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Extracellular calcium modulates *in vitro* bone marrow-derived Flk-1$^+$ CD34$^+$ progenitor cell chemotaxis and differentiation through a calcium-sensing receptor

A. Aguirre [a,b,c], A. González [a], J.A. Planell [a,b,c], E. Engel [a,b,c,*]

[a] Institute for Bioengineering of Catalonia (IBEC), Baldiri Reixac 15-21, 08028 Barcelona, Spain
[b] Dept. of Material Science and Metallurgical Engineering, Technical University of Catalonia (UPC), ETSEIB, Av. Diagonal 647, 08028 Barcelona, Spain
[c] CIBER-BBN, Maria de Luna 11, Ed. CEEI, 50118 Zaragoza, Spain

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ABSTRACT

Angiogenesis is a complex process regulated by many cell types and a large variety of biochemical signals such as growth factors, transcription factors, oxygen and nutrient diffusion among others. In the present study, we found out that Flk-1$^+$ CD34$^+$ progenitor cells (bone marrow resident cells with an important role in angiogenesis) were responsive to changes in extracellular calcium concentration through a membrane bound, G-protein-coupled receptor sensitive to calcium ions related to the calcium-sensing receptor (CaSR). Calcium was able to induce progenitor cell migration in Boyden chamber experiments and tubulogenesis in Matrigel assays. Addition of anti-CaSR antibodies completely blocked the effect, while CaSR agonist Mg^{2+} produced a similar response to that of calcium. Real time RT-PCR for a wide array of angiogenesis-related genes showed increased expression of endothelial markers and signaling pathways involved in angiogenesis. These results suggest calcium could be a physiological modulator of the bone marrow progenitor cell-mediated angiogenic response.

Introduction

Angiogenesis is a complex process of blood vessel growth from pre-existing vessels and it is essential during embryonic development and for tissue repair and organ growth [1]. Many cell types are involved in adult angiogenesis, from mature endothelial cells, mural cells, leukocytes and platelets to endothelial progenitor cells (EPCs) present in the blood and bone marrow [2,3].

EPCs were first isolated from peripheral blood by Asahara et al. [4] as spindle-shaped cells capable of differentiation into a mature endothelial phenotype both *in vitro* and *in vivo*. They participate in post-natal angiogenesis and neovascularization by a homing mechanism directing them to sites undergoing active blood vessel remodelling, thus showing promising potential in vascular regeneration therapies [5,6]. Subsequently, other groups have found EPC-like populations in cord blood and bone marrow [7–9]. Overall, these cells are characterized by expression of several endothelial and stem cell markers such as vWF, CD31, VE-cadherin, Tie-2, CD133, VEGFR-2 and CD34, among others [10], although controversy exists about their identity and role due to their heterogeneity [11].

The calcium-sensing receptor (CaSR) is a seven transmembrane homodimer receptor belonging to the family C of the G-protein-coupled receptor (GPCR) superfamily. The extracellular binding region is sensitive to changes in extracellular calcium ions in the physiological range, although many other agonists have been identified (magnesium, aminoacids, amyloid β-peptides) [12]. Presence of the CaSR was first shown in parathyroid gland cells [13], but since then many cell types expressing functional CaSR have been identified, such as kidney cells, osteoblasts, osteoclasts, endothelial cells, cardiomyocytes, pancreatic cells, haematopoietic stem cells, etc. [14,16]. The CaSR is responsible for playing varied roles in different tissues including proliferation, chemotaxis, differentiation, apoptosis and modulation of the immune response [13,14]. The CaSR plays an important role in localization to the bone marrow of HSCs in mammalian hematopoiesis thanks to high levels of calcium in the endosteal niche [14]. Given all these previous evidence, we decided to investigate the hypothetical role of calcium in chemotaxis and differentiation in a set of HSC-related, bone marrow-derived Flk-1$^+$ CD34$^+$ cells which have been defined as endothelial progenitors [5,9]

Materials and methods

Reagents. Medium 199, calcium chloride, formalin, HEPES, Triton X-100, magnesium chloride, EGTA, heparin, fibronectin,

* Corresponding author. Address: Institute for Bioengineering of Catalonia, Ed. Hélix, Baldiri Reixac 15-21, 08028 Barcelona, Spain. Fax: +34 934020 183.
E-mail address: elisabeth.engel@upc.edu (E. Engel).

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IGF-1, EGF, paraformaldehyde, FITC-conjugated UEA-1, ascorbate and Matrigel were from Sigma–Aldrich (St. Louis, MO). Fetal bovine serum (FBS), Penicillin/streptomycin, L-glutamine, DAPI, trypsin and Alexa-488 secondary antibodies were purchased to Invitrogen (San Diego, CA). Antibodies against CaSR were from Affinity Bioreagents and Santa Cruz, antibodies against vWF, CD31, Flk-1, CD11b and CD34 were from Santa Cruz (Santa Cruz, CA). VEGF and bFGF were purchased from Peprotech (Rocky Hill, NJ). Rat tail collagen I was from BD Bioscience, WST-1 reagent was from Roche (San Jose, CA).

Isolation of rat endothelial progenitor cells from bone marrow. The protocol is a modification of the methods described before by [9,15,16]. Bone-marrow was obtained form the long bones of young Lewis rats 2–4 weeks old (Harlan). The whole cellular fraction was resuspended in M199 supplemented with 20% FBS, 1% Pyr, 1% Pen/Strep, 1% L-glu and 22 μg/ml heparin and was plated in 1 μg/ml fibronectin-coated 6-well plates (Nunc) and allowed to stay for 24 h. Most adherent cells attached to the dish in this period, including MSCs, immature leukocytes, mature ECs from vessels and others. The cells in suspension were then recovered and plated in new dishes for another 24 h. The third day the non-adherent fraction (now highly enriched in hematopoietic cells) was again replated, this time with medium favouring endothelial progenitor cell growth consisting of M199, 20% FBS, 1% Pyr, 1% Pen/Strep, 1% L-glu, 22 μg/ml heparin, 20 ng/ml VEGF, 5 ng/ml bFGF, 20 ng/ml IGF-1, 5 ng/ml EGF and 1 μg/ml ascorbate. Cells reached confluence after 8–9 days and were used from passages 2–6.

Flow cytometry. Trypsinized cells were blocked in 6% BSA solution for 10 min, fixed in 1% paraformaldehyde and incubated in the presence of primary antibodies against CD34, CD45 and CD11b for 30 min on ice. Afterwards, cells were incubated for another 30 min with anti-mouse Alexa-488 secondary antibodies and taken to the flow cytometer. Cells incubated in the absence of primary antibodies were used as negative control.

Immunofluorescence. Cells were cultured on coverslips for 24 h and fixed in 3% paraformaldehyde for 10 min. They were blocked with 6% PBS-BSA and permeabilized with 0.05% TX100 solution for 10 min. Afterwards they were incubated overnight at 4 C with the following primary anti-rat antibodies: CaSR, vWF, CD34, CD31 and Flk-1 followed by incubation with secondary Alexa-488 fluorescent antibodies for 1 h at 37 °C. Cells incubated with secondary antibody alone were used as negative controls. Images were obtained with a Nikon TE300 or with a Leica SPE confocal microscope.

Migration experiments. Transwell chambers with 8 μm pore diameter polycarbonate membranes (Corning) were used to measure cell chemotaxis. Subconfluent cells were trypsinized and 10.000 cells/well were seeded in the upper compartment of the chambers (previously pre-coated with a 10 μg/ml fibronectin solution). The medium used was M199, 1% FBS, 1% L-glu, 1% Pen/Strep, 1% Pyr, 22.5 μg/ml heparin. Lower compartments were loaded with the different conditions: control (culture medium, ~1.5 mM Ca^{2+}), 1.5 mM EGTA, 3 mM Ca^{2+}, 10 mM Ca^{2+}, 20 mM Ca^{2+}, 10 mM Mg^{2+} or 1:100 anti-CaSR antibody. Cells were allowed to migrate for 24 h and then were fixed in 10% formalin and stained with DAPI. Nuclei from migrated cells were counted in 4 different random fields of each sample and averaged. Negative controls were chambers with no gradient (medium in upper and lower chambers without calcium or VEGF addition), while positive control was a 40 ng/ml VEGF gradient. An additional chemokinesis control (with calcium in both compartments to test for increased but random cell motility) was also performed with no significant differences.

Matrigel and collagen I tube formation assay. Tube formation was determined by using Matrigel (Sigma) diluted 1:1 in culture media in 48-well plates and allowed to solidify for 1 h at 37 °C. Cells were then harvested by trypsinization and seeded at 20.000 cells/well in growth factor free M199 with 1% FBS, 1% L-glu, 1% Pen/Srep, 1% Pyr and 22.5 μg/ml heparin. After 4 h and 20 h phase contrast micrographs were taken. Branching was quantified by counting sprouting tube-like structures longer than 100 μm in 4 separate fields in duplicate samples. Cells were treated with 10 mm Ca^{2+}, 1:100 CaSR antibody or 40 ng/ml VEGF were as a positive control. For collagen I tube formation a collagen I solution was diluted in culture media to a final concentration of 1.5 mg/ml collagen I with addition of 1 N NaOH and 20 mM HEPES. This solution was carefully layered over the cells.

WST-1 cell viability and proliferation assay. Cells stimulated with different concentrations of calcium were seeded at a density of 10.000 cells/well in 96-well plates and cultured for 24 h, 3 days and 7 days in complete medium. Afterwards they were incubated for 1 h with 1:10 WST-1 reagent to determine mitochondrial dehydrogenase activity. The supernatant was taken to an ELISA plate reader to measure formazan dye formation by absorption at 450 nm.

Angiogenesis PCR array. RNA was extracted from cells stimulated with 10 mM Ca^{2+} for 3 days using RNeasy Minikit (Qiagen) following the manufacturer instructions. Reverse transcription of the samples was performed with RT^2 First Strand Kit (SABiosciences). 0.5 μg cDNA and RT^2 qPCR SYBR Green Master Mix (SABiosciences) were used in array plates containing a panel of 86 angiogenesis-related genes, 5 housekeeping control genes, a rat genomic contamination control and RT-controls (see Supplemental Table 1). Amplification was performed with a 10 min 95 °C activation step followed by 40 cycles at 95 °C 15 s (denaturation) and 1 min at 60 °C (extension). A cut-off value of ±2-fold expression was used. For gene sequences used see Supplemental Table 1.

Statistical analysis. Data are representative of 3 independent experiments except where otherwise noted. Results are expressed as mean ± S.E.M. Statistical significance was determined by Student's *t*-test.

Results

Obtention and characterization of bone marrow-derived Flk-1$^+$ CD34$^+$ endothelial-like progenitors

Cells were obtained by the procedure described in Materials and Methods. Approximately 5–6 days after initial extraction a population of adherent, spindle-shaped, rapidly proliferating cells was observed. Other contaminating cells were also present at this point, but were mostly eliminated after passage 2 (Fig. 1A). Cells formed typical tube-like structures in the Matrigel assay after 4 h stimulation with 40 ng/ml VEGF and 24 h (data not shown). They also sprouted and formed tube-like structures when covered in a collagen I gel for 3 days (Fig. 1A). Immunofluorescence staining demonstrated a majority of cells expressing Flk-1, weak CD31 and CD34 and diffuse vWF staining at passage 1. Ulex europaeus lectin binding was also positive (Fig. 1B). This phenotype was consistent with an immature, endothelial-like progenitor state as described by others [10,17]. Flow cytometry for CD45 and CD11b was performed on passage 4 cells to assess the purity of the population studied (data not shown). Results showed a small proportion of cells expressing hematopoietic markers (2.12% positive for CD45 and 2.19% for CD11b), confirming very low hematopoietic contamination in regular cultures.

Calcium induces migration through activation of the calcium-sensing receptor in BM-derived Flk-1$^+$ CD34$^+$ progenitors

Immunofluorescence with anti-CaSR antibodies demonstrated that BM-derived progenitors expressed significant amounts of

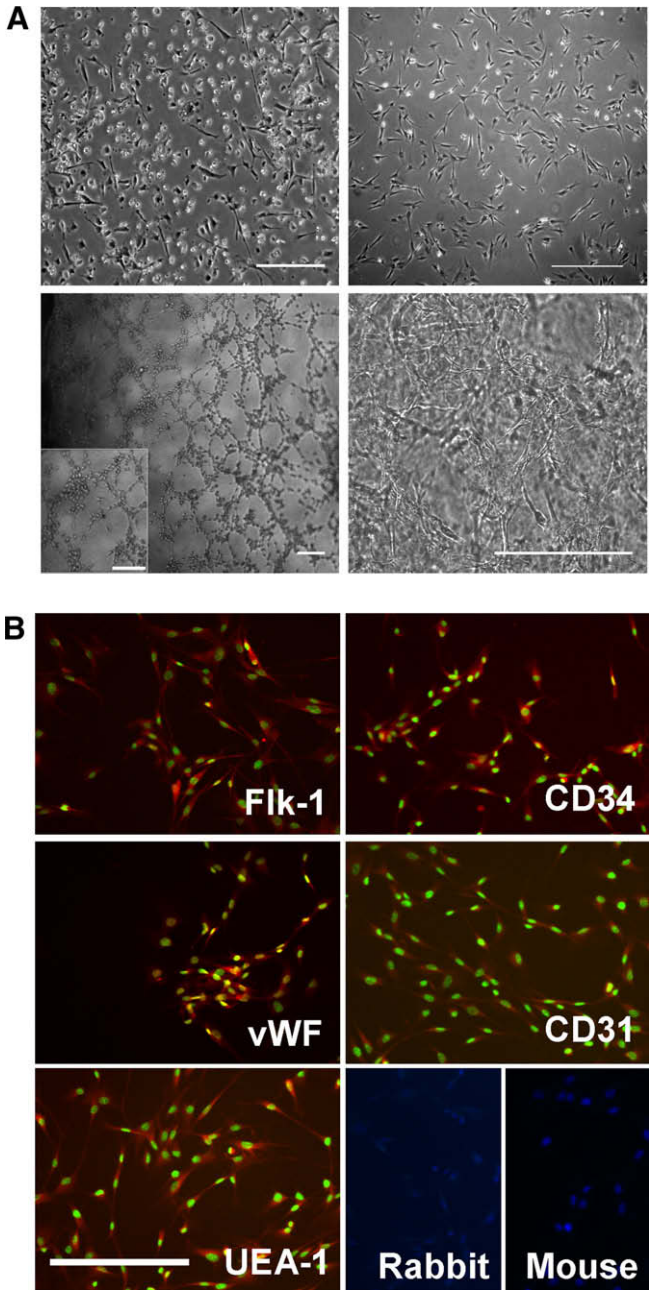

Fig. 1. Characterization of BM-derived progenitors. (A) *Top left*, Adherent cells with spindle-shaped morphology were present after 6 days, as well as other cells of hematopoietic origin. *Top right*, at passage 2 most of the contaminant cells had been removed, while the rapidly proliferating population of spindle-shaped cells remained. *Bottom left*, cells seeded in Matrigel supplemented with 40 ng/ml VEGF formed dense tube-like structures after 4 h. *Bottom right*, cells embedded in a collagen I matrix underwent sprouting and tube formation after 3 days. (B) Immunofluorescent staining showed positive cells for Flk-1/VEGFR-2 and CD34. Weak CD31 expression and diffuse vWF staining were observed. Ulex Europaeus-1 lectin binding was also positive. Bar: 100 μm.

CaSR at the membrane (Fig. 1A). A calcium concentration dependent migratory response was observed after 24 h with a maximal effect achieved at concentrations ranging from 3–10 mM Ca^{2+}, well within the range of the CaSR activation [16]. Culture medium containing ~1.5 mM Ca^{2+} and 40 ng/ml VEGF were used as controls, the latter provoking a strong chemotactic response. Medium treated with the calcium chelator EGTA had no effect. Chemotaxis could be induced by 10 mM Mg^{2+}—a well-known CaSR agonist—[24]. Incubation in the presence of CaSR antibodies completely blocked the response to calcium. WST-1 cell proliferation/survival assay showed no effects of calcium over cell proliferation at any of the time points or concentrations tested (Fig. 2D). We chose a concentration of 10 mM Ca^{2+} for further experiments based on the intensity of the response and the physiologically relevant range of 1–40 mM Ca^{2+} described by other authors [14,17].

Calcium stimulation promotes tubulogenesis in Matrigel

BM-derived Flk-1$^+$ CD34$^+$ progenitors were seeded on Matrigel and treated with 10 mM Ca^{2+}, 40 ng/ml VEGF, anti-CaSR and both Ca^{2+} and VEGF to study the tubulogenic response after 20 h (Fig. 2). Ca^{2+} and VEGF significantly increased the branching response up to 3.3 and 2.9, respectively (Fig. 2B). When cells were simultaneously stimulated with Ca^{2+} and VEGF a stronger tubulogenic response reaching values of 5 could be observed (Fig. 2B). The effects of Ca^{2+} in tube formation could be abolished by treatment with CaSR antibodies.

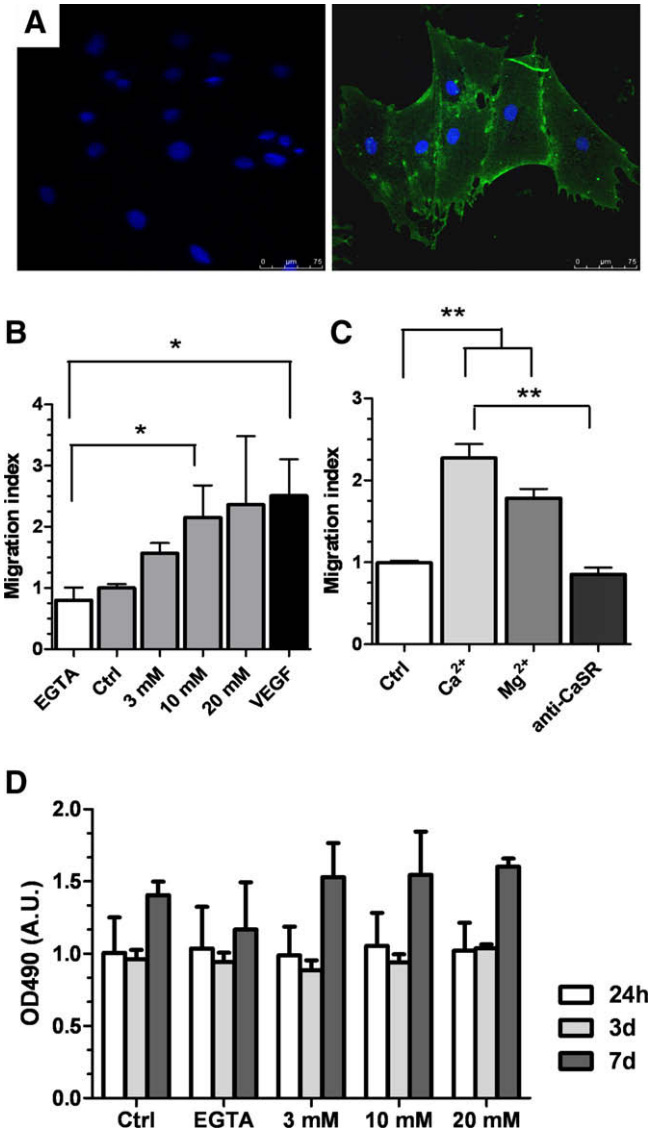

Fig. 2. BM-derived Flk-1$^+$ CD34$^+$ progenitors expressed a functional, membrane bound, CaSR. (A) Immunofluorescent staining with anti-CaSR antibodies demonstrated expression in the plasma membrane. (B) Different calcium concentrations were able to induce BM-EPC migration, while EGTA abolished this response. (C) CaSR agonist Mg^{2+} provoked a similar response to Ca^{2+}, additionally, anti-CaSR antibodies blocked the chemotactic response induced by calcium. (D) Proliferation was unaffected by calcium treatment at any of the time points tested ($n = 3$, $p < 0.05$).

Real time RT-PCR reveals changes in the angiogenesis-related gene expression profile of BM-derived Flk-1$^+$ CD34$^+$ progenitors upon calcium treatment

BM-derived progenitors in the presence of 10 mM Ca^{2+} for 3 days underwent changes in angiogenesis-related gene expression patterns (Fig. 3). Out of 86 genes, 9 were up-regulated and 4 down-regulated. Table 1 summarizes fold expression changes and biological function. Up-regulated transcripts were mainly cell signaling molecules (growth factors and receptors) or adhesion molecules, with the exception of the transcription factor Epas1. Two chemokines, Cxcl2 and Cxcl1, the transcription factor Id1 and Mmp9 were the only down-regulated genes. All changes listed were statistically significant.

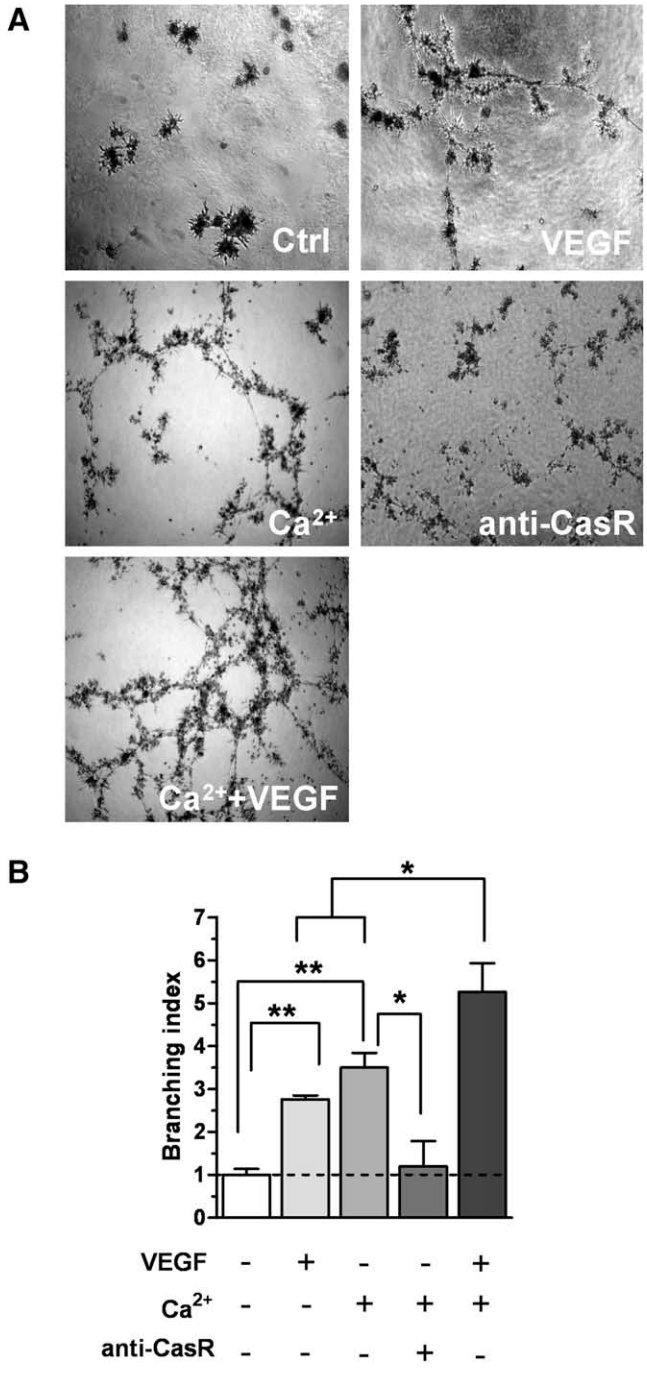

Fig. 3. Calcium promoted tubulogenesis in Matrigel. (A) 10 mM Ca^{2+} and 40 ng/ml VEGF induced tube-like formation but none can be seen in control or Ca^{2+}+anti-CaSR conditions. When Ca^{2+} and VEGF were applied together they had a synergistic effect. (B) Quantification of the tubulogenic response. There were significant differences between control and calcium-stimulated cells, as well as between cells co-treated with VEGF and calcium or antibodies against CaSR ($n = 3$, $p < 0.05$).

Table 1
Real time RT-PCR angiogenesis array for calcium-stimulated BM-derived progenitors at 3 days. Fold differences were normalized to unstimulated cells, a cut-off value of 2 was applied. All changes shown were statistically significant ($n = 4$, $p < 0.05$).

Symbol	Gene name	Biological function	Fold change
Up-regulated			
Edg1 (S1PR1)	Endothelial differentiation gene 1	Sphingolipid G-protein-coupled receptor	2.76
Fzd5	Frizzled homolog 5	Wnt signaling receptor	2.99
Jag1	Jagged 1	Notch1 receptor ligand	2.52
Itgβ3	Integrin β3	Cell-matrix adhesion	2.42
Epas1 (HIF-2α)	Endothelial PAS domain protein 1	Transcription factor	2.15
Thbs4	Thrombospondin 4	Adhesion protein	2.37
Anpep	Alanyl aminopeptidase	Matrix remodelation	2.61
Angpt-2	Angiopoietin 2	Signaling molecule	2.47
IGF-1	Insulin-like growth factor 1	Growth factor	2.03
Down-regulated			
Cxcl2	Chemokine (C-X-C motif) ligand 2	Signaling molecule	−4.31
Cxcl1	Chemokine (C-X-C motif) ligand 1	Signaling molecule	−2.16
Id1	Inhibitor of differentiation 1	Transcription factor	−2.33
Mmp9	Matrix metalloprotease 9	Matrix remodelation	−2.59

Discussion

Extracellular calcium is an important regulator of several biological processes such as differentiation, apoptosis and chemotaxis through activation of the CaSR [12,14]. In endothelial cells the presence of a CaSR has been reported by several groups associated with maintenance of vascular tone, regulation of vessel permeability and vascular inflammation and repair [18,19]. The role of CaSR in the bone marrow niche has also been extensively studied with results indicating a pivotal role in haematopoietic stem cell homing [14,20]. Since BM-derived Flk-1$^+$ CD34$^+$ progenitors are involved in angiogenesis and have the potential to differentiate to mature endothelial cells we speculated whether the CaSR could be playing a role in this process. Immunofluorescence with two different set of antibodies against different CaSR epitopes revealed expression in the membrane of bone marrow-derived progenitors. However, when we analyzed gene expression by real time RT-PCR we were unable to detect a CaSR transcript (data not shown). This opened the possibility of a distinct calcium-sensing receptor being responsible for the effects later observed. This receptor would share high structural homology with the traditional CaSR. Candidates include a possible receptor isoform or one of the other members of the GPCR family C.

The functionality of the receptor could be demonstrated after testing different calcium concentrations and measuring the chemotactic response. Indeed, calcium induced progenitor cell migration in a concentration-dependent manner (Fig. 2C), and magnesium, a well-known CaSR agonist [12] was able to reproduce the effect, albeit with lower intensity than calcium. When CaSR

antibodies were applied in conjunction with calcium the chemotactic response was completely abolished (Fig. 2D). These results supported the presence of a functional CaSR in bone marrow endothelial-like progenitors.

It has been already demonstrated that CaSR has effects over cell proliferation and apoptosis [21,22], however, in our WST-1 experiments calcium had no negative or positive effects over cell proliferation (Fig. 2E).

Cells were seeded on a layer of Matrigel and their tube-forming capacity was evaluated after 20 h treatment with calcium. There was a moderate induction of the tubulogenic response, suggesting a role for calcium in angiogenesis. This response could be blocked with anti-CaSR antibodies, pointing to the involvement of a CaSR-like receptor. Simultaneous treatment with calcium and VEGF potentiated the response (Fig. 3A and B) hinting to the activation of an alternate signaling pathway. CaSR activation leads to NF-κB nuclear translocation, which in turn can increase VEGF synthesis [23,24]. Certainly, when gene expression for VEGF-A was measured at 6 and 24 h after calcium treatment increased levels for this transcript were detected (Supplemental Fig. 1), thus providing a likely mechanism for the proangiogenic action of calcium.

Analysis of gene expression for 86 angiogenesis-related genes after 3 days of calcium stimulation (Table 1) demonstrated 11 up-regulated genes. The up-regulated transcripts were mainly signaling molecules (Jag1, Fzd5, IGF-1, Angpt-2, Edg1), with the exception of the transcription factor Epas1 (HIF-2α). These signaling pathways are involved in Notch, Wnt/β-catenin, tyrosin kinase and G-protein signaling, respectively, thus opening a large network of possible interactions involved in differentiation. The implication of Jag1 and Fzd5 is demonstrated in hematopoietic stem cell specification and development in the embryo [25,26] and could be further involved in BM-derived progenitor biology. The relationship between CaSR and IGF is better established: in knock-out mice a deletion of CaSR results in IGF deficient signaling leading to bone defects [27] and a role for the CaSR in angiogenesis has recently been described in bone marrow cells *in vivo* [28]. Both Edg1 and Angpt-2 have well-established roles in vessel maturation and angiogenesis/inflammation [29,30]. Four down-regulated genes were found. Out of these, two, the chemokines Cxcl2 and Cxcl1, showed very low expression and are usually produced by monocytic-type cells, suggesting the presence of a low number of contaminant cells in culture (this had previously been confirmed by flow cytometry for CD11b). Id1 is a transcription factor crucial in development, cell cycle and cancer [31] whereas Mmp9 (gelatinase B) is a matrix remodelation protein [32].

At sites of injury, inflammation or tissue remodelation increased levels of extracellular calcium are present with reported values in the range of 1–40 mM (e.g. resorbing bone) [14,17]. Several authors have explored how these increased levels of calcium modulate the immune response *in vitro* and *in vivo* [33,34]. Concomitant to the immune response at sites of injury is the angiogenic response, initiated by immune cells and highly dependent on the microenvironment and balance of positive and negative angiogenic regulators [35]. With the evidence presented in this work, we propose that calcium and a calcium-sensing system present in endothelial-like progenitors mobilized from the bone marrow may play a role in initiating and/or modulating angiogenesis, chemotaxis and differentiation at sites of injury, inflammation or tissue remodelation.

Conclusion

Calcium treatment was able to induce up-regulation of important angiogenic cytokines and growth factors through the activation of a CaSR-like receptor. Chemotaxis and tube-like formation also took place under calcium stimulation. These results support a role for calcium in angiogenesis at sites of injury or inflammation with involvement of bone marrow-derived progenitors. This mechanism could be exploited to develop new proangiogenic therapies, particularly in the field of regenerative medicine. However, further research is needed to unequivocally identify the receptor, clarify the signaling pathways involved and explain the physiological significance of this finding *in vivo*.

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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.2010.01.109.

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