Published in final edited form as:

Biochem Biophys Res Commun. 2009 May 1; 382(2): 241–246. doi:10.1016/j.bbrc.2009.02.068.

Calcium and cyclic nucleotides affect TNF- α -induced stem cell migration

Emel Kaplan^{a,e,*}, Jiang-Yong Min^a, Qingen Ke^a, Yu Chen^a, Marc Niethammer^b, Jamal S. Rana^a, Sohail Malek^a, Freek W.A. Verheugt^c, and James P. Morgan^d

^aBeth Israel Deaconess Medical Center, Harvard Medical School, Boston, MA, USA ^bBrigham and Women's Hospital, Harvard Medical School, Boston, MA, USA ^cOnze Lieve Vrouwe Gasthuis, Amsterdam, The Netherlands ^dCaritas St. Elizabeth's Medical Center, Tufts University, Boston, MA, USA ^eDepartment of Cardiology, Radboud University Nijmegen Medical Centre, Geert Grooteplein 10, 6525 GA Nijmegen, The Netherlands

Abstract

The purpose of this study was to study the effect of calcium, cyclic AMP (cAMP) and cyclic GMP (cGMP) on embryonic stem cell (ESC) motility during TNF- α -induced chemotaxis.

ESCs were monitored using a chemotaxis chamber, with different concentrations of calcium or cAMP or cGMP added to the medium. Changes in intracellular calcium ($[Ca^{2+}]_i$) were measured with the fluorescent dye fura-2/AM. We combined migratory parameters in a mathematical model and described it as "mobility".

After adding calcium, a dose-dependant increase in cell speed was found. Cyclic AMP increased mobility as well as the $[Ca^{2+}]_i$. In contrast, adding dbcGMP resulted in a significant decrease in the mobility of the ESCs. During migration ESCs showed an increase in $[Ca^{2+}]_i$. Furthermore, TNF- α dramatically increased the movement as well as the directionality of ESCs.

These results demonstrate that ESCs are highly motile and respond to different concentrations of calcium in a dose-related manner.

Keywords

Stem cell migration; TNF- α ; Calcium; cAMP; cGMP; Fura-2 fluorescence

Introduction

There is increasing interest in stem cell transplantation as a potential therapy for improving the prognosis of patients with cardiac failure [1-3]. We have previously shown that after systemic injection, murine ESCs home to the hearts of mice with inflammation due to viral myocarditis [4]. However, the mechanism by which stem cell migration occurs remains largely unstudied.

Cell migration depends on morphologic changes that are controlled by proteins that are associated with the actin cytoskeleton. Calcium plays an important role in the regulation of actin-binding proteins. Calcium has also been shown to be involved in the stimulation of

myosin II-based contraction [5] and in mediating the amount of integrin available to participate in cell migration [6]. Regulation of cyclic nucleotide levels is an essential element in modulating cell migration. Cyclic adenosine 3,5-monophosphate (cAMP) is a ubiquitous second messenger that regulates many cellular processes and has been shown to be required for efficient cell migration [7]. In addition, cyclic guanosine 3–5' monophosphate (cGMP) has been discovered to modulate cell migration as well and is known to act through different proteins, affecting the extracellular matrix and actin cytoskeleton [8,9]. Tumor necrosis factor- α (TNF- α) is a cytokine that is released after cardiac injury, including myocardial infarction [10]. We recently showed that TNF- α enhanced migration of ESCs *in vitro* [11].

We hypothesized calcium and cyclic nucleotides affect the morphology and membrane deformation of ESCs and in addition TNF- α may play an important role in attracting ESCs. Here, therefore, we studied the effects of changes in extracellular $[Ca^{2+}]$, cAMP and cGMP in the medium on intracellular $[Ca^{2+}]$ and stem cell migration, using a simple mathematical model that allowed us to analyze the overall effect of these second messengers.

Materials and methods

Cell culture

Experiments were conducted using murine ESCs, ES-D3, obtained from the American Type Culture Collection (ATCC, Manassas, VA). The cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM) (Hyclone, Logan, UT), 10% Fetal Bovine Serum (ATCC, Manassas, VA), 1% penicillin–streptomycin (Cellgro, Herndon, VA), 0.1% 2-mercaptoethanol (Invitrogen, Carlsbad, CA) and 1000 U/ml leukemia inhibitory factor (Sigma–Aldrich, Saint Louis, MO). The 6-wells were incubated at 37 °C in a humidified atmosphere of 95% air/5% CO₂. Growth medium was changed twice weekly and cells were subcultured weekly by dissociation with trypsin for 3 min.

Preparation of cells

Before each experiment cells were trypsinized for 3 min and suspended in growth medium. After an incubation period of 30 min the stem cell suspension was centrifuged at 1000g for 3 min. Collected cells were resuspended in DMEM, supplemented with 0.1, 1, 2 or 5 mM of Ca²⁺, just before the start of the experiment. For the experiments with dbcAMP (1 mM) or dbcGMP (1 mM) the medium contained 0.1 mM of calcium.

Dye loading and calcium measurement

ESCs in suspension were loaded for 40 min with 5 μ M/L fura-2/AM (Molecular Probes, Eugene, OR). Extracellular fura-2/AM was washed away and the cells were resuspended in DMEM, with pH adjusted to 7.4. To determine the effect of dbcAMP (1 mM) and dbcGMP (1 mM) on the intracellular calcium concentration, these substances were added to the medium (~40 cells from five separate cultures per condition). Fluorescence intensity was converted to intracellular calcium concentration ($[Ca^{2+}]_i$) using the following equation of Grynkiewicz et al. [12]:

$$[Ca^{2+}]_i = K_d b (R - R_{min}) / (R_{max} - R)$$

where K_d is the dissociation constant of the Ca²⁺/Fura-2 complex (225 nM), $b = F_{380}$ (zero Ca²⁺)/ F_{380} (saturating Ca²⁺), $R = F_{340}/F_{380}$, R_{min} represents the ratio at zero calcium (1 mM EGTA), R_{max} is the ratio at high calcium (1 mM CaCl₂, 5 μ M ionomycin).

Setup of chemotaxis chamber

The Dunn chemotaxis chamber (Weber Scientific International Ltd., Teddington, UK) was filled with the cell suspension and covered by a 22×25 mm coverslip, leaving a narrow gap at one edge for refilling the outer well. The medium was then drained out of the outer well and refilled with medium containing 5 ng/ml of TNF- α . Finally, the gap was sealed using a mixture of hot wax (vaseline, beeswax, paraffin wax 1:1:1).

Recording and tracking cell behavior

A region of the bridge of the Dunn chamber was viewed under an inverted fluorescence microscope at a magnification of $4\times$ (TE-2000-U, Nikon, Tokyo, Japan). Images were obtained every 2 min for a 3-h period with a Photometrics Cool Snap HQ charge-coupled device camera (Roper Scientific, Trenton, NJ).

Fura-2 fluorescence was excited alternately at 340 and 380 nm using a filter wheel and a 75 W Xenon lamp. To reduce photobleaching, a shutter prevented illumination of the cells, except during data acquisition. The fluorescent emissions were acquired through a 510-nm dichroic mirror and 520 nm long pass filter set (Chroma Technology, Brattleboro, VT). Acquired images were generated at 6-s intervals and background was subtracted from each image. The loaded cells were viewed using a $20\times$ objective (Nikon, Tokyo, Japan). Images were analyzed by IP Lab software (Scanalytics Inc., Fairfax, VA). Using the centroid of the cell, each position of the cell was obtained throughout every image to measure cell distance. Speed was calculated by dividing the cell distance by the duration of the movement. The directionality of the migrating cells was determined by using scatter diagrams. In the scatter diagram, the outer well, which contained the TNF- α , is represented by the y direction. The starting point of the cells is the intersection of the y- and x-axes (0, 0), the data points correspond with the final position of the individual cells.

Morphological analysis

The Dunn chamber was set up as described previously (20 cells from five different cultures per condition). Using an inverted fluorescence microscope at a magnification of $40\times$, we were able to study the morphology of ESCs. Images were obtained every 6 s for a period of 60 min.

Statistical analysis

Results are presented as mean $\pm$ S.E.M. Comparison between groups was done using one-way ANOVA. When significant group differences were observed, Student's two-tailed *t*-test for unpaired observations was performed. Differences were considered significant with $P < 0.05$.

Results

Mathematical model

A measure of cell mobility should depend amongst other factors on the speed a cell is traveling at, the duration it is traveling for, as well as the distance traveled in the direction of the chemical gradient. One way to express mobility for a cell population is to compute averages of the aforementioned quantities over the cell population. While this has the benefit of maximal information content it is difficult to interpret. A single scalar quantity as a measure of cell mobility is thus favorable. One way of combining the multiple measured cell state quantities into such a single scalar quantity is by weighing of the individual influence terms. However, this results in various weighing constants that need to be determined. Borrowing from variational mechanics this paper thus uses action as a measure for cell mobility. The action, A , of a system between time point t_1 and t_2 is defined as the time integral over the Lagrangian function $L(x(t), d/dt x(t), t)$, where $x(t)$ denotes position, $d/dt x(t)$ velocity, and t time, i.e.,

$$A = \int_{t_1}^{t_2} L(x(t), \dot{x}(t), t) dt.$$

The action is a measure of the time integral of the dissipated energy (up to a constant). Choosing the Lagrangian as the difference between kinetic (T) and potential energy (U), the action may be written as

$$A = \int_{t_1}^{t_2} T - U dt.$$

Regarding the cell as a point mass, m , with the potential gradient given by the chemical gradient, c , towards the wall the action integral becomes

$$A = \int_{t_0}^{t_1} \frac{1}{2} m \dot{x}^2 - (-cx) dt.$$

To compute the action integral exactly, the cell speed and position need to be measured at every time instant, t . To facilitate computations we assume the cell is traveling for a time t_m at the constant speed v , and that the chemical gradient is constant. With $t_1 = 0$ and $t_2 = T$ (overall measurement time) some algebraic manipulations of the action integral result in

$$A = \frac{1}{2} v t_m \left(v + \frac{c}{m} t_m \right) + \frac{c}{m} d (T - t_m)$$

which is the sought for expression for cell mobility. The first term becomes large for cells traveling at high average speeds over long periods of time. The second favors large displacements in the direction of the chemical gradient over short periods of time. Thus, large values of A will indicate high cell mobility.

Calcium increases migration

To investigate whether calcium has an effect on stem cell migration, we performed the migration assays with different concentrations of extracellular Ca^{2+} ($[\text{Ca}^{2+}]_e$), 0.1, 1, 2 and 5 mM (~60 cells from seven separate cultures per condition). We found a significant concentration-dependent increase in the speed of migration (Fig. 1A). In addition, cells moved significantly further and longer when a higher concentration of calcium was added to the medium ($P < 0.05$; Fig. 1B). Experiments with the fluorescent Ca^{2+} indicator, fura-2/AM, demonstrated that increased $[\text{Ca}^{2+}]_e$ significantly increased $[\text{Ca}^{2+}]_i$ (40 cells from five separate cultures per condition, Fig. 2A). However, the most remarkable increases in $[\text{Ca}^{2+}]_i$ were found in cells that were about to migrate or were migrating (Fig. 2B). These results indicate that calcium plays an important role in the migration of embryonic stem cells.

Cyclic AMP stimulates embryonic stem cell migration

It has previously been shown that cAMP can have different effects on migration of many different cell types. We found that dbcAMP had a positive effect on stem cell migration. With regard to mobility, we found an average of $207 \pm 23 \mu\text{m}$ with 1 mM dbcAMP, compared to a $286 \pm 24 \mu\text{m}$ with only 0.1 mM Ca^{2+} ($P < 0.05$, ~60 cells from seven separate cultures per condition). Interestingly, this computed mobility, was comparable to those with high

extracellular calcium, however speed was not. The speed of migration (Fig. 1A) did not significantly increase in the experiments with 1 mM dbcAMP added ($P > 0.05$). Therefore, speed appears to be dependent on a threshold amount of calcium being present in the medium and proves to be the most sensitive parameter in distinguishing the effect of different calcium concentrations on migration.

We examined whether the effect of cAMP on the migration of stem cells is related to its effect on the intracellular calcium concentration. We found that cAMP significantly increased the $[Ca^{2+}]_i$ from 4.2 ± 0.06 nM to 17.4 ± 0.7 nM in ESCs (Fig. 2A). Also, we found a significant difference in the $[Ca^{2+}]_i$ between 5 mM Ca^{2+} and 1 mM dbcAMP (Fig. 2A). We believe this difference might explain the difference in migratory speed induced by these two agonists.

Cyclic GMP affects the mobility of stem cells

We assessed whether cGMP had an effect on migration of ESC. For this we added 1 mM dibutyryl cGMP to the medium. We found no effect with dbcGMP on cell speed compared to dbcAMP or 0.1 mM calcium ($P > 0.05$, Fig. 1A). Mobility however, was significantly reduced after adding dbcGMP ($P < 0.05$, ~60 cells from seven separate cultures per condition). In order to determine whether this depressant effect of cGMP on the mobility of stem cells was related to the calcium concentration of the cell, we measured the ratio of fura-2 after adding dbcGMP to the medium. We found that dbcGMP did increase $[Ca^{2+}]_i$, but not to the same extent as dbcAMP (Fig. 2A).

TNF- α induction of proteopodia

The addition of 5 ng/ml TNF- α to the outer well of the Dunn chamber, resulted in a dramatic increase in cell movement (Fig. 1). To further demonstrate the role of TNF- α in directing stem cell migration, we plotted cell migration in a scatter diagram (Fig. 3). The data points indicate the final positions of the cells after 3 h of recording and show that TNF- α gradients stimulate ESC chemotaxis (Fig. 3). In TNF- α -free setups, the migration was not directionally orientated.

In experiments where we studied the morphology of ESCs, we found that under the influence of TNF- α , ESCs expressed different types of membrane extensions (~20 cells from five different cultures per condition). We detected lamellipodia (Fig. 4A) and filopodia as well as the less described proteopodia [13], which were either short and club-like or long and slender (Fig. 4B and C). The long proteopodia were able to change rapidly, extending and retracting, in search for contact. However, for migration, the short proteopodia appeared to be an important factor, since only motile cells expressed those.

Discussion

The central role of calcium in mediating the movement of various cell types, including adult and fetal stem cells, is well established [14-17]. This study, to our knowledge, is the first to show that the second messenger pathways mediating movement of more differentiated stem cells are also present and functional in embryonic stem cells. Our results demonstrate that a pathway involving calcium regulates the migration of ESCs. Distinct increases in intracellular calcium levels were observed, immediately before and during migration of ESCs. Increases in extracellular calcium levels also enhanced migration.

Calcium is known for its effect on movement of cells. The normal intracellular calcium concentration is about 0.1 μ M but can rise to 10 μ M after release from internal stores. The change in $[Ca^{2+}]_i$ can result in movement and associated changes in the cytoskeleton. Calcium initiates cytoskeletal changes by binding and changing the activity of a cytoskeletal protein, such as calmodulin or troponin, which possess calcium-binding domains [18,19].

To date, most studies about cardiac repair with stem cells have employed adult stem cells [20-22]. However, experimental and theoretical considerations suggest that ESCs may have advantageous properties for tissue regeneration. Better knowledge of the ways of modulating the homing, transmigration and implantation of ESCs is needed for the attainment of therapeutic goals.

Cyclic nucleotides have been shown to modulate the migration and homing of various cell types and we therefore treated and studied the ESCs with dbcAMP and dbcGMP. We found that dbcAMP increased mobility of the cells. As expected, we found that dbcAMP raised the intracellular calcium concentration. Cyclic AMP is a second messenger that activates protein kinase A (PKA) and is known to be involved in many cell functions, as well as cell shape and organization of actin cytoskeleton. PKA on activation in turn organizes actin in bundles [6,7, 23].

As in the case of cAMP, cGMP has been described in the literature as having inhibitory as well as stimulatory effects on cell migration. Elferink et al. explain this discrepancy by claiming that the concentrations of cGMP used in experiments and conditions such as the existence of extracellular calcium and chemotactic agents, determine whether cGMP will have a stimulatory or inhibitory effect on migration [24]. Our data suggest that cyclic GMP has a depressant effect on stem cell migration. Cyclic GMP is an important second messenger, with several intracellular targets. It can bind to protein kinase G (PKG), which is known to suppress the production of the extracellular matrix proteins, which in turn are needed in the migration and proliferation of several cell types such as smooth muscle cells and HUVEC cells [8,9,25]. Furthermore, cyclic GMP is known for mediating most actions of nitric oxide (NO) that in turn is implicated in inhibiting the formation of F-actin [26,27]. These results are important because they indicate that factors altering the intracellular levels of $[Ca^{2+}]_i$, cAMP and cGMP may modulate the homing, transmigration and attachment of ESCs to desired sites of repair and regeneration in organs, including the heart.

We were able to study the migratory patterns at cellular level by using videomicroscopy. To increase cell speed, morphological changes and minimal adhesion are necessary [28,29]. We achieved the maximum speed by not coating the cells to a coverslip and by using calcium to change the morphology. Since we found that the presence of a threshold amount of $[Ca^{2+}]_e$ is necessary for reaching high speeds, calcium can be valued as an important factor for the homing of ESCs. As for directionality, the influence of TNF- α on stem cell migration was proven by their chemotactic response to concentration gradients of TNF- α (Fig. 3), hence confirming the previous findings of our group that TNF- α attracts ESCs [11]. Using videomicroscopy, we were able to study the morphology of the moving cells. We distinguished several forms of extensions, such as filopodia, lamellipodia and proteopodia. Previously, proteopodia have been described in a study of haematopoietic stem cells (HSC), where they were associated with directed motility, homing and engraftment of HSCs [13]. In our setup, these proteopodia were only observed when TNF- α was used in the experiments, implicating an important role of TNF- α in the expression of these proteopodia and in inducing stem cell migration.

In summary, we have shown that calcium and cyclic nucleotides play an important role in mediating the movement of embryonic stem cells. We plan to further investigate the actions and interactions of calcium, cAMP and cGMP to understand these cellular mechanisms in anticipation of being able to use these to enhance the use of stem cell transplantation.

References

- [1]. Hill JM, Dick AJ, Raman VK, Thompson RB, Yu ZX, Hinds KA, Pessanha BS, Guttman MA, Varney TR, Martin BJ, Dunbar CE, McVeigh ER, Lederman RJ. Serial cardiac magnetic resonance imaging of injected mesenchymal stem cells. *Circulation* 2003;108:1009–1014. [PubMed: 12912822]

- [2]. Abbott JD, Giordano FJ. Stem cells and cardiovascular disease. *J. Nucl. Cardiol* 2003;10:403–412. [PubMed: 12900745]
- [3]. Xu C, Police S, Rao N, Carpenter MK. Characterization and enrichment of cardiomyocytes derived from human embryonic stem cells. *Circ. Res* 2002;91:501–508. [PubMed: 12242268]
- [4]. Malek S, Kaplan E, Wang JF, Ke Q, Rana JS, Chen Y, Rahim BG, Li M, Huang Q, Xiao YF, Verheugt FW, Morgan JP, Min JY. Successful implantation of intravenously administered stem cells correlates with severity of inflammation in murine myocarditis. *Pflugers Arch* 2006;452:268–275. [PubMed: 16715295]
- [5]. Stossel TP. On the crawling of animal cells. *Science* 1993;261:1086–1094. [PubMed: 8493552]
- [6]. Sanchez-Madrid F, del Pozo MA. Leukocyte polarization in cell migration and immune interactions. *EMBO J* 1999;18:501–511. [PubMed: 9927410]
- [7]. Howe AK. Regulation of actin-based cell migration by cAMP/PKA. *Biochim. Biophys. Acta* 2004;1692:159–174. [PubMed: 15246685]
- [8]. Smolenski A, Poller W, Walter U, Lohmann SM. Regulation of human endothelial cell focal adhesion sites and migration by cGMP-dependent protein kinase I. *J. Biol. Chem* 2000;275:25723–25732. [PubMed: 10851246]
- [9]. Dye NB, Boerth NJ, Murphy-Ullrich JE, Chang PL, Prince CW, Lincoln TM. Cyclic GMP-dependent protein kinase inhibits osteopontin and thrombospondin production in rat aortic smooth muscle cells. *Circ. Res* 1998;82:139–146. [PubMed: 9468184]
- [10]. Li D, Zhao L, Liu M, Du X, Ding W, Zhang J, Mehta JL. Kinetics of tumor necrosis factor alpha in plasma and the cardioprotective effect of a monoclonal antibody to tumor necrosis factor alpha in acute myocardial infarction. *Am. Heart J* 1999;137:1145–1152. [PubMed: 10347344]
- [11]. Chen Y, Ke Q, Yang Y, Rana JS, Tang J, Morgan JP, Xiao YF. Cardiomyocytes overexpressing TNF- α attract migration of embryonic stem cells via activation of p38 and c-Jun N-terminal kinase. *FASEB J* 2003;17:2231–2239. [PubMed: 14656985]
- [12]. Grynkiewicz G, Poenie M, Tsien RY. A new generation of Ca^{2+} indicators with greatly improved fluorescence properties. *J. Biol. Chem* 1985;260:3440–3450. [PubMed: 3838314]
- [13]. Frimberger AE, McAuliffe CI, Werme KA, Tuft RA, Fogarty KE, Benoit BO, Dooner MS, Quessenberry PJ. The fleet feet of haematopoietic stem cells: rapid motility, interaction and proteopodia. *Br. J. Haematol* 2001;112:644–654. [PubMed: 11260067]
- [14]. Dedieu S, Poussard S, Mazeres G, Grise F, Dargelos E, Cottin P, Brustis JJ. Myoblast migration is regulated by calpain through its involvement in cell attachment and cytoskeletal organization. *Exp. Cell Res* 2004;292:187–200. [PubMed: 14720518]
- [15]. Munevar S, Wang YL, Dembo M. Regulation of mechanical interactions between fibroblasts and the substratum by stretch-activated Ca^{2+} entry. *J. Cell Sci* 2004;117:85–92. [PubMed: 14627625]
- [16]. Henschler R, Piiper A, Bistran R, Mobest D. SDF-1 α -induced intracellular calcium transient involves Rho GTPase signaling and is required for migration of hematopoietic progenitor cells. *Biochem. Biophys. Res. Commun* 2003;311:1067–1071. [PubMed: 14623290]
- [17]. Ueda S, Mizuki M, Ikeda H, Tsujimura T, Matsumura I, Nakano K, Daino H, Honda Zi Z, Sonoyama J, Shibayama H, Sugahara H, Machii T, Kanakura Y. Critical roles of c-Kit tyrosine residues 567 and 719 in stem cell factor-induced chemotaxis: contribution of src family kinase and PI3-kinase on calcium mobilization and cell migration. *Blood* 2002;99:3342–3349. [PubMed: 11964302]
- [18]. Bray, D. *Cell Movements: From Molecules to Motility*. second ed.. Garland Publishing; New York: 2001.
- [19]. van Haelst C, Rothstein TL. Cytochalasin stimulates phosphoinositide metabolism in murine B lymphocytes. *J. Immunol* 1988;140:1256–1258. [PubMed: 2830337]
- [20]. Wang FS, Trester C. Bone marrow cells and myocardial regeneration. *Int. J. Hematol* 2004;79:322–327. [PubMed: 15218958]
- [21]. Chiu RC. Bone-marrow stem cells as a source for cell therapy. *Heart Fail. Rev* 2003;8:247–251. [PubMed: 12878833]
- [22]. Menasche P. Cell therapy of heart failure. *CR Biol* 2002;325:731–738.
- [23]. Glenn HL, Jacobson BS. Cyclooxygenase and cAMP-dependent protein kinase reorganize the actin cytoskeleton for motility in HeLa cells. *Cell Motil. Cytoskeleton* 2003;55:265–277. [PubMed: 12845600]

- [24]. Elferink JG, vanUffelen BE. The role of cyclic nucleotides in neutrophil migration. *Gen. Pharmacol* 1996;27:387–393. [PubMed: 8919662]
- [25]. Deguchi A, Thompson WJ, Weinstein IB. Activation of protein kinase G is sufficient to induce apoptosis and inhibit cell migration in colon cancer cells. *Cancer Res* 2004;64:3966–3973. [PubMed: 15173009]
- [26]. Agullo L, Garcia-Dorado D, Escalona N, Inserte J, Ruiz-Meana M, Barrabes JA, Mirabet B, Pina P, Soler-Soler J. Hypoxia and acidosis impair cGMP synthesis in microvascular coronary endothelial cells. *Am. J. Physiol* 2002;283:917–925.
- [27]. Torreilles J. Nitric oxide: one of the more conserved and widespread signaling molecules. *Front. Biosci* 2001;6:D1161–D1172. [PubMed: 11578977]
- [28]. Walmod PS, Hartmann-Petersen R, Prag S, Lepekhin EL, Ropke C, Berezin V, Bock E. Cell-cycle-dependent regulation of cell motility and determination of the role of Rac1. *Exp. Cell Res* 2004;295:407–420. [PubMed: 15093740]
- [29]. Sroka J, Kaminski R, Michalik M, Madeja Z, Przestalski S, Korohoda W. The effect of triethyl lead on the motile activity of walker 256 carcinosarcoma cells. *Cell Mol. Biol. Lett* 2004;9:15–30. [PubMed: 15048148]

Fig. 1.

Calcium and cAMP but not cGMP stimulate TNF- α induced migration of ESCs. (A) TNF- α and calcium increased ESCs speed of migration compared to controls. Administration of cAMP or cGMP did not affect cell speed. Speed of migration was determined for each condition by dividing total distance by the total time of migration. (B) Calcium and cAMP stimulated and cGMP diminished ESC mobility. ESCs subject to TNF- α were significantly more mobile than the control cell population. (Mean $\pm$ S.E.M., * P < 0.05 vs. control, ** P < 0.05 vs. TNF- α plus 0.1 mM Ca²⁺, *** P < 0.05 vs. TNF- α plus 1 mM Ca²⁺, ~60 cells from seven separate cultures per condition).

Fig. 2. Changes in intracellular calcium [Ca²⁺]_i concentration. (A) Increase of intracellular [Ca²⁺]_i, induced by extracellular calcium, dbcAMP and dbcGMP. At least 40 cells from five separate cultures were analyzed per condition, ratio was calculated every 6 s. **P* < 0.05 vs. control. (B) Changes in [Ca²⁺]_i of migrating stem cells. The increase in [Ca²⁺]_i compared to quiescent cells demonstrates to be of temporary nature as it is pumped out of the cell during the time of migration. (Mean ± S.E.M., ~20 cells from five separate cultures).

Fig. 3. Effect of TNF- α on the directional migration of ESCs. The starting point for each cell is at the intersection between x and y axes (0, 0) and data points show the final location of individual cells after the 3-h recording period. The source of TNF- α (5 ng/ml) is at the top. Data of five separate cultures are shown, at least 50 cells per condition.

Fig. 4. Representative images of migrating embryonic stem cells with different membrane extensions. The experiments were performed in the presence of $\text{TNF-}\alpha$ as chemotactic agent. (A) Embryonic stem cell expressing lamellipodia (40 $\times$). (B) ESC with short proteopodia (40 $\times$). (C) Embryonic stem cell expressing long proteopodia (40 $\times$).