**In Vitro** Characterization of the Molecular Machinery Regulating Umbilical Cord Blood Mesenchymal Stem Cell Angiogenesis: A Step Towards Multipotent Stem Cell Therapy for Vascular Regeneration

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**Abstract**

Umbilical cord blood (UCB)-derived mesenchymal stem cells (MSCs) promote vascular growth in vivo. Here we examined the intracellular regulatory machinery involved in the *in vitro* angiogenic behaviour exhibited by UCBMSCs. Angiogenic activity was measured in the standard Matrigel-based culture assay after treatment of cells with known modulators of early growth response factor (Egr-3) and endothelial cell (EC) angiogenesis. Egr-3 expression was assessed by quantitative RT-PCR and indirect immunofluorescence, and specifically abrogated using small interfering RNA (siRNA) technology. While addition of phorbol-12-myristate-13-acetate (PMA) promoted angiogenic capacity (*P*<0.001), selective inhibitors of PKC/MAPK/ERK abrogated this capacity (*P*<0.016) in UCBMSCs. Treatment with PMA increased Egr-3 mRNA and protein levels (*P*<0.001). However, cyclosporine A (CsA) and vascular endothelial growth factor (VEGF) affected neither Egr-3 levels nor the formation of polygonal cell networks. PMA also induced ERK1/2 phosphorylation, which was abolished by the selective inhibitor U0126 (*P*=0.021 and *P*=0.014, respectively). Marked inhibition of network-forming capacity was observed in siEgr-3-transduced cells (*P*<0.001). Taken together, our results highlight that Egr-3 is commonly involved in the molecular machinery regulating mature EC and multipotent MSC angiogenesis. This knowledge may be applied to increase therapeutic efficacy against human vascular diseases.

**Keywords:** Umbilical cord blood; Mesenchymal stem cells; Endothelial cell; Angiogenesis; Egr-3

**Abbreviations:** Ab: Antibody; AAD: 7-Amino-Actinomycin D; BCA: Bicinchoninic Acid Assay; Cal C: Calphostin C, Ca2+: Cyclopentoridine A; DMSO: Dimethyl Sulfoxide; Egr-3: Early Growth Response 3; ERK: Extracellular-signal Related Kinase; FBS: Fetal Bovine Serum; GAPDH: Glyceraldehyde 3-phosphate Dehydrogenase; HUVEC: Human Umbilical Vein Endothelial Cell; ISO: Isoproterenol; MAPK: Mitogen-activated Protein Kinase; MSC: Mesenchymal Stem Cell; PBS: Phosphate-buffered Saline; PKC: Protein Kinase C; PMA: Phorbol-12-myristate-13-acetate; RT: Room Temperature; SDF-1: Stromal Cell-derived Factor 1; SDS-PAGE: Sodium Dodecyl Sulphate-polyacrylamide Gel Electrophoresis; siRNA: Small Inhibitory RNA; SD: Standard Deviation; UCB: Umbilical Cord Blood; VEGF: Vascular Endothelial Growth Factor

**Introduction**

In order to maintain homeostasis, tissues of multicellular organisms are endowed with a vascular system responsible for gas and nutrient exchange. Maintenance of this paramount function is dependent on endothelium integrity. Our view of the endothelium has changed gradually over the years. Whereas the endothelium used to be considered a simple inert barrier between blood vessels and subjacent tissues, it is now recognized as a specialized layer of cells - endothelial cells (ECs) - [1]. It is clear that ECs have critical roles in organism homeostasis and in moving nutrients, gases and waste products to and from cells [2-4]. Therefore, loss of vascular endothelial homeostasis critically contributes to the origin and/or progression of numerous ischemic, inflammatory, infectious and immune diseases in humans [5].

There is compelling evidence that vascular maintenance arises due mainly to two non-exclusive mechanisms: local maintenance by pre-existing or mature (terminally differentiated) ECs (sprouting angiogenesis) [6] and distant maintenance by specific recruitment of vascular precursors from bone marrow (vasculogenesis) [7]. A variety of both cellular and extracellular molecules, including numerous growth and early response factors, chemokines, interleukins and metalloproteinases, take part in active angiogenesis [8,9]. These molecules include the early growth response factor (Egr-3), which is a transcription factor rapidly induced by extracellular stimuli that regulates proliferation and differentiation in multiple cell types such as ECs [10]. Moreover, Egr-3 has been reported as one of the most strongly activated genes by vascular endothelial growth factor (VEGF) and its specific suppression attenuates EC growth [11,12]. At the molecular level, Egr-3 has been related to intracellular signaling mediated by protein kinase C (PKC) [13,14] and mitogen-activated protein kinase (MAPK)/extracellular-signal related kinase (ERK) [15,16]. Notably, Li

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et al. [15] described Egr-3 as the precise effector molecule of MAPK-ERK signaling involved in enduring memory formation in activated neurons.

In recent years, umbilical cord blood (UCB) has emerged as a promising source of cells for human regenerative medicine [17] and UCB-derived mesenchymal stem cells (MSCs) have been shown to promote vascular growth in vivo [18]. However, further understanding of the molecular mechanisms regulating UCBMSC angiogenesis should provide insights that may help develop more effective therapies against human diseases that involve vascular deficits. Particularly, we hypothesized that Egr-3 was a key mediator of UCBMSC angiogenesis because its expression increased as cells gained EC characteristics and self-organized to form branched polygonal cell networks similar to those developed by bona fide ECs [18].

Thus, with the overarching aim of improving their therapeutic efficacy, we here examined the intracellular regulatory machinery involved in the angiogenic behaviour exhibited by UCBMSCs by: (1) exposure to known modulators of both Egr-3 and EC angiogenesis; (2) assessment in the standard Matrigel assay; and (3) specific suppression of Egr-3 expression using small interfering RNA (siRNA) technology.

Materials and Methods

Cell culture and treatments

Isolation and culture of UCBMSCs were previously described in detail [14,18,19]. Briefly, a total of 25 UCB samples (60-100 ml) from the umbilical cord vein were processed 12 h post-extraction. Blood cells were clarified by previous centrifugation and resuspended in 30 ml calcium- and magnesium-free phosphate-buffered saline (PBS) (Invitrogen). The cell suspension layered over 1.077 g/ml Lymphoprep (Nycodem) was centrifuged at 400 g for 30 minutes. Mononuclear cells recovered by centrifugation were eliminated from contaminant red blood cells by incubation with lysis reagent Pharm-Lyse (BD Biosciences) for 15 minutes. Recovered cells were grown in α-MEM supplemented with 30% fetal bovine serum (FBS), 1% penicillin/streptomycin (Invitrogen), 1 mM L-glutamine and 10 μM dexamethasone (Sigma) at 37°C in 5% CO₂ in air. Adherent cells were seeded in 5% CO₂ in air. Adherent cells were selected at day 10 following Oil red O (Sigma) staining, which detects the presence of intracellular lipid accumulation.

Differentiation assays

Adipogenic induction: UCBMSCs were cultured in α-MEM (Sigma) supplemented with 10% FBS, 1 mM L-glutamine and 1% penicillin/streptomycin. Differentiation samples were fixed at day 14 following Oil red O (Sigma) staining. Deposition of calcium matrix was then detected by specific staining with Alizarin red S (Sigma).

Quantitative RT-PCR

Whole RNA content was isolated from cells using the QuickPrep Extraction Kit (Amersham). cDNA from 2 μg of RNA was obtained using random hexamers and the Script™ One-Step RT-PCR Kit (Bio-Rad) following the supplier’s protocol. Subsequently, 2 μl of cDNA were amplified in a final volume of 50 μl containing 25 μl TaqMan PCR Master Mix and 2 μl of the following FAM-labelled primer/probes (Applied Biosystems): CD31 (Hs00169777_m1), CD34 (Hs00990732_m1), CD36 (Hs00169267_m1), CD102 (Hs00163841_m1), sarcomeric α-actinin (Hs00241650_m1) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (Hs99999905_m1). Each sample was run in duplicate in the same experiment and amplification data from three independent experiments were analyzed on the ABI Prism 7000 Sequence Detection System. The Δ threshold cycle (Ct) method was used to quantify the relative expression for each gene using GAPDH as endogenous reference [20].

Indirect immunofluorescence

Egr-3 protein expression was analyzed following fixation with 4% paraformaldehyde (Sigma) for 10 min at room temperature (RT). Cells were permeabilized with magnesium-free PBS supplemented with 10% horse serum and 0.5% Triton X-100 (Sigma) during 2 h at RT. A specific rabbit Ab against human Egr-3 (2 μg/ml; Santa Cruz Biotechnology) was then applied. Cell nuclei were also counterstained with Hoechst 0.5 μg/ml (Sigma) for 10 min at RT. A donkey anti-rabbit Alexa Fluor 688-conjugated Ab (1 μg/ml; Invitrogen) was finally used to detect labelled cells under a Leica confocal laser scanning microscope (TCS SP5).

Western blotting

Whole cell extracts were obtained using the Mammalian Cell
Protein levels were quantified using the Quantity One 1-D Analysis knockdown Egr-3 gene expression. All cell transductions were 3-5 target-selective 19-25 nucleotides in length designed to efficiently siEgr-3 (Santa Cruz Biotechnology). The siEgr-3 consisted of a pool of Therapy criteria (standard surface antigen pattern and multipotency) recognized as MSCs according to the International Society for Cellular

statistical tests were considered significant when to determine significance among more than two groups. Descriptive way analysis of variance with Tukey's B post-hoc analysis was applied or transduced with siRNA sequences, were detached using non-

inhibitors added to the cultures were kept in the samples for the pre-coated with 200µl ECMatrix (Matrigel). Agonists and selective inhibitors added to the cultures were kept in the samples for the duration of the experiment. The cell networks that developed were examined from 10 images. Angiogenic capacity was measured as the number of network circles per mm², a measurement equivalent to the classic determination of total network length [21]. Three independent cultures were tested per condition.

siRNA transfection

Cells were transduced with 2 µg siControl-GFP, siControl A and siEgr-3 (Santa Cruz Biotechnology). The siEgr-3 consisted of a pool of 3-5 target-selective 19-25 nucleotides in length designed to efficiently knockdown Egr-3 gene expression. All cell transductions were performed via a nonviral Nucleofector™ procedure (Amansa® Cell Line Optimization Nucleofector® Kit; Lonza Cologne AG) with the program U23 for transfection of human MSCs according to the manufacturer’s instructions [22].

Statistical analysis

Statistical analysis was performed using two-tailed Student’s t-tests. Values are expressed as mean ± standard deviation (SD). One-way analysis of variance with Tukey’s B post-hoc analysis was applied to determine significance among more than two groups. Descriptive statistics were performed using SPSS Statistics 15.0.1 (SPSS Inc.), and statistical tests were considered significant when P<0.05.

Results

Modulation of Egr-3 expression by PKC/MAPK/ERK in UCBMSCs

Primary cultures of elongated fibroblast-like cells were established from human UCB samples. Cultured cells were homogeneously recognized as MSCs according to the International Society for Cellular Therapy criteria (standard surface antigen pattern and multipotency) [23]. The cells were strongly positive for CD105 (18.5 ± 0.14) and CD90 (60 ± 11), and consistently negative for CD45 (1 ± 0), CD34 (1.2 ± 0.2) and CD14 (1 ± 0) (Figure 1A). Induction of UCBMSCs with adipogenic and osteogenic media resulted in intracellular accumulation of lipid droplets and in high extracellular deposition of calcium, respectively (Figure 1B).

We had previously demonstrated the following: 1) Egr-3 expression was highly promoted when human UCBMSCs differentiated towards the EC lineage; 2) in Matrigel, similar to mature ECs such as human umbilical vein ECs (HUVECs), UCBMSCs had the ability to rapidly migrate developing well-organized vascular-like networks; and 3) developed networks showed high amounts of Egr-3 within the nuclei of aligned cells [18].

In accordance with this background, we further investigated the molecular machinery regulating the angiogenic behaviour of UCBMSCs and whether Egr-3 was mechanistically involved. Thus, cells were then exposed to PMA, CsA and VEGF, known modulators of Egr-3 expression and EC angiogenesis, and seeded in Matrigel. In this standard assay of angiogenesis, which is often preferred over other in vitro assays because of its ease of use, rapidity and the ability to measure key steps in angiogenesis, cells rearrange and align forming a polygonal cell network resembling growing blood vessels from which angiogenic capacity may be evaluated, e.g. by measuring the number of network circles per unit area [24-26]. Egr-3 gene expression level was also assessed using real-time PCR, which is the most direct, sensitive, reproducible and accurate method to quantify gene activity [27].

While PMA induced increases in Egr-3 mRNA and protein and promoted the capacity to form well-organized vascular-like networks in comparison with untreated UCBMSCs (P<0.001), the addition of either CsA nor VEGF significantly affected any of these properties (Figure 2). In addition, exposure to calphostin C (Cal C), which is a commonly-used inhibitor of PKCa, drastically reduced both PMA-induced Egr-3 gene transcription (P<0.001) and the Matrigel-mediated formation of cell network circles by UCBMSCs (P<0.001; Figure 2). Stimulation of cells with the G protein-coupled receptor agonist isoproterenol (ISO) [28] also doubled Egr-3 gene expression in comparison with untreated cells (P=0.013; data not shown). In contrast to the effect of each agent individually, combined ISO and PMA treatment had a reduced effect in comparison with PMA alone regarding Egr-3 activation and network-forming capacity (Figure 2). U0126, which is a selective pharmacological inhibitor of MAPK/ERK signaling [29], suppressed both Egr-3 transcriptional activation and cell network organization in treated cells (P=0.015 and P=0.016, respectively; Figure 2). Moreover, the presence of Cal C in the cell culture medium significantly reduced PMA-induced angiogenesis as well as Egr-3 activation in UCBMSCs (P<0.001; Figure 2). Neither cell network-forming ability nor Egr-3 expression in UCBMSCs were affected by addition of U0124, an inactive structural analogue of U0126 (Figure 2).

Importantly, analysis of both Annexin V expression and 7-amino-actinomycin D (7-AAD) staining by flow cytometry showed that the observed changes in the angiogenic behaviour of UCBMSCs did not result from variations in cell apoptotic rate and/or viability following each cell treatment (Figure 3). Western blot experiments also evidenced that the addition of PMA to UCBMSCs induced an increase in ERK1/2 phosphorylation (P=0.021), and that this effect was significantly abolished by treatment with U0126 (P=0.014; Figure 4).

Collectively, these observations suggest so far that the modulation of Egr-3 expression and the promotion of network-forming capacity in UCBMSCs are mediated through PKC/MAPK/ERK signaling.
Specific suppression of Egr-3 abrogates cell network capacity of UCBMSCs

To clearly demonstrate that Egr-3 directly regulates UCBMSC angiogenic behaviour, cells were transduced with a specific siRNA against Egr-3. This strategy resulted in a marked reduction of both Egr-3 mRNA and protein levels in siEgr-3-transduced cells relative to those transduced with control siRNA (siControlA) (P<0.001; Figures 5A and 5B). Moreover, the number of developed network circles was inhibited by 85% in siEgr-3-transduced UCBMSCs (Figure 5B). Interestingly, specific suppression of Egr-3 expression had no effect on either cell apoptotic rate or cell viability (Figure 3). Data from quantitative RT-PCR analysis of siEgr-3-transduced UCBMSCs finally indicated that the expression pattern of EC markers was not broadly different in comparison with that exhibited by siControlA-transduced cells. Only CD36 expression was reduced by 50%, whereas CD102 activation increased (2-fold) in siEgr-3-transduced cells (Figure 5C). Thus, the transcriptional profile observed in siEgr-3-transduced UCBMSCs finally indicated that the expression pattern of EC markers was not broadly different in comparison with that exhibited by siControlA-transduced cells. Only CD36 expression was reduced by 50%, whereas CD102 activation increased (2-fold) in siEgr-3-transduced cells (Figure 5C). Thus, the transcriptional profile observed in siEgr-3-transduced UCBMSCs finally indicated that the expression pattern of EC markers was not broadly different in comparison with that exhibited by siControlA-transduced cells. Only CD36 expression was reduced by 50%, whereas CD102 activation increased (2-fold) in siEgr-3-transduced cells (Figure 5C).

Discussion

The presented results identify Egr-3, which is a transcriptional factor highly expressed during mature, terminally differentiated EC angiogenesis [11,30], as a key mediator of UCBMSC angiogenesis. In particular, we find that a central PKCα/MAPK/ERK pathway common to mature ECs regulates Egr-3 in UCBMSCs. Moreover, selective suppression of Egr-3 significantly abrogates the angiogenic capacity of UCBMSCs. Following Egr-3 inhibition, the reduction in network circles developed by UCBMSCs was similar to that previously found in ECs [10]. Thus, our findings suggest the existence of a conserved regulatory machinery regulating angiogenesis between ECs and multipotent MSCs (Figure 6). This knowledge should provide insights that may help develop more effective therapies against human vascular diseases.

In addition, our results agree with the traditional concept that PKC has an essential role in angiogenesis, based on the findings that...
Phorbol esters, by mimicking the natural PKC activator diacylglycerol, induce angiogenesis [31-33] and that specific knockdown of PKC prevents PMA-induced angiogenesis [13]. In our experiments, Egr-3 expression and cell network-forming capacity of UCBMSCs was not affected by VEGF levels or by the powerful immunosuppressive agent CsA, which are known to modulate VEGF-mediated EC angiogenesis and Egr-3 levels in hematopoietic-lineage cells such as lymphocytes T respectively [11,13,30,34-37]. In regards to both VEGF and CsA, the

Figure 2: Modulation of Egr-3 expression levels and angiogenic capacity by UCBMSCs. Representative images and quantification of the cell network-forming capacity, measured as the number of network circles per unit area, of UCBMSCs after 2 h in Matrigel following treatment with a specific agonist (PMA) and inhibitors (Cal C and U0126) of PKCo/MEK/Egr-3 signaling. Scale bars: 100 µm. Histogram represents the relative levels of Egr-3 gene expression and cell network formation compared with those in control or untreated cells. DMSO and U0124 were used as vehicle and negative control, respectively. Note that there is equivalence in the scales of gene expression and network circles/mm², maybe indicating the close relationship between these two cellular events. Data are from three independent experiments and all values are expressed as mean ± SD. Specific detection of Egr-3 protein in control, PMA- and U0126-treated UCBMSC cultures by indirect immunofluorescence. *P<0.001, **P=0.016 and †P=0.015. Scale bars: 20 µm. A minimum of 10 microscopic fields per condition and experiment (n=3) were analyzed with similar results.
**Figure 3: Analysis of cell viability.** Raw data from representative analysis of Annexin (Ann) V expression and 7-AAD staining in UCBMSCs treated with camptothecin, used as positive control for cell apoptosis, calphostin C and U0126, and following transduction with siEgr-3. Ann+7-AAD- and 7-AAD+ label early and late apoptotic cells, respectively. The percentage of live cells under each condition is indicated in the corresponding dot plot. Treatment with camptothecin induced a moderate amount of programmed cell death, indicative of intrinsic UCBMSC robustness. The 10-fold increase in the fluorescence emitted by calphostin C-treated cells did no effect measured levels of cell death. N=2 for each experimental condition with similar results.

**Figure 4: PMA induces phosphorylation of ERK1/2 in UCBMSCs.** Western blot panels show that ERK1 (44 kDa) and ERK2 (42 kDa) were phosphorylated following exposure to PMA. In contrast, addition of U0126 suppressed PMA-mediated ERK1/2 phosphorylation. Histogram depicts protein levels as arbitrary units (A.U.), which were determined by densitometry in three independent experiments. A 3.6-fold activation of ERK1/2 was detected in PMA-treated relative to control cells. N=3 for each experimental condition with similar results. Values are expressed as mean ± SD. *P=0.021 and **P=0.014.
Figure 5 Inhibition of UCBMSC angiogenic capacity by specific suppression of Egr-3. (A) Representative images showing internalization of control sequence (siControl-GFP conjugate) (left), and specific detection of Egr-3 in siControlA- (middle) and siEgr3-transduced (right) cells. Scale bars: 20 µm. (B) Representative images of the cell network developed by UCBMSCs transduced with siControlA and siEgr3 after 5 h in Matrigel. Histogram indicates the number of network circles and the transcriptional levels of Egr-3 in siControlA- and siEgr3-transduced cells. A minimum of 10 microscopic fields per condition and experiment (n=3) were analyzed. Scale bars: 100 µm. Values are expressed as mean ± SD and *P<0.001. (C) Transcriptional profile exhibited by siEgr3-transduced UCBMSCs showing the relative changes (compared with siControlA-transduced cells) in specific (CD31, CD34, CD36, CD102 and VEGFA) and non-specific (SDF-1α and sarcomeric α-actinin) EC genes. N=3 with similar results. Values are expressed as mean ± SD. *P<0.001 and **P<0.05.
lack of response by UCBMSCs may be due, in part, to the fact that these cells do not express baseline levels of VEGFR-2 [18] and do not belong to this particular cell lineage. However, Egr-3 levels declined and vascular-like network formation was inhibited following treatment with Cal C and U0126, known inhibitors of PKCα and MAPK/ERK (MEK) signaling [10,15,38,39]. ISO, which has been related to MEK activation [28], also produced a paradoxical effect; stimulating Egr-3 expression in UCBMSCs when used alone, but having an attenuating effect when used in combination with PMA. Although increase in Egr-3 level following ISO treatment could be a mere artifact arising from the fact that isoproterenol and modulators of Egr-3 gene expression utilize common signaling pathways, this result could support previous observations of a regulatory mechanism based on crosstalk between G protein-coupled receptors and components downstream of receptor tyrosine kinases such as MAPK [40]. Thus, taken together, it would appear that independent of CsA and VEGF, Egr-3 mediated UCBMSC angiogenic behaviour through the integration of distinct signals which efficiently modulate the level of its downstream response. Further studies would be necessary to determine whether Egr-3 expression is also modulated through both CXCR-4- and p38 mitogen-activated kinase-mediated signaling in UCBMSCs, as is the case in T lymphocytes [39]. For instance, inhibition of Egr-3-mediated activation through the p38 pathway could enhance cell differentiation towards the cardiomyogenic lineage, as suggested by previous findings using human embryonic stem cells [41].

In conclusion, we confirm that UCBMSCs constitute a valuable model for the analysis of angiogenic cell migration and assembling into growing vascular structures [18]. In a clinical context, the understanding that effective repair of the vast majority of tissues must be accompanied by the provision of an adequate vascular system has prompted an active search for cell types that could facilitate this process during therapy. Recently, together with MSCs derived from bone marrow and adipose tissue [42], UCB has been shown to be a valuable source for human regenerative medicine [17]. Particularly, UCBMSCs exhibit high capacity for migration and development of an extensive, well-organized polygonal cell network that is equivalent to that produced by HUVECs in vitro [18]. Furthermore, in mice, UCBMSCs...
induces the generation of microvasculature connected with the host circulatory system. When implanted with fibrin in a myocardial infarct model, UCBMSCs also organized into vascular networks, reduced scar size and increased the blood vessel-occupied area in the subjacent myocardium [18]. The findings presented here contribute to a better understanding of the molecular machinery regulating angiogenesis by multipotent MSCs [43]. Notably, we highlight that Egr-3 is commonly involved regulating mature EC and multipotent MSC angiogenesis through a central PKCα/MAPK/ERK via; this knowledge may be applied to increase efficacy of cell therapies against human diseases with vascular deficit, including cardiac alterations of ischemic origin.

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References


