Original Research

Knockdown of stromal interaction molecule 1 attenuates hepatocyte growth factor-induced endothelial progenitor cell proliferation

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Abstract

Increased Ca^{2+} entry through store-operated Ca^{2+} channels (SOCCs) plays an essential role in the regulation of hepatocyte growth factor (HGF)-induced cell proliferation. Stromal interaction molecule 1 (STIM1) is thought to transmit endoplasmic reticulum (ER) Ca^{2+} store depletion signals to the plasma membrane (PM), causing the opening of SOCCs in the PM. However, the relationship between HGF and STIM1 in endothelial progenitor cell (EPC) proliferation remains uncharacterized. The objective of this study was to evaluate the potential involvement of STIM1 in HGF-induced EPC proliferation. For this purpose, we used cultured rat bone marrow-derived EPCs and found that HGF-induced EPC proliferation at low concentrations. Store-operated Ca^{2+} entry (SOCE) was elevated in HGF-treated EPCs, and the SOCC inhibitors 2-aminoethoxydiphenyl borate (2-APB) and BTP-2 inhibited the HGF-induced proliferation response. Moreover, STIM1 mRNA and protein expression levels were increased in response to HGF stimulation and knockdown of STMI1 decreased SOCE and prevented HGF-induced EPC proliferation. In conclusion, our data suggest that HGF-induced EPC proliferation is mediated partly via activation of STIM1.

Keywords: stromal interaction molecule 1, hepatocyte growth factor, endothelial progenitor cell, proliferation

Experimental Biology and Medicine 2010; **235**: 317–325. DOI: 10.1258/ebm.2009.009237

Introduction

Endothelial damage is a major contributing factor to atherosclerosis and postangioplasty re-stenosis.[1,2] Although endothelial cell (EC) repair mechanisms are considered to be mediated by adjacent mature ECs,[3] vascular ECs only regenerate moderately in physiological conditions.[4] Thus, if the endothelium persists under several risk factors, such as smoking, hypercholesterolemia, hyperglycemia or hypertension, local endothelial repair is incomplete and defective.[5] More recently, circulating, bone marrow-derived endothelial progenitor cells (EPCs) have been found to play an important role in endothelial regeneration after vascular injury.[6–9] The function of EPCs can be regulated by many factors such as vascular endothelial growth factor (VEGF), fibroblast growth factor and estrogen.[10–13] Moreover, hepatocyte growth factor (HGF) has been demonstrated to promote the proliferation and survival of human CD34+ hematopoietic progenitors[14] and induce angiogenesis in injured lungs through mobilizing EPCs from bone marrow.[15]

HGF, initially identified as a powerful stimulatory agent for primary cultured hepatocytes, is a multifunctional cytokine that posses a variety of biological activities.[16–18] It regulates growth, motility and morphogenesis of various cell types.[19–22] Serum HGF levels are elevated in response to hypertension,[23] acute myocardial infarction,[24] diabetes mellitus with hypertensive complications,[25] peripheral arterial occlusive diseases[26] and carotid atherosclerosis.[27] Several lines of evidence have implied that HGF is expressed locally in the arterial wall after injury and promotes the survival and proliferation of EPCs and ECs, suggesting that HGF is involved in arterial repair and atherogenesis.[22,28,29] HGF promotes cell growth by stimulating the tyrosine kinase activity of the HGF-specific receptor encoded by the c-Met proto-oncogene.[30,31] Following HGF activation of c-Met, phospholipase C-γ (PLC-γ) is phosphorylated.[32] Phosphorylation of PLC-γ results in IP3-mediated Ca^{2+} store depletion and subsequent Ca^{2+} influx across the plasma membrane (PM). Endoplasmic reticulum (ER)

Ca^{2+} depletion activates PM-localized Ca^{2+} influx channels known as store-operated Ca^{2+} channels (SOCCs).[33] In various non-excitable cells, store-operated Ca^{2+} entry (SOCE) is considered a common and ubiquitous mechanism of regulating Ca^{2+} influx into cells.[34,35] Ca^{2+} signaling through SOCCs activates the expression of specific genes necessary for the regulation of cell growth and cell division.[36–38]

SOCC activation relies exclusively on store depletion. Thus, the ER must communicate with SOCCs within the plasma to signal Ca^{2+} store depletion. After years of research, a membrane-spanning protein named stromal interaction molecule 1 (STIM1) was revealed as the key molecule required for the activation of SOCCs.[39,40] Although STIM1 is likely to be the 'sensor' of Ca^{2+} within ER Ca^{2+} stores and disperses on the ER membrane in quiescence, Ca^{2+} store depletion results in the rapid translocation of STIM1 into puncta close to the PM.[40] This relocalization of the protein is thought to act as the store depletion signal to the SOCCs in the PM, subsequently leading to the opening of SOCCs.

However, the role of STIM1 in EPCs is not well understood. Moreover, it is not known whether STIM1 is involved in HGF-induced EPC growth, which is an important event for the vascular repair process. Therefore, we used cultured rat bone marrow-derived EPCs transfected with STIM1 siRNA and investigated whether STIM1 had a role in HGF-induced EPC proliferation.

Material and methods

Study approval

All procedures were carried out in accordance with the guidelines of the Animal Research Committee of the Third Military Medical University, Chongqing, China.

Animals

Sprague–Dawley rats (1–2 months old, 180–200 g weight) were obtained from the Experimental Animal Center of the Third Military Medical University.

EPCs isolation and characterization

EPCs were obtained and characterized according to previously described techniques in our laboratory.[11,41] Briefly, total bone marrow-derived mononuclear cells (BMNCs) were isolated by density gradient centrifugation (Lymphoprep 1.083, Tianjing, China) at 400 *g* for 20 min. After three washes, cells were plated on gelatin-coated cell culture flasks and propagated in low glucose Dulbecco's modified Eagle's medium (Gibco, California, USA) supplemented with 20% fetal calf serum (Gibco), 10 ng/mL recombinant human VEGF (R&D Systems, Inc, Minnesota, USA), 100 IU/mL penicillin and 100 μg/mL streptomycin. Cells were maintained at 37°C in a humidified atmosphere (95% air and 5% CO_2). Twenty-four hours later, non-adherent cells were transferred to a new flask to remove adherent hematopoietic cells and mature ECs. After another three or four days in culture, unattached cells were removed and adherent cells were continuously cultured and used for further experiments. Media were refreshed every three days. For characterization, differentiating cells were incubated with acLDL-DiI (10 mg/mL, Invitrogen, California, USA) for 4 h, fixed with 4% paraformaldehyde and then incubated with fluorescein isothiocyanate-labeled lectin (Ulex europaeus agglutinin-1 [UEA-1], 10 mg/mL, Sigma-Aldrich, Missouri, USA) for 1 h. Dual-stained cells positive for both acLDL-DiI and UEA-1 were identified as EPCs. Additionally, flow cytometry (FACS) analysis was performed using antibodies against rat CD133, CD34, VEGFR-2 and the corresponding isotype control antibodies (Bios, Beijing, China).

Cell proliferation studies

EPCs were pre-conditioned in serum/VEGF-free media for 24 h to minimize the influence by serum or cytokines. [^{3}H]thymidine incorporation was used to measure the mitogenic response of EPCs as previously described.[42] Briefly, EPCs were trypsinized from the cultures and replaced into fibronectin-coated 96-well plates (2×10^4 cells/mL). Cells were treated with various concentrations of HGF (5, 10 and 20 ng/mL). Each group contained eight parallel wells. After treatments, cells were added with 1 μCi/mL [^{3}H]thymidine and incubated for an additional 6 h and then washed three times with 10% TCA for 20 min to remove unincorporated [^{3}H]thymidine. Next, cells were incubated with 0.5 mol/L NaOH for 30 min at 37°C and the incorporation of [^{3}H]thymidine was determined by a liquid scintillation counter.

RNA isolation and quantitative realtime polymerase chain reaction

Total RNA was extracted from EPCs using the TRIzol (Invitrogen) reagent. cDNA was synthesized using oligo (dT) and M-MLV reverse transcriptase (Takara, Dalian, China) according to the manufacturer's protocol. For quantitative realtime polymerase chain reaction (RT-PCR) analyses, the ABI PRISM 7000 Sequence Detection System (Applied Biosystems, Foster City, CA, USA) and SYBR Green PCR Master Mix (Takara) were used with the following primers: STIM1: 5′ ccagacacaccatctccagtt 3′ (forward) and 5′ tagagccattatcctcctcagc 3′ (reverse); glyceraldehyde 3-phosphate dehydrogenase (GAPDH): 5′ accacagtccatgccatcac 3′ (forward) and 5′ tccaccaccctgttgctgta 3′ (reverse). All primers were synthesized by Takara and were of high purity and salt-free. The PCR parameters used for STIM1 amplification were: 30 s of denaturation at 94°C, 30 s of annealing at 59°C and 1.5 min of extension at 72°C. The parameters for GAPDH amplification were: 30 s of denaturation at 94°C, 30 s of annealing at 57°C and 1.5 min of extension at 72°C. SYBR Green I fluorescence emission was monitored after each cycle. The results were expressed as mean $\pm$ SD for the relative expression levels. A melting curve analysis was performed after each run to verify that the PCR products were the same, and that primer dimers were absent.

Western blot analysis

After treatment, cells were lysed in lysis buffer (50 mmol/L Tris, pH 8.0, 150 mmol/L NaCl, 5 mmol/L EDTA, 5% glycerol, 1% Triton X-100, 25 mmol/L NaF, 2 mmol/L Na_2VO_4 and 10 μg/mL of each aprotinin, leupeptin and pepstatin). The cell lysates were further centrifuged at 14,000 *g* for 10 min at 4°C to pellet insoluble material. Protein concentrations of cell extracts were measured using the Bradford method. Equal amounts of protein (100 μg) were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (10% polyacrylamide gel) and electrophoretically transferred onto a polyvinylidene fluoride membrane. The membranes were blocked with 5% non-fat milk solution in tris-buffered saline with 0.5% Tween-20 and incubated in STIM1 primary antibody purchased from BD (NJ, USA), at 4°C for 4 h. Finally, membranes were incubated with anti-rabbit horseradish peroxidase-conjugated IgG for 1 h. Protein bands were visualized by chemiluminescent detection (Amersham Biosciences, Uppsala, Sweden) and quantified by a gel image analysis system. Anti-GAPDH monoclonal antibody (Cell Signaling, Boston, USA) was used to test for equal protein loading.

Fluorescence cell imaging

EPCs placed in a special chamber were loaded with the Ca^{2+} indicator Fluo-3/AM (5 μmol/L; Beyotime, Jiangshu, China) and continuously cultured at 37°C for 30 min in a 5% CO_2 incubator. The labeled cells were washed with D-Hanks to remove the unloaded Fluo-3/AM. After cells were loaded with the fluorescence probe, changes in intracellular Ca^{2+} ($[Ca^{2+}]_i$) of individual cells were measured using a digital imaging system equipped with a laser confocal scanning microscope (Olympus, FV-300, Japan) with an excitation wavelength of 488 nm. The SOCC-mediated influx of Ca^{2+} following stimulation with 2 μmol/L thapsigargin (an ER Ca^{2+} ATPase inhibitor, TG) during the change from a Ca^{2+}-free condition to 5 mmol/L Ca^{2+} was estimated as described.[36] Additionally, 5 μmol/L nifedipine was added to the solution to block voltage-dependent L-type channel activity.

Knockdown of STIM1 by siRNA

Ad-si/STIM1 and non-silencing control (NSC) were a gift from Dr Guo.[43] The selected siRNA duplex sequences specifically targeted rat STIM1 (rSTIM1, NM_001108496), and showed no homology to any other sequences by a blast search. The two sequences used in this study are (i) start nucleotide 935, GCAUGGAAGGCAUCAGAAGUG UAUA and (ii) start nucleotide 970, GGAUGAGGUGAUA CAGUGGCUGAUU. An NSC sequence was designed according to the sequence of a negative control.

Statistical analysis

All values from our experiments were expressed as mean $\pm$ SD of at least three independent experiments. SPSS 12 software was used for statistical analysis. Comparisons between groups were analyzed by two-tailed Student's *t*-test or analysis of variance. $P < 0.05$ was taken to be statistically significant.

Results

EPC isolation and characterization

Freshly plated BMNCs possessed a rounded shape. After three to five days in culture, adherent cells formed cluster- or cord-like structures. The number of EPCs continually increased and the cells elongated to possess a spindle shape over the next seven days. EPC colony-forming units became obvious after 10 days in culture. EPCs cultured 14 days formed a tubular-like appearance and grew to confluence with a cobblestone shape at 21 days (Figure 1a). For further characterization, after seven days in culture, attached cells were analyzed with immunofluorescence and FACS. Cells which took up Dil-Ac-LDL, bound lectin, or expressed endothelial/stem cell markers, including CD133, CD34 and VEGFR-2 were EPCs (Figure 1b).

Effects of HGF on EPC proliferation

The effect of HGF on EPC proliferation was examined using [^{3}H]thymidine incorporation after 48 h exposure to different quantities of HGF (range 5–20 ng/mL). The proliferation effect was strongly dose-dependent and increased in HGF-treated EPCs but not in control cells (Figure 2). Thus, our results suggest that HGF accelerates EPC proliferation. Because the maximum proliferation effect occurred even at a concentration as low as 10 ng/mL, we used 10 ng/mL HGF as the proliferation stimulus in the subsequent experiments.

Increased SOCE in HGF-treated EPCs

In order to test whether SOCE plays a role in HGF-induced EPC proliferation, the long-term effect of HGF on SOCC activation in EPCs was assessed after being treated with HGF for 48 h. Changes in $[Ca^{2+}]_i$ in individual cells were estimated and compared before and after store depletion between control and HGF-treated cells using fluorescence microscopy. As show in Figure 3a, Ca^{2+} stores were initially depleted by inhibition of ER Ca-ATPase activity with 2 μmol/L TG in the absence of extracellular Ca^{2+}. After addition of TG, transient cytosolic Ca^{2+} release from the ER occurred, confirming the emptying of ER Ca^{2+} stores. Following restoration of extracellular Ca^{2+} ($CaCl_2$; 5 mmol/L), a rapid increase of Ca^{2+} influx was observed, which was due to SOCC activation. The maximum amplitude in $[Ca^{2+}]_i$ caused by SOCE was greatly increased in HGF-treated EPCs compared with control cells (approximately 150% versus control) (Figure 3b).

To assess further the dependence of SOCE on HGF-induced EPC proliferation, we investigated the effects of SOCC inhibitors on EPC proliferation. As shown in Figure 3c, this inhibition was evaluated by [^{3}H]thymidine incorporation after 12 h of pre-incubation with SOCC inhibitors (2-aminoethoxydiphenyl borate [2-APB], 100 μmol/L and BTP-2, 10 μmol/L). Both BTP-2 and 2-APB, compared with the control- and HGF-treated groups, inhibited the proliferation response significantly. $^*P < 0.05$ versus control group and $^{**}P < 0.05$ versus HGF-treated group. These common effects clearly

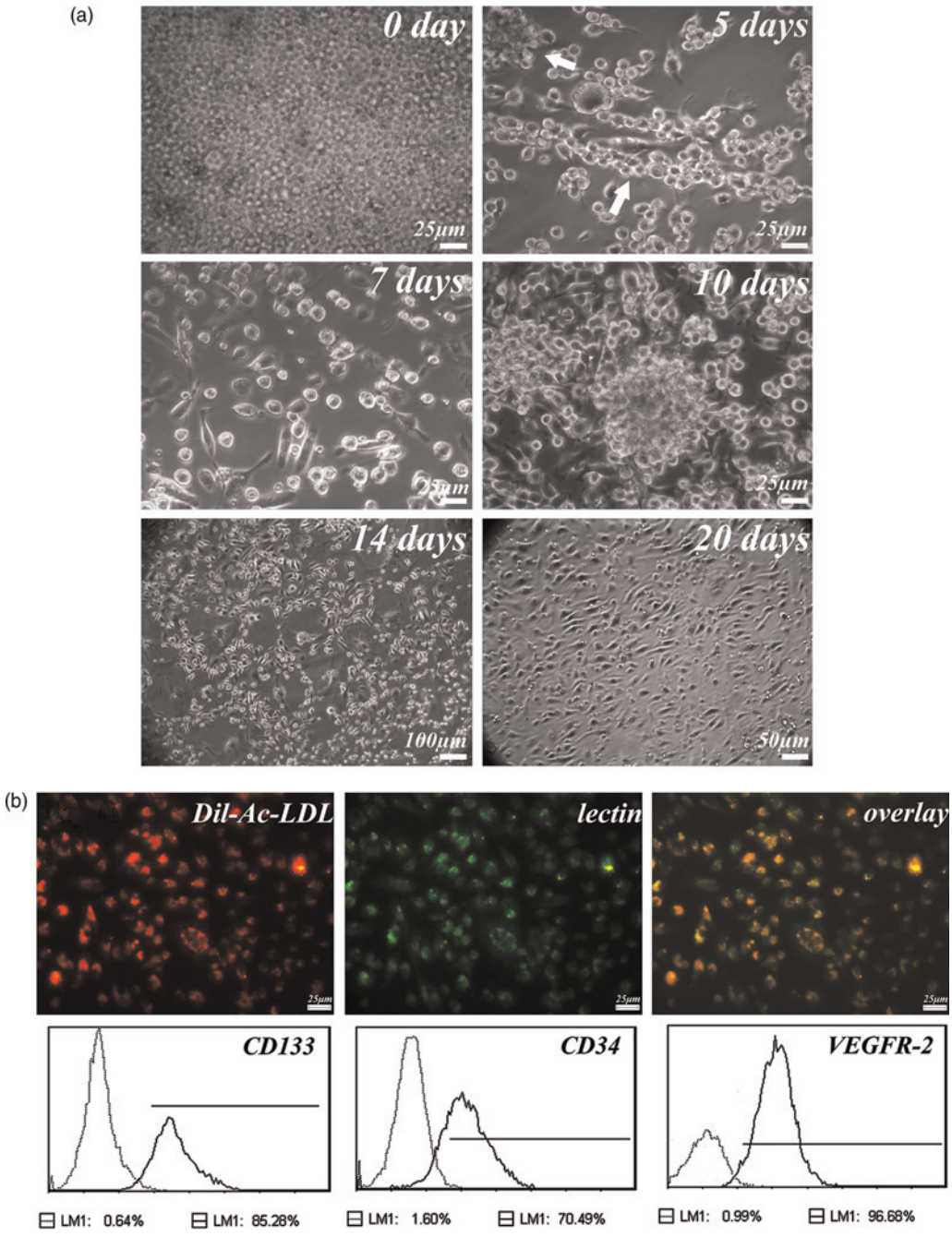

Figure 1 Endothelial progenitor cell (EPC) isolation and characterization. (a) Freshly plated bone marrow-derived mononuclear cells (BMNCs) possessed a rounded shape. After three days in culture, EPCs formed cluster- or cord-like structures. EPC number continuously increased and the cells acquired a spindle shape over the next seven days. EPC colony-forming units became obvious after 10 days in culture. Fourteen-day cultured EPCs formed tubular-like structures. After 20 days, EPCs grew to confluence, with a cobblestone-like appearance. (b) EPCs showed uptake of acetylated LDL and lectin binding. Cells labeled with fluorescent antibodies CD133, CD34 and VEGFR-2 are shown in the right areas of each box. The left area in each box represents corresponding negative control labeling. The line represents the positive gate. The numbers are the percentage of positive cells

suggest that SOCE is needed for HGF-induced EPC proliferation.

Expression of STIM1 in HGF-treated EPCs

To test whether HGF affects the expression of STIM1, quantitative realtime PCR and Western blotting were performed after HGF stimulation (Figure 4a). STIM1 mRNA expression level was very low at resting state, but greatly increased after 12 h of HGF treatment (2.37-fold compared with the 0 h) and reached a maximum of 3.86-fold greater than 0 h level after 24 h of HGF treatment. All values for STIM1 mRNA expression have been normalized to the value at 0 h. Western blot analysis indicated that the STIM1 protein levels increased significantly after 24 and 48 h of treatment with HGF (Figure 4b). These results indicate that STIM1 expression is upregulated in EPCs after HGF stimulation.

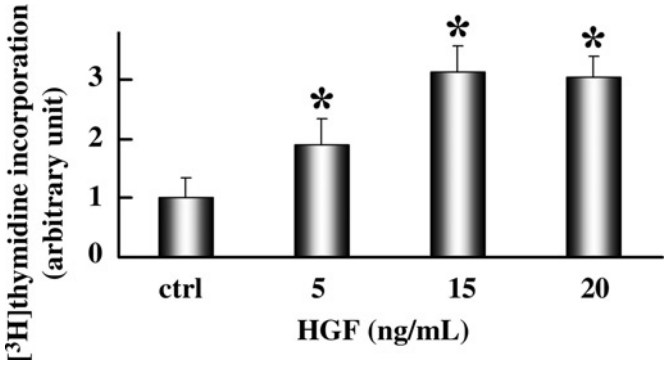

Figure 2 Effects of hepatocyte growth factor (HGF) on endothelial progenitor cell (EPC) proliferation. The [^{3}H]thymidine incorporation was used to assess the effect of HGF on EPC proliferation. EPCs were treated with the different concentrations of HGF (5, 10 and 20 ng/mL) for 48 h. The data are presented as the mean ± SD, $^*P < 0.05$ compared with the control group. The stimulatory activity was dose-dependent, with a maximum effect occurring at 10 ng/mL. ctrl, control

Knockdown of STIM1 expression attenuates SOCE and HGF-induced EPC proliferation

To determine whether endogenous STIM1 affects SOCE and HGF-induced EPC proliferation, we delivered adenovirus constructs expressing si/rSTIM1 to knockdown STIM1 protein levels. Cells treated with HGF but transfected with a random sequence were defined as the non-silencing group (nsRNA) and used as a control to monitor non-sequence-specific effects. Forty-eight hours after transfection, the level of STIM1 protein decreased by 66% compared with the control. HGF stimulation for 48 h increased the STIM1 protein level to 441% compared with the control group. In the siRNA + HGF group, STIM1 protein expression decreased by 79% compared with the HGF group (Figure 5a). Furthermore, we measured the effect of a siRNA targeted against rSTIM1 on SOCE. In this test, cells pretreated with 2-ABP and BTP-2 were used as the control (2-APB, 100 μmol/L and BTP-2, 10 μmol/L). The maximum increase in $[Ca^{2+}]_i$ caused by SOCE robustly decreased in siRNA-treated EPCs compared with the nsRNA group. 2-ABP and BTP-2 presented similar effects (Figure 5b and Figure 5c). Cell growth measured by [^{3}H]thymidine incorporation in the siRNA group was significantly lower compared with the nsRNA group (Figure 5d). Taken together, these results indicate that STIM1 molecule play a key role in the proliferation response of EPCs.

Discussion

Recent experimental and clinical studies indicate that EPCs play an important role in neoangiogenesis and rejuvenation of the endothelial monolayer.[6–9] The number and function of circulating EPCs inversely correlate with risk factors for coronary artery disease and predict the occurrence of cardiovascular events and death.[44,45] Thus, increasing attention has been focused on the improvement of EPC function.[46] The present *in vitro* study identify that STIM1 has a critical role in HGF-induced EPC proliferation. The result was based on several lines of evidence. First, the growth effect of HGF was evident in EPCs even at low concentrations. Meanwhile, the SOCE was increased in HGF-treated

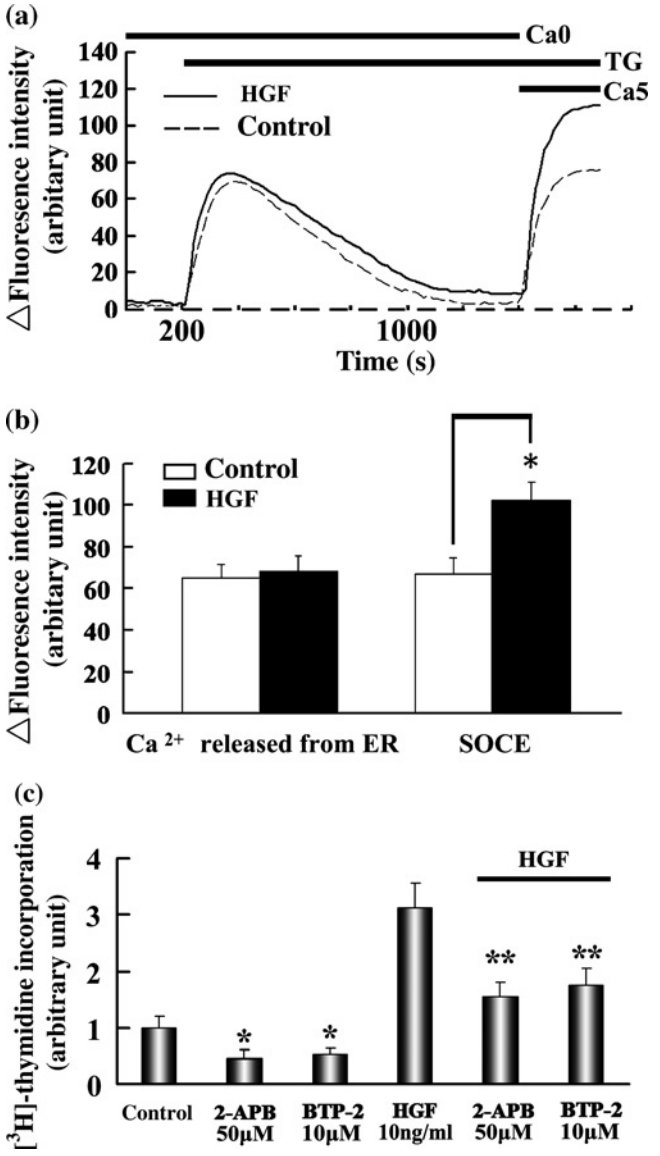

Figure 3 Increased store-operated Ca^{2+} entry (SOCE) in hepatocyte growth factor (HGF)-treated endothelial progenitor cells (EPCs). (a, b) The effect of HGF on store-operated Ca^{2+} channel (SOCC) activation was assessed after cells were treated with HGF for 48 h. Changes in $[Ca^{2+}]_i$ in individual cells were compared between control and HGF-treated cells. The maximum amplitude in $[Ca^{2+}]_i$ caused by SOCE was greatly increased in HGF-treated EPCs compared with control cells. The results of 30 control and 35 HGF-treated EPCs are presented as the mean ± SD. $^*P < 0.05$ versus control group. (c) SOCC inhibitors suppressed HGF-induced EPC proliferation significantly. EPCs were pretreated with 2-aminoethoxydiphenyl borate (2-APB) or BTP-2 before [^{3}H]thymidine incorporation. $^*P < 0.05$ versus control group, $^{**}P < 0.05$ versus HGF treated group

EPCs, and SOCC inhibitors 2-APB and BTP-2 inhibited the HGF-induced proliferation response. Second, STIM1 mRNA and protein expression increased significantly in response to HGF stimulation, and knockdown of STMI1 decreased SOCE and reduced HGF-induced EPC proliferation. These data suggest that the expression and function of STIM1 contribute to HGF-induced EPC proliferation.

As an important factor responsible for angiogenesis and the repair of injured blood vessel walls, HGF has a protective function role in the processes of endothelial repair

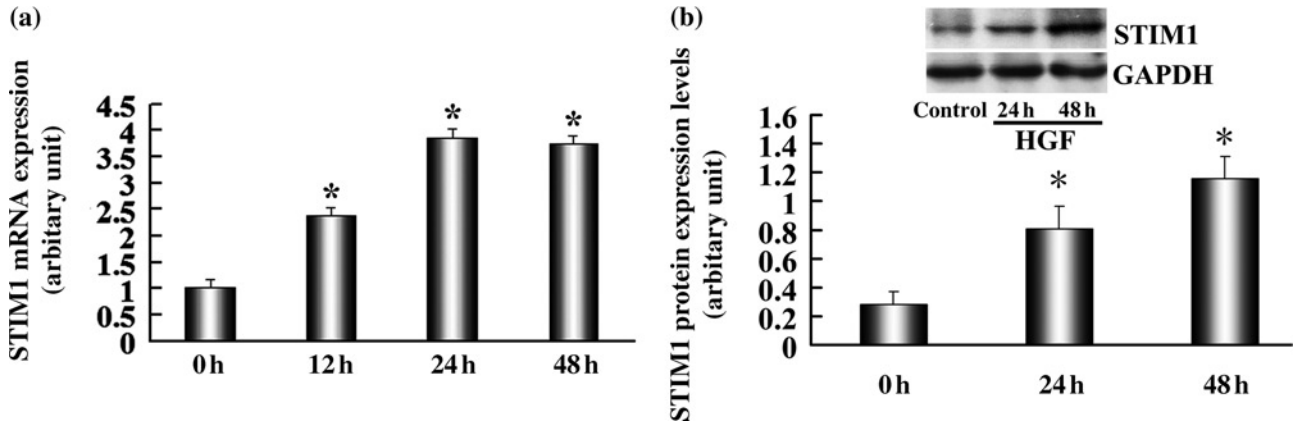

Figure 4 Expression of stromal interaction molecule 1 (STIM1) in hepatocyte growth factor (HGF)-treated endothelial progenitor cells. (a) STIM1 mRNA expression was at a very low level during the resting state, but increased significantly 12 h after HGF treatment, and reached a maximum of 3.86-fold greater than 0 h level after 24 h of treatment. STIM1 was expressed relative to glyceraldehyde 3-phosphate dehydrogenase (GAPDH). $^{*}P < 0.05$ versus 0 h group. All values for STIM1 mRNA expression have been normalized to the value at 0 h. (b) At the protein level, Western blot analysis indicated that after 24 and 48 h treatment with HGF, STIM1 protein levels increased significantly. $^{*}P < 0.05$ versus control group

and prevents the development of atherosclerosis.[22] One of the essential signaling events involved in this protective function is Ca^{2+} influx from SOCCs. After binding to its receptor (Met), HGF induces Met dimerization and autophosphorylation. Then, phosphorylated Met generates a multidocking site at the intracellular C-terminus, leading to the activation of PLC-γ.[47] Activation of PLC-γ results in two major responses which induce Ca^{2+} entry from the extracellular space. The first is direct activation of PM Ca^{2+} entry channels by second messengers such as

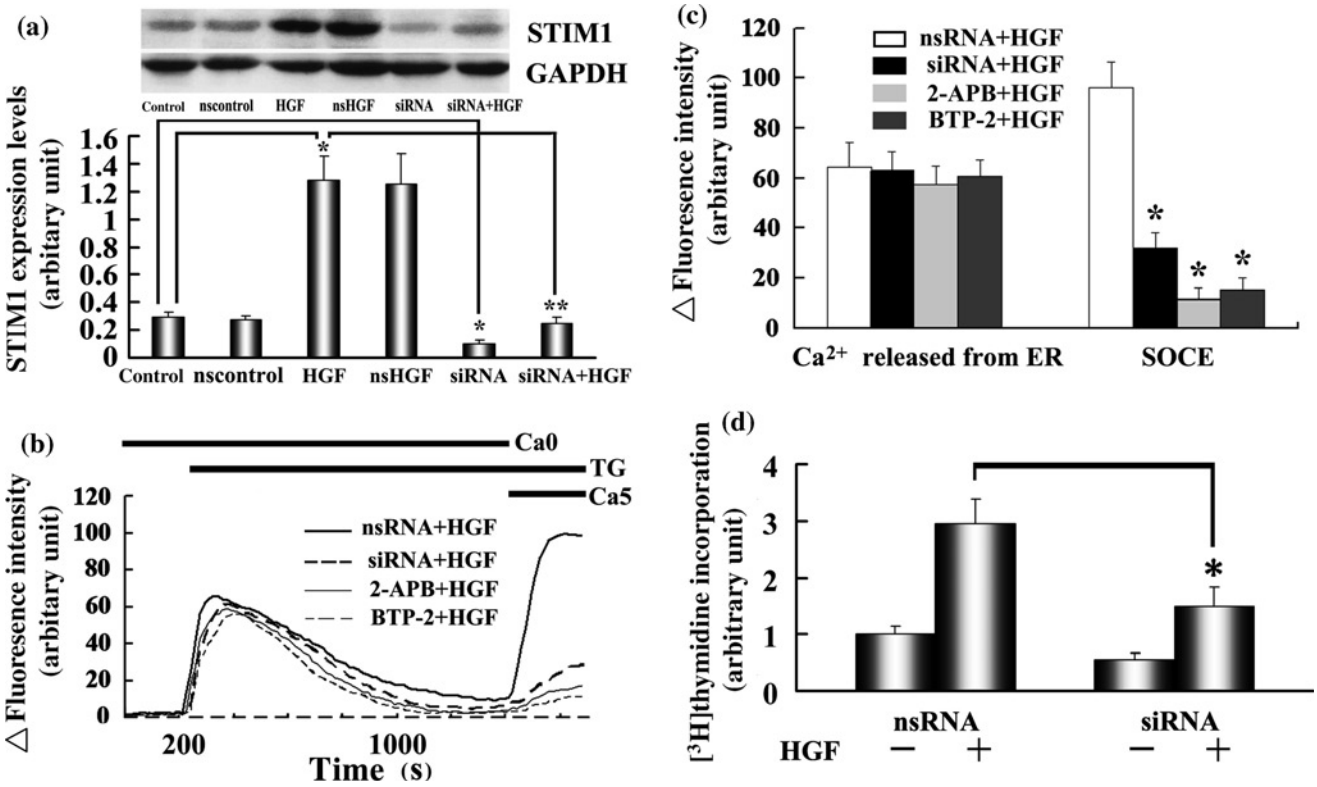

Figure 5 Inhibition of stromal interaction molecule 1 (STIM1) expression attenuates store-operated Ca^{2+} entry (SOCE) and cell viability. (a) Non-silencing groups showed no difference relative to their corresponding groups. Forty-eight hours after transfection, the level of the STIM1 protein level decreased by 66% compared with the control. Hepatocyte growth factor (HGF) stimulation for 48 h increased the STIM1 protein level by 441% compared with the control group. In the siRNA + HGF group, STIM1 protein expression decreased by 79% compared with the HGF group. (b, c) The maximum increase in $[Ca^{2+}]_i$ caused by SOCE robustly decreased in siRNA-treated EPCs compared with the nsRNA group. The results of 40 siRNA and 40 nsRNA-treated EPCs are presented as the mean $\pm$ SD. $^{*}P < 0.05$ versus HGF-treated cells. (d) The cell growth measured by $[^3H]$thymidine incorporation in the siRNA group was significantly lower compared with the nsRNA group. $^{*}P < 0.05$ versus nsRNA group

diacylglycerol and IP3. These channels are receptor-operated channels. Second, ER Ca^{2+} store is depleted via IP3 receptors and this effect results in the subsequent activation of other PM-localized Ca^{2+} channels known as SOCCs[48] (Figure 6). Ca^{2+} influx from SOCCs can influence several critical functions within the cell. First, Ca^{2+} influx via the SOCCs replenishes the ER Ca^{2+} stores following a release event. Second, Ca^{2+} concentrations within the ER must be maintained at sufficient levels for the organelle to carry out many of its fundamental functions. Third, Ca^{2+} influx through SOCCs first accesses the cytoplasm before entering the ER and such an event results in sustained elevation in Ca^{2+} influx levels, which is significant for some signaling pathways.[49] Thus, SOCE are necessary for many aspects of cell biology.[50]

In the present study, we revealed that HGF significantly induced proliferation of EPCs even at low concentrations. Furthermore, its proliferative effect was partially dependent on the augmentation of calcium signals in the cytoplasm via SOCE. Several previous reports suggested that increased Ca^{2+} influx through SOC channels might have a role in growth factor-induced proliferation in a variety of cell types. For example, Abdullaev *et al*. reported that VEGF elicited SOCE in human umbilical vein endothelial cells (HUVECs), and knockdown of STIMI decreased SOCE and inhibited HUVEC proliferation.[36] In fact, agonists, binding to either G protein-coupled receptors (such as thrombin)[36] or tyrosine kinase-linked receptors (such as VEGF and HGF), can activate PLC-γ, leading to the Ca^{2+} influx from SOCCs in the PM.[47,51] Thus, HGF and VEGF are two import activators of SOCCs. For a long time, some canonical transient receptor potential (TRP) channels have been considered molecular candidates of SOCCs. We noticed that in HUVECs treated with HGF, cDNA microarray analysis of the gene expression found that a number of cell cycle control proteins and genes involved in the regulation of mitosis were upregulated, including TRP protein isoform TRPC4.[52] It is worth mentioning that TRPC1 and TRPC4 have always been regarded as SOCCs (or subunits).[53,54] Meanwhile, in human hepatocytes, HGF causes intracellular Ca^{2+} elevation in the early stage of the cell cycle, which is due to the release of Ca^{2+} from intracellular stores and acts as a stimulus for cell growth.[55] Furthermore, El Boustany *et al*.[37] found that HGF increased TRPC6 expression and SOCE amplitude, which were correlated with Huh-7 cell proliferation. In our study, we also observed that SOCE in HGF-treated EPCs was increased significantly compared with normal EPCs. Moreover, cell proliferation in HGF-stimulated EPCs was diminished strongly by 2-APB and BTP-2, which both inhibited SOCCs.[36] These results suggest that SOCE is essential for HGF-induced EPC growth.

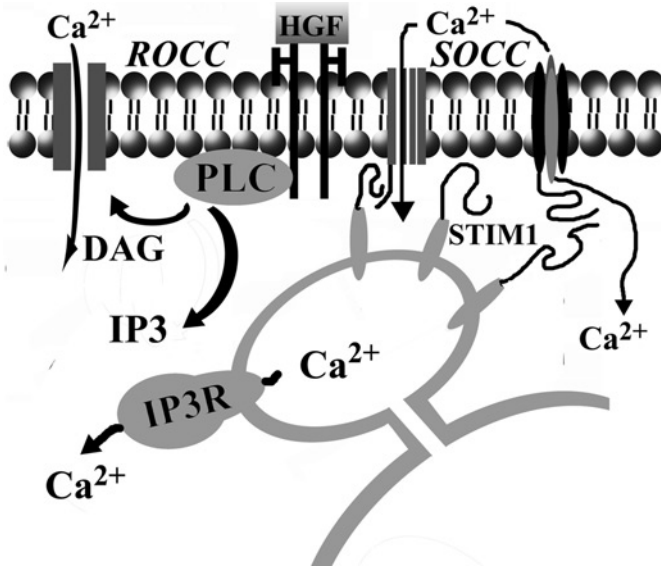

Figure 6 Model for the activation mechanism of store-operated Ca^{2+} entry (SOCE) by hepatocyte growth factor (HGF). After binding to its receptor (Met), HGF induces Met dimerization and autophosphorylation, leading to generate a multidocking site at the intracellular C-terminus, which results in the activation of phospholipase C-γ (PLC-γ). Following PLC-γ activation, two major responses of Ca^{2+} entry from the extracellular space take place. The first is direct activation of plasma membrane (PM) Ca^{2+} channels by second messengers such as diacylglycerol (DAG); these channels are receptor-operated Ca^{2+} channels (ROCCs). Second, Ca^{2+} is released from endoplasmic reticulum (ER) via IP3 receptors and this effect results in Ca^{2+} influx through PM-localized Ca^{2+} channels known as store-operated Ca^{2+} channels (SOCCs) (some parts of this figure are from Smyth *et al*.)[50]

Since the inception of the concept of SOCE, several distinct mechanisms have been proposed to explain the interaction between intracellular stores and the SOCCs in the PM, including (i) diffusible messengers,[56] (ii) conformational coupling and secretion-like coupling,[57] (iii) vesicle secretion[58] and (iv) removal of Ca^{2+} inhibition.[59] However, the nature of this signal remained uncertain until recently.[60] It now appears that STIM1 is the key molecule which is involved in regulation of SOCCS. Indeed, STIM1 possesses a Ca^{2+} sensing domain (called the EF-hand) on its N-terminal ER luminal portion.[61] Mutation of the EF-hand or knockdown of STIM1 changes the functions of SOCCs in mammalian cells.[43,62] In our work, we confirmed that the HGF/STIM1 pathway was involved in EPC proliferation. We showed that STIM1 was present at low levels in quiescent EPCs, but was rapidly upregulated upon HGF stimulation. HGF stimulation increased SOCE along with enhanced STIM1 expression. Gene silencing of STIM1 attenuated SOCE and HGF-induced EPC activity. These findings strongly suggest that HGF increases SOCE via upregulation of STIM1, implying that STIM1 plays an important role in the HGF-induced proliferation responses. However, since the involvement of STIM1 in SOCCs was a new breakthrough in the last five years,[60] what the mechanism was through which STIM1 expression was increased by HGF was unclear. Several recent literatures identified that the expression of STIM1 protein was increased in the aorta from stroke-prone spontaneously hypertensive rats, serum-induced proliferating human coronary artery smooth muscle cells and vascular injury SD rats.[43,63,64] We hypothesize that a possible mechanism is that Ca^{2+} release activates calmodulin which further activates other downstream transcriptional factors.[65] These events may result in STIM1 gene expression. Further studies are needed to understand more detailed regulatory mechanisms.

In conclusion, our results provide the first evidence that STIM1, a molecule required for the activation of SOCCs, has a critical role in HGF-induced EPC proliferation, and may be

an important regulator in neovascularization and re-endothelialization mediated by EPC proliferation.

Author contributions: YS is responsible for study design, cell culture, realtime PCR and paper writing. MS is responsible for study design, proliferation analysis and proofreading. RG is responsible for knockdown of STIM1 by siRNA. HW is responsible for confocal scanning microscope. PG is responsible for Western blot. WS is responsible for statistical analysis. LH is the corresponding author and responsible for all work. YS and MS contributed equally to this work.

ACKNOWLEDGEMENTS

This study is supported by grants from the National Natural Science Foundation of China (No. 30700889, 30770852 and 06j013). We also thank Huali Kang and Mengyang Deng for their kind help in this work.

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(Received August 8, 2009, Accepted November 25, 2009)