Extracellular calcium increases CXCR4 expression on bone marrow-derived cells and enhances pro-angiogenesis therapy

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Abstract

Cell surface receptors play major roles in the mobilization and homing of progenitor cells from the bone marrow to peripheral tissues. CXCR4 is an important receptor that regulates homing of leucocytes and endothelial progenitors in response to the chemokine stromal cell-derived factor-1 (SDF-1). Ionic calcium is also known to regulate chemotaxis of selective bone marrow cells (BMCs) through the calcium-sensing receptor, CaR. Here we show that calcium regulates CXCR4 expression and BMC responses to SDF-1. CaCl2 treatment of BMC induced a time- and dose-dependent increase in both the transcription and cell surface expression of CXCR4. BMC subpopulations expressing VEGFR2, CD34 and cKit/Sca-1 were especially sensitive to calcium. The effects were blocked by calcium influx inhibitors, anti-CaR antibody and the protein synthesis inhibitor cycloheximide, but not by the CXCR4 antagonist AMD3100. Calcium treatment also enhanced SDF-1-mediated CXCR4 internalization. These changes were reflected in significantly improved chemotaxis by SDF-1, which was abolished by AMD3100 and by antibody against CXCR4. Calcium pre-treatment improved homing of CD34 BMCs to ischemic muscle in vivo, and enhanced revascularization in ischemic mouse hindlimbs. Our results identify calcium as a positive regulator of CXCR4 expression that promotes stem cell mobilization, homing and therapy.

Keywords: CXCR4 - SDF-1 - calcium - progenitor cells - bone marrow

Introduction

CXCR4 is a member of the seven-transmembrane family of cell surface receptors that are coupled to G-proteins though specific chemokine ligands [1, 2]. CXCR4 is expressed by cells of haematopoietic and non-haematopoietic lineage and is required for stromal cell-derived factor-1 (SDF-1) chemotaxis of neutrophils, lymphocytes and CD34 progenitor cells [2, 3]. CXCR4 mediates the mobilization and homing of progenitor cells by its ligand, SDF-1. The SDF-1/CXCR4 interaction has been shown to play an important physiological role during embryogenesis in haematopoiesis [4], vascular development, cardiogenesis [5] and cerebellar development [6].

Chemotaxis by SDF-1 correlates with the amount of surface CXCR4 expression [2]. Like most cell surface receptors, CXCR4 expression on the cell surface is dynamic. Freshly harvested bone marrow derived cells (BMCs) have low surface CXCR4 expression; however, the level can be induced by culture [7–11]. Previous studies have described complex patterns of CXCR4 gene regulation that include induction by growth factors, cAMP, prostaglandin E2 and nitric oxide, and repression by a calcium-calcineurin-dependent pathway [12]. Because ionic calcium is elevated in the bone marrow, and has been shown to regulate the migration of haematopoietic precursors during embryonic development [13–15], we asked whether calcium also contributes to stem cell chemotaxis by modulating the CXCR4/SDF-1 signalling pathways in adult BMC. Here we report that extracellular calcium significantly controls surface CXCR4 expression and regulates both homing and therapeutic responses of progenitor cells in vivo. These results have important implications for progenitor cell therapy for ischemic disease.

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Materials and methods

Reagents

CXCR4 antagonist AMD3100, nitric oxide synthase (NOS) inhibitor Nω-monomethyl-L-arginine (L-NMMA), protein synthesis inhibitor cycloheximide and monoclonal anti-CaR (calcium-sensing receptor), antibody were purchased from Sigma Chemical (Saint Louis, MO, USA). PI3K inhibitor LY294002 was from EMD Biosciences (San Diego, CA, USA). Dulbecco’s phosphate buffered saline (PBS) contains 1.54 mM KH2PO4, 2.71 mM Na2HPO4 7H2O and 155 mM NaCl (pH 7.2) was from Invitrogen (Frederick, MD, USA).

BMCS harvesting and treatment

All animals were treated in accordance with the NIH Guide for the Care and Use of Laboratory Animals, and the procedures were approved by the University of Miami Animal Care and Use Committee. Mice (C57B/L6J Huxb13, The Jackson Laboratory), age 6 to 10 weeks, were anaesthetized with ketamine (100 mg/kg) and xylazine (10 mg/kg) intramuscularly (i.m.). Femurs and tibias were removed. Bone marrow was flushed with PBS. After filtration with BD Falcon™ 70-g Cell Strainer, BMCS were collected by centrifugation at 200 × g for 5 min. at 4°C, and resuspended in 1 ml red cell lysis buffer (Sigma) for 5 min. incubation at room temperature. After washed by PBS, the cells were incubated in PBS with or without CaCl2 in specified concentration at 4°C or 37°C for specified time.

FACS analysis of surface CXCR4

BMCS (1 × 10^6) in 100 μl PBS containing 1% bovine serum albumin (BSA, Sigma) were incubated with 2 μg FITC-conjugated rat anti-mouse CXCR4 mAb (BD Pharmingen) for 60 min. at 4°C. After two PBS washes, the cells were analysed by flow cytometry (FACS Calibur with CellQuest Pro 4.0,2 software, Becton Dickinson, San Jose, CA, USA). Control cells were incubated with isotype antibody to show background fluorescence. To study the inhibitor effect on CXCR4 expression, inhibitors AMD3100 (10 μM), LY294002 (20 μM), L-NMMA (300 μM) and Ab against CaSR (6 μg/ml) were added to the cell mixture prior the 4-hr incubation.

Multi-colour FACS (LSR2, BD Phamingen) was used to examine the surface expression of CXCR4 on the different BMC subpopulations. BMCS (1 × 10^6) with or without 4-hr calcium treatment were incubated at 4°C for 60 min. with a cocktail of antibodies: 5 μg APC-conjugated rat anti-mouse C-kit Ab (BD Pharmingen), 5 μl PE-conjugated rat anti-mouse Sca-1 Ab (BD Pharmingen), 5 μl PE-conjugated rat anti-mouse VEGFR-2 Ab (BD Pharmingen), 5 μl PE-conjugated rat anti-mouse CD34 Ab (BD Pharmingen) and 2 μl FITC-conjugated rat anti-mouse CXCR4 mAb (BD Pharmingen). Isotype and single colour controls were used for multi-colour FACS.

FACS analysis of intracellular CXCR4

The cell surface CXCR4 was blocked by incubation of BMCS with FITC-conjugated anti-CXCR4 mAb at 4°C for 1 hr as described above. The cells were then fixed with 2% paraformaldehyde for 20 min. at room temperature, followed with PBS wash. The cells were permeabilized with 0.1% saponin at room temperature for 10 min. After PBS wash, 5 μl PE-conjugated anti-mouse CXCR4 mAb (BD Pharmingen) was added to100 μl cell suspension containing 0.1% saponin and 1% BSA. The mixture was incubated at room temperature for 30 min. The surface and cytoplasmic CXCR4 were measured by two-colour flow cytometry.

FACS analysis of CXCR4 internalization

BMCS (1 × 10^6 cells) were incubated with 0.5 mM CaCl2 in PBS at 37°C for 4 hrs, then washed with PBS, re-suspended in 200 μl PBS containing recombinant mouse SDF-1α (final concentration 500 ng/ml, R&D Systems), and incubated at 37°C for 2 hr to allow SDF-1 to bind to the CXCR4 and the internalization of CXCR4/SDF-1 complex. The BMC were then washed with PBS at 4°C and subjected to FITC-conjugated rat anti-mouse CXCR4 mAb and FACS analysis as described above. The decrease in surface CXCR4 after incubation with SDF-1 reflects CXCR4 internalization.

RNA preparation and quantitative PCR

The first-strand cDNA was synthesized from the total RNA isolated from BMCS. CXCR4 mRNA was reverse-transcribed by use of TaqMan Reverse Transcription Reagents (Applied Biosystems, Foster City, CA, USA). Relative expression levels were quantified by real-time PCR with primers and probes for CXCR4 and the housekeeping gene hypoxanthine–guanine phosphoribosyltransferase (HPRT) along with the ABI PRISM 7700 sequence detection system (Applied Biosystems). The quantitation of CXCR4 mRNA relative to HPRT was carried out using the comparative threshold cycle (Ct) method (2^ΔΔCt) [16].

Calcium influx measurement

For intracellular calcium measurement, BMCS (1 × 10^7/ml) were loaded with the calcium indicator dye Fluo-4/AM (Invitrogen) at a final concentration of 5 μM for 30 min. In Ca^2+ flux assay buffer (Hank’s balanced salt solution containing 20 mM HEPES and 0.2% BSA, pH 7.4) at room temperature in the dark. Cells were then washed three times with the same buffer and maintained in darkness until use. After a 100-sec. baseline monitoring was performed by flow cytometry, sample aspiration was briefly paused and CaCl2 or CaCl2 plus BAPTA in Ca^2+ flux assay buffer were quickly added. The Ca^2+ response was measured against the change in green fluorescence intensity of the cells as a function of time.

Migration assay

A modified Boyden chamber assay was performed as described [17]. In brief, BMCS (1 × 10^3) pre-incubated with PBS with or without 0.5 mM CaCl2 for 4 hrs at 37°C were plated on the upper chambers. The lower chambers were filled with DMEM medium supplemented with 0.5% BSA plus 100 ng/ml SF-1. The migration after 6 hrs incubation was quantified by counting the cells that migrated to the other side of the membrane. To study the inhibitor effect on cell migration, AMD3100 (10 μM), LY294002 (20 μM), L-NMMA (300 μM) and anti-CXCR4 antibody (1:200) were added to the upper chamber along with BMCS.
Mouse hindlimb ischemic model

The entire right superficial femoral artery and vein of male mice (C57BL/6J), aged 8 to 10 weeks, were excised as described [18]. SDF-1 protein (100 ng in 0.1 ml PBS) was injected i.m. into the ischemic abductor muscle at two locations immediately after the surgery and once daily for the next 2 days. BMCs (1 × 10⁶ in 0.1 ml PBS) that had been incubated in PBS with or without 0.5 mM CaCl₂ at 37°C for 4 hrs were injected via the tail vein into mice with the Surgically created hindlimb. Five groups (six mice per group) were examined with different injections: (i) 0.1 ml saline (0.9% NaCl, i.m.); (ii) BMCs; (iii) calcium-treated BMCs; (iv) BMCs and SDF-1 and (v) calcium treated BMCs and SDF-1. Mice were killed 21 days after vessel resection, and the ischemic muscles were harvested and cryopreserved for capillary staining or fixed in 10% formalin and embedded in paraffin for immunohistochemical staining.

To study BMC homing (n = 6), injected BMCs were isolated from GFP mice with BL6 background (The Jackson Laboratory). Ischemic muscles were recovered after the mice were killed 7 days after cell injection. Cryopreserved sections were observed under the fluorescent microscope. Cells with green fluorescence were counted under high power of magnification.

Laser Doppler perfusion images

Laser Doppler perfusion images was performed as described [18] to record blood flow over a course of 3 weeks post-operatively. Relative perfusion data were expressed as the ratio of the ischemic (right) to normal (left) limb blood flow.

Statistics

Results are expressed as mean ± S.D. Statistical significant differences between groups were compared with ANOVA for multi-groups using GraphPad (San Diego, CA, USA) or two-tailed unpaired Student’s t-test for two groups only. Significance was attributed to a P-value of less than 0.05.

Results

Calcium-induced CXCR4 surface expression

CXCR4 expression on the surface of mouse BMC was analysed by FACS (Fig. 1A). On freshly isolated BMCs CXCR4 expression was 10.5 ± 0.6% and this increased fourfold to 43.5 ± 6.6% after 4 hrs of calcium treatment. The corresponding mean fluorescent intensities (MFIs) increased from 11.2 ± 1.9 to 25.17 ± 3.98 (P < 0.01, n = 6). The calcium effect was optimal after 4 hrs (Fig. 1B) and was maintained between 0.5 and 4 mM extracellular calcium (Fig. 1C). Treatment of BMC with MgCl₂ within the same concentration range did not change CXCR4 expression (data not shown). To investigate the subpopulations of calcium-responsive cells in the bone marrow, mononuclear cells were labelled with antibodies against VEGFR2, CD34, cKit, Sca-1 and CXCR4. As indicted in Fig. 1D, CXCR4 expression was enriched in VEGFR2, CD34 and cKit/Sca-1 cells and these cells were more sensitive to calcium stimulation. In particular, the VEGFR2 cells expressed 10-fold more surface CXCR4 after calcium treatment compared with the VEGFR2 population. Therefore, there is a differential response of selective subpopulations of progenitors to calcium-induced expression of CXCR4.

Calcium influx is required for enhanced CXCR4 surface expression

Fluo-4 AM was used to quantify calcium influx in BMC. A rapid and transient increase in the intracellular calcium concentration was observed after treatment with CaCl₂ (Fig. 2A). The calcium influx was inhibited by chelating calcium with BAPTA-AM (Fig. 2B). Inhibition of calcium influx reduced calcium-induced CXCR4 surface expression by 50% (Fig. 2C). This suggests that CXCR4 expression requires elevated intracellular calcium. To investigate possible mechanisms of calcium uptake, we blocked the dihydropyridine calcium channel with nifedipine and the CaR with a specific blocking antibody. Nifedipine did not inhibit CXCR4 expression but anti-CaR antibody reduced expression by the same amount as BAPTA-AM (Fig. 2C). Therefore the CaR may be involved in calcium-induced activation of CXCR4.

Enhanced CXCR4 expression involves synthesis of new protein

To determine whether calcium-induced CXCR4 surface expression involves new protein synthesis, we measured mRNA levels by real time PCR (RT-PCR) and protein level by FACS, and determined the effects of the translation inhibitor cycloheximide. CXCR4 mRNA levels in the CaCl₂ treated cells were unchanged after 2 hrs but increased by 2.2 ± 0.7-fold relative to untreated cell after 4 hrs (P < 0.05, n = 3). Inclusion of cycloheximide at the onset of calcium treatment eliminated CXCR4 induction (Fig. 3). These results suggest that calcium-induced CXCR4 surface expression requires new protein synthesis. To determine the intracellular CXCR4 and calcium effect on the distribution of CXCR4, antibodies labelled with FITC or PE were used to quantify surface and intracellular CXCR4 before and after cell permeabilization as described in the section ‘Materials and methods’. We found that intracellular CXCR4 was also significantly increased by CaCl₂ treatment (20.9 ± 2.3 versus 26.0 ± 1.9) and the increase was sensitive to
cycloheximide (Fig. 3B and C). These results suggest that calcium promotes synthesis and translocation of CXCR4.

Calcium promotes SDF-1-mediated CXCR4 internalization

Receptor internalization is a function of ligand binding and receptor activation. To determine whether calcium stimulated the generation of active receptors we measured CXCR4 internalization in response to SDF-1 binding. BMCs were incubated with CaCl$_2$ for 4 hrs, and then exposed to SDF-1. Receptor internalization was quantified by fluorescent antibodies. In the absence of treatment, the MFI of anti-CXCR4 binding decreased from 12.1 ± 0.7 to 8.8 ± 2.1 after incubating with SDF-1. For the calcium-stimulated group, the corresponding MFI decreased from 24.7 ± 3.3 to 11.7 ± 3.4 (Fig. 4A). The intracellular CXCR4 increased in a reciprocal manner as expected (Fig. 4B). These data confirm that SDF-1 induces CXCR4 internalization and calcium stimulates the production of active CXCR4 receptors.

BMC mobility is enhanced by calcium treatment

BMC mobilization by SDF-1 was determined using a modified Boyden chamber assay. Cell migration towards SDF-1 increased twofold after calcium treatment relative to control (32.9 ± 8.4 versus 15.6 ± 2.9; P < 0.05, n = 4, Fig. 5A). The mobility of calcium treated BMCs was significantly reduced by addition of PI3K inhibitor LY294002, or nitric oxide synthase inhibitor L-NNMA, CXCR4 antagonist AMD3100, or anti-CXCR4 antibody (Fig. 5A; all P < 0.05). None of these inhibitors had a direct effect on the surface CXCR4 expression (Fig. 5B). These results indicate that cell mobility is regulated by calcium through SDF-1/CXCR4 interaction, and supports roles for PI3-kinase and nitric oxide in the regulation of mobility.

Calcium-increased BMCs homing and angiogenesis

To examine whether calcium-induced CXCR4 expression facilitates BMC homing and angiogenesis in vivo, BMCs from GFP mice...
were incubated for 4 hrs with or without calcium, and injected into the tail veins of mice after surgical implementation of hindlimb ischemia as described in the section ‘Materials and methods’. SDF-1 protein was also injected into the ischemic hindlimb muscle to enhance BMC homing. Significantly more GFP+/H11001 cells were detected in the ischemic muscles of the mice that received calcium-treated BMCs than PBS-treated cells in the absence of SDF-1 (Fig. 6). With exogenous SDF-1, both cells homes more efficiently. But calcium-treated BMC still homed more efficiently to the ischemic site than the control BMC.

To determine whether calcium-enhanced CXCR4 activity translates into functional responses in vivo, we quantified blood flow by Doppler imaging, capillary density and cell homing by direct CD34+/H11001 immunostaining. Injection of calcium-treated BMC promoted significantly improved reperfusion (Fig. 7A and D) and higher capillary density (Fig. 7B and E) in ischemic limbs at 3 weeks after surgery both in the presence and absence of SDF-1 injections. The improved therapy was paralleled by significantly more CD34+ cells in the ischemic muscles following injections of calcium treated BMC (Fig. 7C and F).

**Discussion**

Our results show that calcium promotes a time and dose-dependent induction of CXCR4 expression in bone marrow mononuclear cells and selectively targets VEGR2+, CD34+ and cKit/Sca-1+ subpopulations. The induction involved increased transcription of the CXCR4 gene and new protein synthesis. Cell surface CXCR4 expression increased maximally by fourfold and was accompanied by a profound amplification of responses to SDF-1. CXCR4

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**Fig. 2** Correlation of calcium influx with CXCR4 expression. Calcium influx into BMC was measured by flow cytometry with Fluo-4/AM staining. Arrows indicate the time when CaCl2 (A), or CaCl2 + BAPTA (B) were added. CXCR4 surface expression under the different conditions was measured by FACS (C). *P < 0.05 versus PBS group, #P < 0.05 versus calcium treated group.

**Fig. 3** CXCR4 expression at different conditions. (A) FACS analysis of CXCR4 surface expression. (B) FACS analysis of intracellular CXCR4. (C), Quantitation of CXCR4 expression at different conditions.
Internalization in response to SDF-1 was increased in calcium-stimulated cells compared with controls indicating that the receptors are biologically active. Chemotaxis in response to SDF-1 was increased by twofold. The chemotactic response, but not CXCR4 expression was prevented by PI3-kinase or NO inhibitors, or by selective antagonism of the CXCR4 receptor. These effects are consistent with the known signalling pathways activated by SDF-1 through CXCR4 [19], [20]. Calcium stimulation also enhanced homing of CD34+/H11001 cells and promoted angiogenesis in vivo in a mouse ischemic hindlimb model. Homing of cells to ischemic muscle was amplified by calcium stimulation and enhanced further by SDF-1 treatment of the recipient muscle. The enhanced response to calcium and SDF-1 pre-treatment included increased blood flow, angiogenesis and homing of CD34+ cells to the ischemic limb.

Previous reports have documented an effect of culture on CXCR4 surface expression [7, 8, 10, 11, 21]. Freshly harvested BMCs have low surface CXCR4 expression that is increased by culture [7–11]. Evidence has also been presented that cultured bone marrow are therapeutically more active than uncultured cells [22, 23]. The effects may be selective for endothelial progenitor cells where culture has profound effects on CXCR4 expression [2, 8, 20, 24–27]. One mechanism for this proposes that CD34+/CXCR4+ cells harbour intracellular CXCR4 that is mobilized to the cell surface in response to cytokine stimulation [28]. We present here the first direct evidence that exposure to extracellular calcium is a major component responsible for the effects of culture on CXCR4 expression. Our results show that calcium alone in the range 0.5 to 4 mM induces a fourfold increase of cell surface CXCR4 expression with consequent activation of each step in the homing response to SDF-1 both in vitro and in vivo. The requirement for calcium uptake and inhibition by a selective CaR antibody suggests a novel pathway for calcium uptake and communication between CXCR4 and CaR. This is also consistent with previous work showing a requirement of CaR for homing and retention of haematopoietic cells in the bone marrow during ontogeny as well as demonstrations that CaR is expressed in sub-populations of BMCs [29]. Autocrine effects of secreted SDF-1

Fig. 4 Internalization of CXCR4 after binding with SDF-1. BMC were incubated with PBS with or without CaCl2 for 4 hrs and then mixed with SDF-1 for 1 hr at 37°C. The surface (A) and intracellular (B) CXCR4 were measured by FACS. Surface CXCR4 was decreased and intracellular CXCR4 was increased after binding with SDF-1. *P < 0.05.

Fig. 5 BMC migration towards SDF-1. (A) BMCs were pre-incubated with PBS with or without CaCl2 for 4 hrs at 37°C and transferred to the upper chamber of inserts in a 24-well plate containing DMEM and 100 ng/ml SDF-1. Migrated cells were counted after 6 hrs incubation at 37°C. Inhibitors AMD3100, LY294002, L-NMMA and anti-CXCR4 antibody were added to the upper chamber along with BMCs. *P < 0.05 versus Calcium treated group; #P < 0.05 versus other groups. (B) Effect of the inhibitors on CXCR4 surface expression. BMC were incubated at 37°C for 4 hrs in PBS without or with 0.5 mM CaCl2 plus specified inhibitors, and subjected same FACS analysis as in Fig. 1. Amount of surface CXCR4 on BMC was expressed as MFI.
Fig. 6 Homing of injected BMC in ischemic site. BMCs from GFP mice were incubated in PBS with or without CaCl$_2$ at 37°C for 4 hrs, and then intravenously injected into WT BL6 mice with ischemic limb. SDF-1 protein (100 ng) was injected into the ischemic hindlimb muscle twice at consecutive days after the surgery. The hindlimb muscles were recovered 7 days after the cell injection, stained with DAPI for nucleus (blue), and examined for GFP cells incorporation under fluorescent microscopy. (A) Untreated BMC, (B) Ca-treated BMC, (C) BMC + SDF-1, (D) BMC/Ca + SDF-1. (E) The incorporated GFP cells were quantified as cells per high power field (HPF). * $P < 0.05$, and ** $P < 0.01$ versus all other groups ($n = 6$).
have been reported to play a role in culture-induced CXCR4 expression [10, 30]. Our finding that the CXCR4 antagonist AMD3100 had no effect on calcium-induced CXCR4 expression (Fig. 5B) and that calcium treatment did not alter SDF-1 expression in BMC (data not shown) suggest that this is not a component of calcium induced CXCR4 expression.

We found that calcium stimulated CXCR4 gene transcription, and enhanced surface expression required new protein synthesis (Fig. 3). Previous work using different cell populations, demonstrated that CXCR4 mRNA levels can be enhanced by cAMP [31] and protein kinase C [12]. Other studies reported cytokine stimulation of BMC increased the surface CXCR4 perhaps through activation of NF-kB [11, 28, 32]. The CXCR4 gene promoter contains multiple transcription factor binding sites that include positive NFκB, Sp1, NFκB, HIF-1α and CBP and negative YY1. A previous study reported that calcium influx down-regulated CXCR4 expression in human T lymphocytes. This effect was attributed to enhanced binding of the negative YY1 factor to the CXCR4 promoter [12]. They also showed that regulation of YY1 by calcium may be suppressive or inductive depending on cell type [12]. Therefore it is possible that activation of YY1 by calcium in BMCs plays a positive role in BMC. Whereas we have not ruled out a possible role for PKC in the induction of CXCR4 expression, the effects appear to be independent of P3-kinase or NOS. Both of the latter signalling pathways are required for chemotaxis and migration but not for CXCR4 expression (Fig. 5). These results are also consistent with previous finding describing roles for P3-kinase, Akt, ERK and NOS in CXCR4/SDF-1 signalling [33–37].

Our results indicate a critical role for extracellular calcium in the activation of CD34⁺ cells that may have important implications for stem cell therapy for coronary and peripheral artery diseases. Results from clinical trials of autologous bone marrow derived mononuclear cells to treat lower limb ischemia have usually been positive although in some cases only marginally so and do not appear to be optimal [38–43]. Multiple studies have demonstrated that enhanced CXCR4 expression increases both survival and homing of haematopoietic and endothelial progenitor cells [2, 10, 11, 44–47]. Therefore optimizing CXCR4 cell surface expression for cell therapy is an important goal. Our studies show that calcium pre-treatment for 4 hrs dramatically improves the homing of injected BMCS as well as angiogenesis. We report significantly improved reperfusion and higher capillary density supported by calcium-treated BMCS (Fig. 7). These results also indicate that CD34⁺, VEGF receptors may be the ultimate targets for calcium-stimulated CXCR4 expression. Calcium pre-treatment of BMCS promotes improved neovascularization of ischemic muscle, and may enhance clinical therapeutic angiogenesis.

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