# Silence of STIM1 attenua=tes the proliferation and migration of EPCs after vascular injury and its mechanism

Xin−Peng Cong[1], Wen−Hui Wang[1], Xi Zhu[2], Can Jin[2], Liang Liu[2*], Xin−Min Li[2*]

[1]Affiliated Shanghai East Hospital of Tongji University,  Shanghai 200120, China
[2]Shanghai Zhoupu Hospital, Shanghai 201318, China

## ARTICLE INFO

## ABSTRACT

**Objective:** To investigate the effect of stromal interaction molecule 1(STIM1) knockdown on the proliferation and migration of endothelial progenitor cells (EPCs) after vascular injury and its mechanism. **Methods:** The rat bone marrow derived EPCs were divided into three groups: adenovirus negative control (group NSC), rat STIM1 adenovirus vector transfection group (group si/rSTIM1) and rat &human recombinant STIM1 adenovirus transfection group (group si/rSTIM1+hSTIM1). The STIM1 expressions in each group were detected by reverse transcription PCR after transfection; the cell proliferation was tested by [$^3$H] thymidine incorporation assay ($^3$H−TdR); Cell cycle was analyzed by flow cytometry; the cells′ migration activity was detected by Boyden assay; Calcium ion concentration was detected by using laser confocal method. **Results:** 48 h later after transfection, the expression level of STIM1 in si/rSTIM1 cells was significantly lower than that in NSC group (0.21±0.12 *vs* 1.01±0.01, $P<0.05$); EPCs that stayed in $G_1$ phase in si/rSTIM1 group [(93.31±0.24)%] were significantly more than that in NSC group [(78.03±0.34)%, $P<0.05$]; EPCs′ migration activity in si/rSTIM1 group (10.03±0.33) was significantly lower than that in NSC group: (32.11±0.54, $P<0.05$); EPCs calcium ion concentration changes in EPCs in si/rSTIM1 group (38.03±0.13) was significantly lower than that in NSC group  (98.11±0.34, $P<0.05$). While there was no significant difference between si/rSTIM1+hSTIM1 group and NSC group on the four indexes above. **Conclusions:** Silence of STIM1 attenuates EPCs proliferation and migration after vascular injury, by mediating the calcium ion concentration in EPCs.

## 1. Introduction

Vascular injury is one of the critical causes of atherosclerosis and stenosis after vascular surgery[1]. When a blood vessel is injured, the adjacent endothelial cells can repair it[2]. However, since the endothelial cells were highly differentiated, the repair capacity is limited. It was found recently that it is the EPCs that migrate and recruited into the injury, and achieve vascular repair finally after vascular injury[3,4]. Therefore, it is beneficial to promote its application in clinical practice by investigating the mechanism of the proliferation and migration activity of endothelial progenitor cells (EPCs). So far, it is known that the function of EPCs is regulated by many known factors, such as vascular endothelial growth factor, fibroblast growth factor, and estrogen level of apolipoprotein and CCNI[5−7]. However, there is not yet a thorough theory to explain the mechanism regulating EPCs′ proliferation, migration, especially in the view of ion channel. It has been confirmed that the calcium ion is involved with the regulation of cell proliferation, migration function. According to the latest report, calcium is also involved in the regulation of proliferation and migration of EPCs. We conclude that the function of EPCs may be subject to the regulation of the calcium ion channels (SOCs). SOCs are mainly composed of three families, among which stromal interaction molecule 1(STIM1) is the major family since STIM1 is the receptor of

SOCs. STIM1 plays a key role to accept, transfer internal and external information of the cells. It has been demonstrated that STIM1 silencing can attenuate the calcium influx of the vascular smooth muscle cells (SMCs), resulting in impaired muscle cell proliferation, thereby inhibiting neointimal thickening after vascular injury[8,9]. Meanwhile it is reported that knockdown of STIM1 could inhibit the proliferation and migration of EPCs induced by hepatocyte growth factor. Additionally, a new study has been found that silencing STIM1 inhibited the proliferation and migration of EPCs[10,11]. Nevertheless, it is still lack of satisfactory reports regarding how EPCs regulated by STIM1 and its mechanism. In this study, we try to explore the regulation and mechanism of STIM1 on EPCs through analyzing changes in cell behavior after the knockdown of STIM1 and detecting (calcium) changes on molecular level. The study provides a more thorough mechanism for modulating the biological properties of EPCs and indicates that STIM1 may be a new target for inducing vascular repair by EPCs.

## 2. Materials and methods

### 2.1. Isolation and characterization of EPCs

Isolation and identification of rat bone marrow stem cell process was described in Figure 1. Mononuclear cells were extracted from the bone marrow of femur and tibia by using concentration gradient centrifugation (400 g, 2 min). Then those cells were be cultured in DMEM−L selective medium. Finally EPCs were identified if the cell was positive for both Dil−AcLDL and fluorescein isothiocyanate−labeled lectin.

### 2.2. Adenovirus transfection

All of the animal experiments were approved by the Care of Experimental Animals Committee of our school. Three kinds of adenovirus NSC, rSTIM1, hSTIM1 (Ad) vector were constructed in our laboratory. The isolated EPCs were divided into three groups, and were transfected with Ad−si/rSTIM1, Ad−si/rSTIM1+hSTIM1 and NSC respectively, which were used in the subsequent experiments 48 h later.

### 2.3. Detection of STIM1 mRNA level using reverse transcript PCR.

The total RNA were extracted using Taco RNA Extraction Kit (provided by Harbin De Yuan Science and Technology Development Co Ltd), which was followed by cDNA synthesis with Clontech Kit (provided by Clonetech), PCR and electrophoresis. The primers used were as follows: STIMI1 upstream primer 5′−GACCCATTCCGATTC−3′, downstream primer 5′−GGCTATGAGAATGGGAAGA−3′; using rat B−actin as the reference. All primers were synthesized by Invitrogen company.

### 2.4. Cell proliferation off[3H] thymidine incorporation method

[3H] concentration was detected to measure the synthesis of DNA in EPCs. EPCs were cultured in serum free culture for 24 h, then EPCs in each group were seeded onto the 96−hole plate and cultured for 40 h. Each hole then was added with 1 $\mu$ Ci of[methyl −3H] thymidine. After another 8 h of culture, 3H−methyl thymidine was precipitated by 10% three trichloroacetic acid. With a liquid scintillation counter, eventually, cells with 3H were counted at 0, 24, 48 and 72 h, respectively, with a liquid scintillation counter. All groups of experiments were performed in triplicate.

### 2.5. Analysis of cell cycle with flow cytometry

EPCs were digested by the trypsin, following by 1 500 $g$ centrifugation for 5 min, PBS cleaning, finally, fixation overnight with 70% alcohol under 4 ℃. Then the nucleus was again with PBS, and was incubated with 0.1% sodium citrate in the chamber at room temperature (0.05 mg PI and 100 mg/mL RNase) for 30min. subsequently, the analysis of the fluorescence was done with the flow cytometry. The number of cells in each phase were analyzed with automatic analysis software (Cell−FIT software), which is specific for analyzing the cell cycle.

### 2.6. Cell migration detected with Boyden assay

48 hours after transfection, the EPCs ($2×10^5$/mL) were dissolved in DMEM−L without serum, and then they were put into the upper ward of modified Boyden chambers. Afterwards, the lower chamber was placed with 20% FCS and DMEM−L. After 6 h, micro membrane was removed and washed with PBS. And then cells in the lower chamber were fixed with ethanol and stained with hematoxylin for 3 min. The cells that migrated from the upper chamber to the lower chamber were counted with the micro device. The average value of each five randomly selected vision was calculated as the final value and all experimental groups were performed in triplicate.

### 2.7. Calcium ion concentration in EPCs tested by confocal laser

The EPCs were incubated in Fluo−3/AM ($Ca^{2+}$ indicator) solution for 30 min within the condition of 37 ℃ and 5% $CO_2$. After rinsing the cells with D−Hanks liquid, those cells were cultured under the same conditions for 30 min. Eventually the fluorescence intensity of Fluo−3/AM was recorded at room temperature with micro laser confocal microscope (LSCM) when the excitation wavelength was 488 nm. Changes in the intensity of fluorescence in EPCs demonstrated the changes of the concentration of $Ca^{2+}$ in EPCs. In addition, before the experiment was done 5 mM nifedipine was added to the EPCs to inhibit L− channel activity. All groups of experiments were performed in triplicate.

## 2.8. Statistical analysis

Data from at least three independent experiments were expressed as mean±SD and SPSS 11.0 software was used for statistical analysis. Data were analyzed in pairs (test and control) using $t$–tests. $P<0.05$ was considered to be statistically significant.

## 3. Results

### 3.1. Expression of STIM1 in the EPCs–the transfection efficiency of STIM1

After 4–7 days of culture, EPCs were identified by flow cytometry and LSCM. Cells that were positive both for Dil–AcLDL and lectin weighted for (91.4±1.67)% (Figure 2). And expressed endothelial/stem cell markers including CD133 (90.86%), VEGFR–2 (90.12%), CD34 (78.62%).

The EPCs were divided into three groups (10 MOI): Ad–si/rSTIM1, (5 MOI+5 MOI) Ad–si/rSTIM1+ hSTIM1 and (10 MOI) and NSC.48 h later. The expression of STIM1 in EPCs was detected by semi quantitative reverse transcription PCR. The results were shown at Figure 3: Compared with NSC group (1.01±0.01), STIM1 mRNA in Ad–si/rSTIM1 group (0.20±0.12) was decreased obviously ($P=0.000$, $t=5.734$). (5 MOI+5 MOI) mRNA level of STIM1 in Ad–si/rSTIM1+ hSTIM1 group was (0.95±0.13) reversed to the level of NSC group.

### 3.2. STIM1 silencing inhibited the proliferation and migration of EPCs and extended the Gl phase of the cell cycle

EPCs were divided into three groups: Ad–si/rSTIM1, Ad–si/rSTIM1+hSTIM1 and NSC. After 48 h, the EPCs′ proliferation was detected by of the intaked $^3$H–TdR. Cell cycle changes were observed by flow cytometry and migration of EPCs by using Boyden assay. $^3$H–TdR test results: $^3$H–TdR incorporation in EPCs of Ad–si/rSTIM1 group (2 439.03± 120.32) was significantly lower than that of the NSC group (6 890.43±256.43). The difference was statistically significant ($P=0.002$, $t=5.208$). While $^3$H–TdR incorporation in group of Ad–si/rSTIM1+hSTIM1 (6 782.00±267.36) was as much as that in NSC group ($P=0.12$), which indicated that the silencing of STIM1 suppressed EPCs′ proliferation ability.

Flow cytometry result: the number of cells that distributed in $G_1$ phase[(93.31±0.24)%] in the Ad–si/rSTIM1 transfection group was significantly higher than that in the NSC group (78.03±0.34)%. The difference was statistically significant ($P=0.0001$, $t=6.234$). Moreover, the number of cells in S phase (2.14±0.03) was significantly lower than that of NSC group (14.56±0.23), and the difference was statistically significant ($P=0.0001$, $t=7.120$). The number of cells in Ad–si/rSTIM1+hSTIM1 group distribution in $G_1$ and S phase[(79.00 ±0.43)%; (14.54±0.06)%] showed no significant difference ($P=0.2310$, $t=6.513$) compared with the NSC group.

Subsequently, modified Boyden chambers were used to assess the effects of STIM1 on EPC migration. The number of EPCs migration cells in Ad–si/rSTIM1 transfection group (10.03±0.33) was significantly lower than that in NSC group (32.11±0.54), and the difference was statistically significant ($P=0.003$, $t=5.423$), while the number of migration cells in Ad–si/rSTIM1+hSTIM1 group (33.21±0.65) was close to that of NSC group. These results demonstrated that knockdown of STIM1 inhibited the migration of EPCs in vitro.
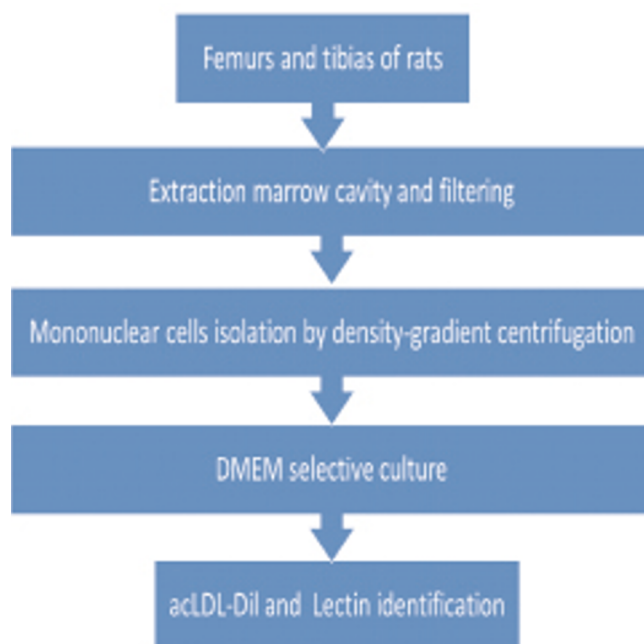


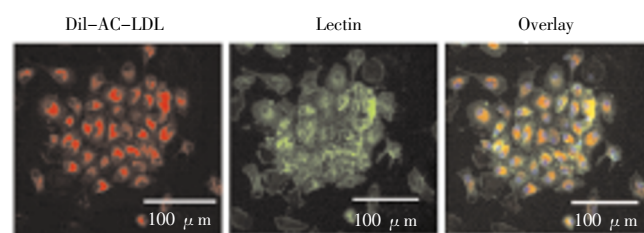**Figure 1.** Schematic of the isolation of EPCs.



**Figure 2.** Characterization of EPCs.
Note: EPCs showing uptake of acetylated LDL (red) and lectin (green) binding[(91.4±1.67)%], $n$= 3; 3 random fields per well.
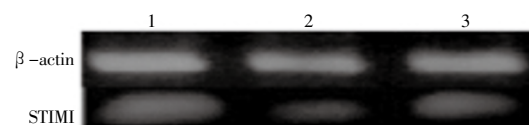


**Figure 3.** Expression of STIM1 in EPCs.
1: NSC group, 2: Ad–si/rSTIM1 group, 3: Ad–si/rSTIM1+ hSTIM1 group.
Note: the expression of STIM1 mRNA was studied using semi–quantitative RT–PCR. Transduction of EPCs with Ad–si/rSTIM1 significantly degraded STIM1 mRNA expression at 48 h post–transduction. On the rSTIM1–knockdown background, hSTIM1 re–expression reversed the effects of STIM1 knockdown on STIM1 mRNA expression levels. Co–transfection of Ad–hSTIM1 (MOI 5 pfu/cell) with Ad–si/rSTIM1 (MOI 5 pfu/cell) restored the expression of mRNA and protein in EPCs ($n$=3).

### 3.3. Change of cell calcium concentration

48 h after transfection, finally, we evaluated the effect of si/rSTIM1 on SOCE, which was activated by the depletion of intracellular $Ca^{2+}$ stores. Then extracellular $Ca^{2+}$ was added to EPCs culture to a concentration of 5 mmol/L. The changes of calcium ion concentration were detected by the laser confocal fluorescence. The results showed that: the change of $Ca^{2+}$ concentration in EPCs of Ad–si/rSTIM1 transfected group (38.03±0.13) was lower than that in NSC group (98.11±0.34), and the difference was statistically significant ($P$=0.000, $t$=15.007). Interestingly, there was no significant difference between the $Ca^{2+}$ change of Ad–si/rSTIM1+hSTIM1 group (97.75±0.25) and that of NSC group. These results demonstrated that 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. These results demonstrated that SOCE might play a key role in the proliferation and migration of EPCs.

## 4. Discussion

This study mainly evaluated the potential involvement of STIM1 in EPCs cell function and its mechanism through RNA interference silencing STIM1. According to this purpose, detections were performed as following described. Firstly, the proliferation of the EPCs was detected by testing the[³H] thymidine incorporation. Additionally, cell cycle of the EPCs of each group was further investigated by flow cytometry. Meanwhile, we studied the changes of EPCs migration activity after knockdown of STIM1 by using Boyden assay. Additionally, to figure out the mechanism that the STIM1's regulation on EPCs, we also detected the $Ca^{2+}$ concentration change with confocal laser scanning method after silencing STIM1 in EPCs. We hope to explore the effects of STIM1 on the proliferation and migration of EPCs cells, thus achieve more comprehensive understanding of EPCs on the repair of vascular injury to provide a new theoretical guidance for clinical.

### 4.1. Mechanism of the vascular endothelial injury

The bad repair of endothelial injury and vascular injury are two of the most common pathological and physiological basis of coronary heart disease and other vascular injury diseases[12,13]. It is a crucial step to prevent and cure the disease to repair the damaged endothelial, promoting re endothelialization. However, vascular injury repair process is complex and it is generally considered to be a variety of mechanisms involved in this process. Among them, intimal neovascularization slowly re–endothelialization is an important pathological mechanism of adverse cardiovascular remodeling[14,15]. Therefore, the prevention and cure of the vascular endothelial injury can be achieved by promoting re–endothelialization as soon as possible, which meanwhile inhibited the proliferation of the smooth muscle cells. So

far, research on EPCs has confirmed that the proliferation, migration, homing characteristics of EPCs doomed its function on repairing the vascular endothelial injury[16–18]. However, few thorough researches were reported on the regulation of STIM1 on EPCs and its mechanism, which has seriously hindered the clinical application of EPCs. Therefore, further study of the molecular mechanism of the biological behavior of EPCs is of great importance on providing a more detailed regulatory mechanism that may induce new drug targets.

### 4.2. Analysis of the EPCs proliferation, migration and $Ca^{2+}$ concentration after knockdown of STIM1

After silencing STIM1, the ³H–TdR intake of the EPCs was significantly decreased ($P$<0.05), the number of cells that stay in $G_1$ phase increased significantly compared with the control group ($P$<0.05), and the EPCs migration ability also significantly decreased ($P$<0.05). The results showed that knockdown of STIM1 can inhibit the proliferation and migration of EPCs, which is consistent with the Oh–Hora's report[19]. He reported that STIM1 regulated the normal development on various cells. When T cells were lack of STIM1, the secretion of cytokines and nuclear factor were significantly reduced, therefore the T cells growth was inhibited. Guo *et al*[20] also reported that inhibiting expression of STIM1 by RNA interference in smooth muscle cell resulted in lower $Ca^{2+}$ concentration than that of the control group. Lacking of $Ca^{2+}$, smooth muscle cells were arrested in the $G_0/G_1$ phase, which eventually led to the inhibition of the proliferation of smooth muscle cells, and also migration. In this study by silencing of STIM1 in EPCs, the proliferation and migration of EPCs were decreased and the expression of human STIM1 had reversed these effects. These results further demonstrated that, STIM1 is a critical regulatory factor in EPCs biological behavior.

In addition, the results also showed that the silence of STIM1 could reduce $Ca^{2+}$ internal flow, but the expression of human STIM1 could restore it. This suggested that STIM1 may regulate EPCs' proliferation and migration through the regulation of $Ca^{2+}$. As is known calcium is essential for the biological behavior of second messenger in cell proliferation, migration, differentiation[21–24]. EPCs calcium channel mainly is based on SOCs[25]. STIM is an important part of the SOCs. By inference, silencing of STIM1 is likely to inhibit the activity of the SOCs in EPCs, and then $Ca^{2+}$ influx was inhibited. With $Ca^{2+}$ as the second messenger pathway restricted, cell proliferation and migration pathways were surely to be affected.

### 4.3. Problems and prospects of the study

It is unclear yet that weather STIM1 is acting together with other factors to regulate the internal flow of $Ca^{2+}$. Konstantinos also speculate that STIM1 may involve with cAMP signal pathways[26–28]. Therefore, further studies are needed to understand more detailed regulatory mechanisms

which referring to other signaling pathways in combination with STIM1.

## 4.4. Conclusions

In summary, silencing of STIM1 can inhibit the proliferation and migration of EPCs after vascular injury. The effect of STIM1 on the regulation of EPCs mainly achieved through the regulation of calcium intracellular implementation. This is the first study to better elucidate the mechanism of EPCs repair after vascular endothelial injury, providing a theoretical basis for the clinical new therapeutic targets.

## Conflict of interest statement

We declare that we have no conflict of interest.

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