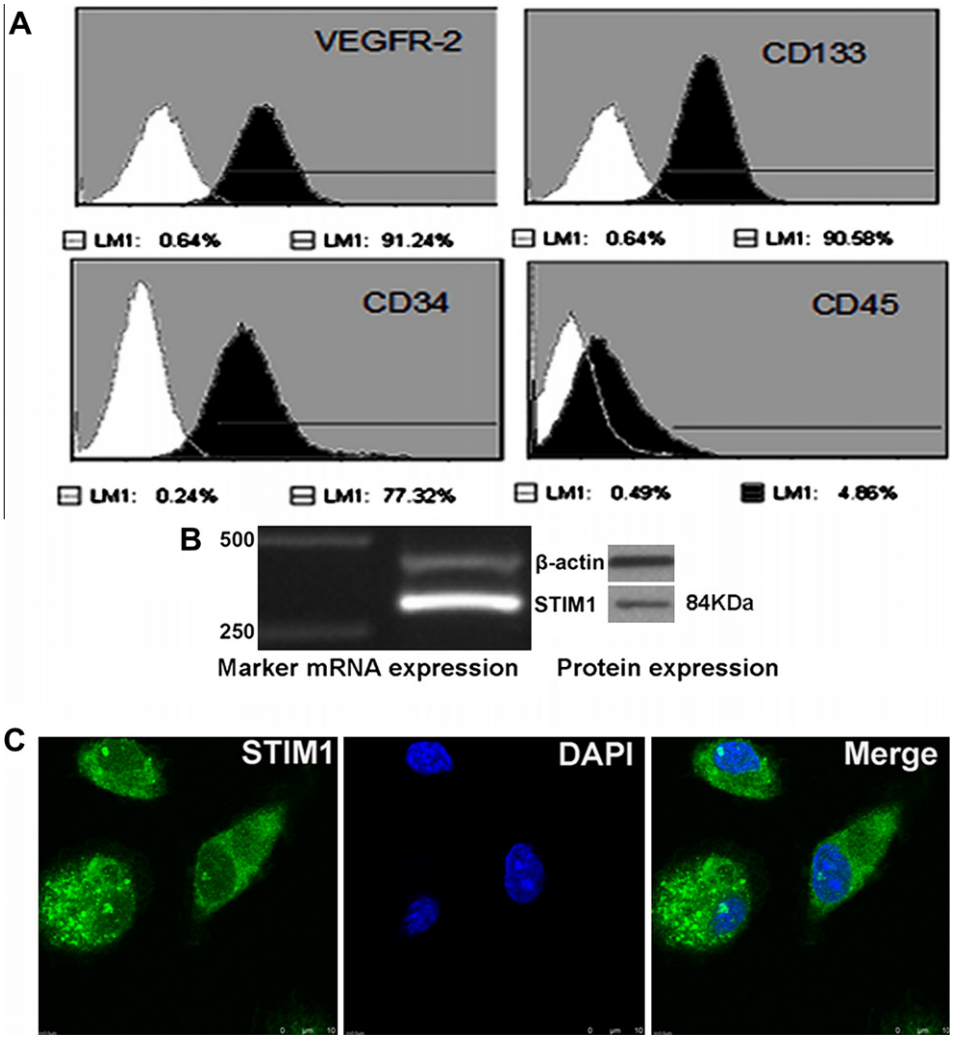

Fig. 1. Stromal interaction molecule 1 (STIM1) is expressed in primary EPCs. (A) Flow cytometry analysis of primary EPCs cultured for 4–7 days in low-glucose DMEM supplemented with 20% FCS and 10 ng/mL VEGF. Cells were labeled with fluorescent antibodies recognizing CD133, CD34, VEGFR-2 and CD45, which are shown as black areas. The white area on each box represents the corresponding negative control labeling, and the line denotes a positive gate. Numbers are the percentage of positive cells. (B) mRNA and protein expression of STIM1 in the EPCs. Semi-quantitative RT-PCR products from cultured rat bone marrow-derived EPCs were amplified using primers specific for rat STIM1 and β-actin (left). Data shown for β-actin and STIM1 are from two separate sections of the same gel. STIM1 protein and β-actin were detected in cultured rat bone marrow-derived EPCs using western blot analysis (right). STIM1 (84 kDa) was expressed in EPCs ($n = 3$). (C) Subcellular localization of STIM1 in EPCs. EPCs were fixed and incubated with primary anti-STIM1 polyclonal antibody and FITC-conjugated secondary antibodies. Green fluorescence (STIM1) was observed in the cytoplasm of EPCs, indicating that STIM1 localized to the cytoplasm. The scale bar represents 10 μm.

This assay showed that STIM1 co-precipitated with TRPC1, indicating a molecular complex formed between STIM1 proteins and TRPC1 channels in rat EPCs. Interestingly, more TRPC1 co-precipitated with STIM1 in cells subjected to store depletion via thapsigargin treatment as compared with the unstimulated cells. These data suggest that during store depletion, the interaction of STIM1 with TRPC1 is enhanced in rat EPCs. To further investigate how TRPC1 is regulated by STIM1, we tested IP3 levels by ELISA 48 h after NSC, hSTIM1 and/or si/rSTIM1 transfection. The results were not significantly different when the NSC and si/rSTIM1 treatments were compared, nor were they significantly different when the si/rSTIM1 and si/rSTIM1 + hSTIM1 groups were compared ($P > 0.05$; Fig. 3D). These findings indicate that the function of TRPC1–SOC incorporates the regulation of SOCE in EPCs via STIM1.

4. Discussion

The present results demonstrate that STIM1 is a powerful regulator of cell proliferation and migration in rat EPCs. STIM1 may have a critical role in the re-endothelialization process mediated by EPCs. We deduced this role of STIM1 based on several independent lines of evidence. First, STIM1 was expressed in quiescent EPCs and predominantly localized to the cytoplasm. Second, knockdown of endogenous STIM1 by adenoviral delivery of siRNA significantly suppressed the proliferation and migration of EPCs, which was reversed by STIM1 replenishment. Finally, TRPC1 was expressed in EPCs, and STIM1 knockdown inhibited SOCE and TRPC1 expression in EPCs *in vitro*, which was also reversed by STIM1 replenishment, and TRPC1–SOC was involved in Ca^{2+} influx through STIM1 regulation in EPCs. These observations suggest that STIM1 expression and function may be involved in the re-endothelialization process mediated by EPCs. Furthermore, Ca^{2+} influx through SOCs has an important function in EPC proliferation and migration.

STIM1, as a major component of SOCs, contains 685 amino acids that comprise an N-terminal EF hand, a transmembrane domain, and two coiled-coil domains that lie in the intracellular space. The EF hand domain is predicted to function in Ca^{2+} binding [9]. For the first time, our results demonstrate that STIM1 is expressed in the cytoplasm of EPCs. Accumulating evidence has revealed that

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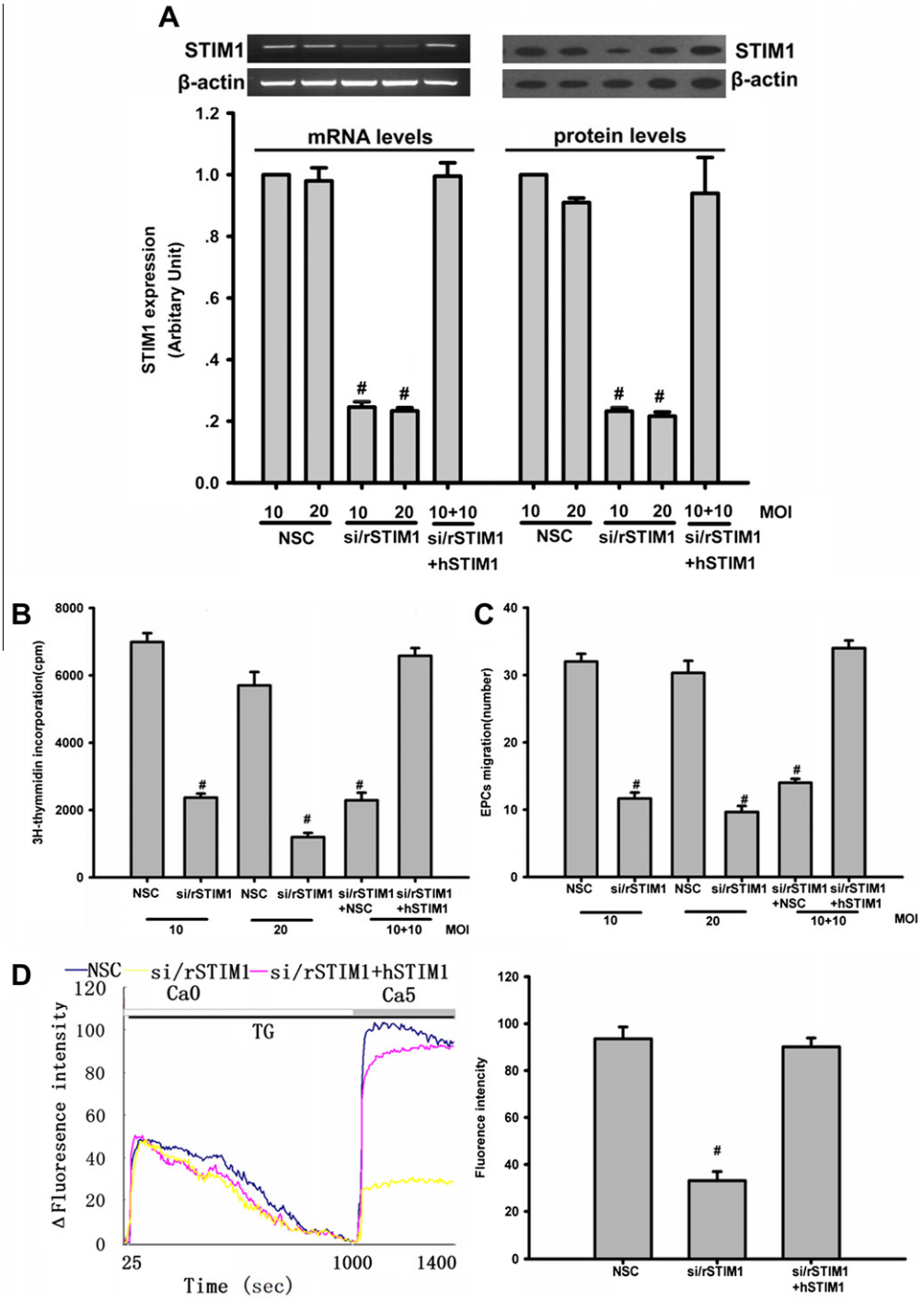

Fig. 2. siRNA-induced STIM1 silencing inhibits proliferation, migration and SOCE in EPCs. (A) Top: the expression of STIM1 mRNA and protein was studied using semi-quantitative RT-PCR and western blotting. Transduction of EPCs with increasing MOIs of Ad-si/rSTIM1 significantly degraded STIM1 mRNA and protein expression at 48 h post-transduction. On the rSTIM1-knockdown background, hSTIM1 re-expression reversed the effects of STIM1 knockdown on STIM1 mRNA and protein expression levels. Co-transfection of Ad-hSTIM1 (MOI 10 pfu/cell) with Ad-si/rSTIM1 (MOI 10 pfu/cell) restored the expression of mRNA and protein in EPCs ($n = 3$). Bottom: densitometric analysis of STIM1 mRNA and protein expression levels relative to β-actin were determined by the Quantity One program. The results are expressed as mean ± SEM. $^{\#}P < 0.05$ vs. NSC and si/rSTIM1 + hSTIM1. (B) [^{3}H]-Thymidine incorporation was used to assess the effect of STIM1 on EPC proliferation. Transfection of EPCs with Ad-si/rSTIM1 significantly decreased the uptake of [^{3}H]-thymidine by EPCs 48 h after infection. The co-transfection of cells with recombinant hSTIM1 reversed the effects of STIM1 knockdown on [^{3}H]-thymidine uptake. The data are presented as the mean ± SEM ($n = 3$). $^{\#}P < 0.05$ vs. NSC and si/rSTIM1 + hSTIM1. (C) Migration of EPCs was analyzed using the modified Boyden chamber assay. Transfection of EPCs with Ad-si/rSTIM1 decreased the number of migrating EPCs to a large extent, whereas transfection of Ad-hSTIM1 (MOI 10 pfu/cell) + Ad-si/rSTIM1 (MOI 10 pfu/cell) restored the effects of STIM1 knockdown. The data are presented as the mean ± SEM ($n = 5$). $^{\#}P < 0.05$ vs. NSC and si/rSTIM1 + hSTIM1. (D) We measured the SOC-mediated influx of Ca^{2+} following stimulation with 1 μM thapsigargin during the change from Ca^{2+}-free conditions to 2 mM Ca^{2+}. Left: The maximum amplitude in $[Ca^{2+}]_i$ caused by SOCE greatly decreased after infection with Ad-si/rSTIM1 (MOI 20 pfu/cell) 48 h after infection in EPCs compared with NSC. Co-transfection of cells with hSTIM1 reversed the effects of STIM1 knockdown on $[Ca^{2+}]_i$ in the SOCs of EPCs. Right: Statistical analysis of SOCE in different groups. The data are presented as the mean ± SEM ($n = 6$), $^{\#}P < 0.05$ vs. NSC and si/rSTIM1 + hSTIM1.

STIM1 is critical for normal development and that it functions in multiple cell types, such as by regulating T-cell development and SOC-mediated functions [13] involved in inherited thrombocytopenias [14]. Our previous study demonstrated that knockdown

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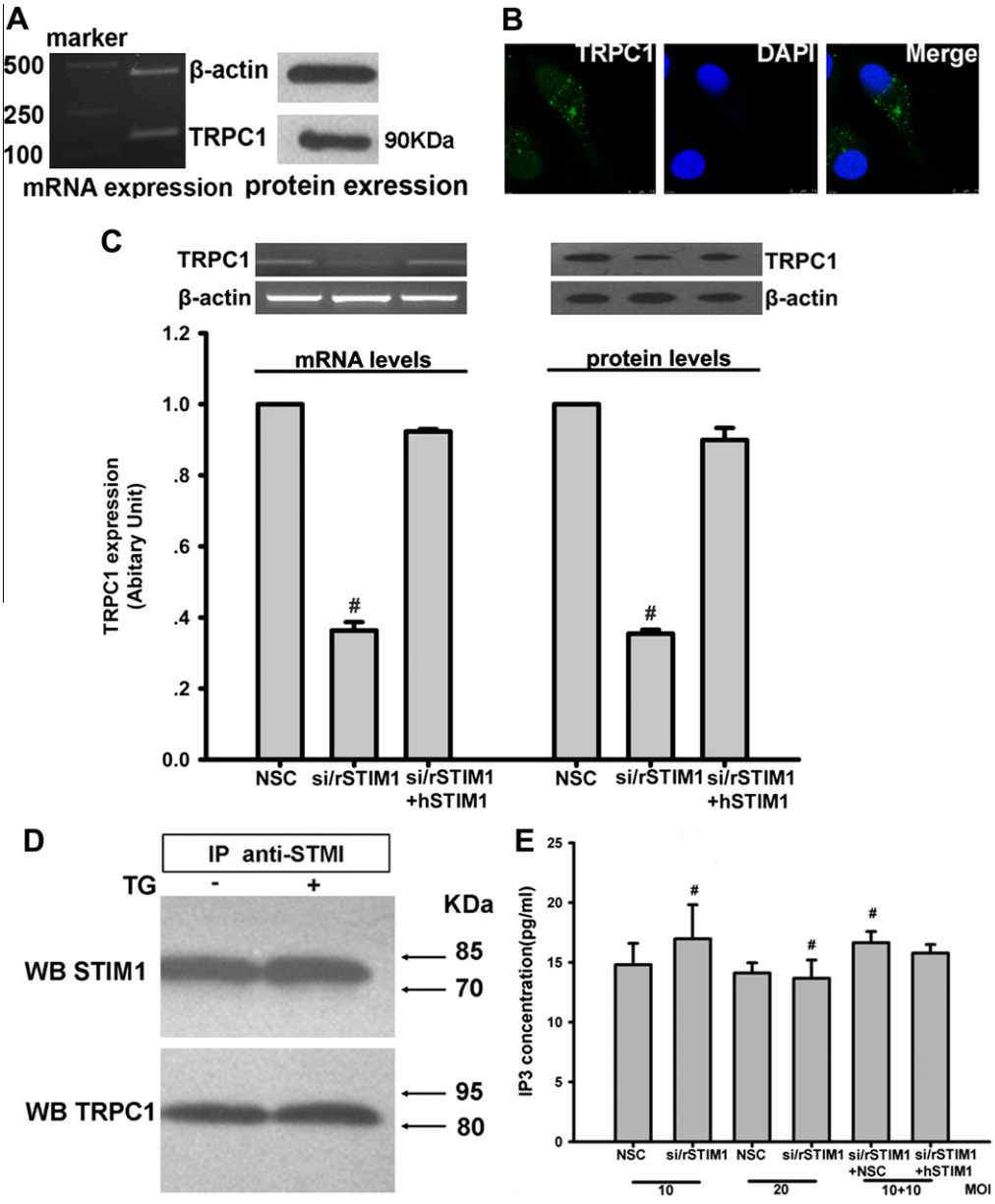

Fig. 3. TRPC1–SOCs cooperate with STIM1 in mediating SOCE of EPCs. (A) The expression of TRPC1 mRNA and protein (90 kDa) was detected in the EPCs using semi-quantitative RT-PCR and western blotting, respectively. (B) Cellular localization of TRPC1 in EPCs. EPCs were fixed and incubated with anti-TRPC1 polyclonal antibody and FITC-conjugated secondary antibodies. Green fluorescence (TRPC1) indicated that TRPC1 localized to the plasma membrane and the cytoplasm of EPCs. The scale bar represents 7.5 μm. (C) Top: The expression of TRPC1 mRNA and protein was studied using semi-quantitative RT-PCR and western blotting, respectively. Transduction of EPCs with Ad-si/rSTIM1 (MOI 20 pfu/cell) clearly decreased TRPC1 mRNA and protein expression at 48 h post-transduction. Transfection of Ad-hSTIM1 (MOI 10 pfu/cell) with Ad-si/rSTIM1 (MOI 10 pfu/cell) restored the expression of TRPC1 mRNA and protein. The data shown are representative of three different experiments. Bottom: densitometric analysis of TRPC1 mRNA and protein expression levels, normalized to β-actin, were determined by the Quantity One program. The results are expressed as mean ± SEM of three experiments. $^{\#}P < 0.05$ vs. NSC and si/rSTIM1 + hSTIM1. (D) The interaction of STIM1 with TRPC1 in EPCs. TRPC1 was co-precipitated using an anti-STIM1 antibody in thapsigargin-stimulated or non-stimulated EPCs. The interaction of STIM1 with TRPC1 was enhanced in rat EPCs after thapsigargin stimulation ($n = 3$). (E) Detection of IP3 concentration among different groups. Supernatants from EPCs transfected with NSC, hSTIM1 and/or si/rSTIM1 were collected at 48 h after transfection and subjected to ELISA, using IP3-specific ELISA kits. The results are expressed as the mean ± SD of six experiments ($n = 6$). $^{\#}P > 0.05$ vs. NSC and si/rSTIM1 + hSTIM1.

of STIM1 suppressed neointima hyperplasia [11], which was related to hepatocyte growth factor-induced EPC proliferation [15]. In this study, we further explored the effect STIM1 may exert on the proliferation and migration of EPCs. We silenced and re-expressed STIM1 in rat EPCs *in vitro*. [^{3}H]-Thymidine uptake and the number of migrating cells significantly decreased after STIM1 knockdown, suggesting that the proliferation and migration of EPCs was inhibited; however, these effects were reversed by STIM1 replenishment. Additionally, the influx of extracellular Ca^{2+} was inhibited by siRNA knockdown of endogenous STIM1 in the EPCs, which was also reversed by STIM1 replenishment. As reported [10,11], STIM1 knockdown inhibited the proliferation and migration of SMCs and endothelial cells. Our results suggest that STIM1 has a direct role in the regulation of migration and proliferation of EPCs by controlling the influx of extracellular Ca^{2+}.

STIM1 is an ER Ca^{2+} sensor that aggregates and relocates into clusters at the ER-plasma membrane junctions, where it functionally interacts with and activates plasma membrane TRPC channels that mediate SOCE under store depletion [16]. We demonstrated that TRPC1 was expressed and localized both at the plasma membrane and at intracellular sites in primary EPCs, similar to previous reports [17–19]. Furthermore, our results also indicate that si/

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rSTIM1 inhibited the expression of TRPC1, which was also reversed by STIM1 replenishment. TRPC1 has an NFAT-binding sequence in its promoter; therefore, up-regulation was facilitated through a feed-forward mechanism. Knocking down STIM1 halts this feed-forward mechanism and inhibits TRPC1 expression [20]. The above results suggest that TRPC1 participates in regulating the influx of extracellular Ca^{2+} mediated by STIM1 in the EPCs. However, the mechanism by which STIM1 regulates TRPC1 in EPCs is unknown. We demonstrated that STIM1 interacts with TRPC1 in rat EPCs by co-immunoprecipitation. Interestingly, more TRPC1 co-precipitated with STIM1 in EPCs under store depletion after treatment with thapsigargin compared with untreated cells. This finding suggests that the interaction of STIM1 with TRPC1 is enhanced during store depletion in rat EPCs. A similar phenomenon has been previously suggested for TRPC1–STIM1 modulation of SOC activity [21]. Many studies suggest that the activation of TRPC1 channels leads to increased Ca^{2+} entry from the extracellular space through two major mechanisms, receptor-operated Ca^{2+} influx (ROC) or SOC [22–25]. The first mechanism involves the direct activation of TRPC1 channels by their binding to the IP3 receptor in response to IP3, and these channels are referred to as TRPC1–ROC. In the second mechanism, TRPC1 is recruited by STIM1 to lipid raft domains in the surface membrane, resulting in a store-dependent mode of TRPC1 channel activation, which is referred to as TRPC1–SOC. Our observations show that STIM1 possibly regulates TRPC1–SOCs in EPCs. We detected the protein levels of IP3 by ELISA, but the results were not significantly different in NSC vs. si/rSTIM1 or in si/rSTIM1 vs. si/rSTIM1 + hSTIM1 treatments. This revealed that STIM1 knockdown did not affect IP3 protein expression; therefore, TRPC1–ROC was not mediated by STIM1 silencing. We hypothesized that the function of TRPC1–SOC involves the proliferation and migration of EPCs through STIM1. As recently revealed by other researchers, STIM1 hetero-multimerizes with TRPC channels and converts TRPC1 from a ROC to a SOC, which moves TRPC1 in and out of lipid rafts [19]. A previous report stated that gating of TRPC1 by STIM1 is mediated by intermolecular electrostatic interactions between the conserved, negatively charged aspartate residues in TRPC1 (^{639}DD^{640}) and the positively charged lysines of STIM1 (^{684}KK^{685}) [26]. The mechanisms by which STIM1 gates TRPC1–SOC need to be more extensively investigated in EPCs.

To date, the molecular basis of SOC is incompletely understood in EPCs, although it is clear that TRPC1 and STIM1 are essential to the biological functions of EPCs. Other possibilities have emerged concerning the mechanism of STIM1 activity. Our previous study [11] demonstrated that STIM1 knockdown induces suppression of VSMC proliferation in association with p21. Konstantinos et al. proposed the existence of a pathway connected to cAMP signaling through a process that involved STIM1 [27]. Therefore, further studies will be required to determine whether other signaling pathways, together with STIM1, are involved in regulating the biological functions of EPCs.

In summary, we showed that STIM1 is a critical regulator of EPC proliferation and migration and may be involved in the re-endothelialization process mediated by EPCs after vascular injury.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2010.06.088.

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