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Rafs constitute a nodal point in the regulation of embryonic endothelial progenitor cell growth and differentiation

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Abstract

Mouse embryonic endothelial progenitor cells (eEPCs) acquire a mature phenotype after treatment with cyclic adenosine monophosphate (cAMP), suggesting an involvement of Raf serine/threonine kinases in the differentiation process. To test this idea, we investigated the role of B-Raf and C-Raf in proliferation and differentiation of eEPCs by expressing fusion proteins consisting of the kinase domains from Raf molecules and the hormone binding site of the estrogen receptor (ER), or its variant, the tamoxifen receptor. Our findings show that both B- and C-Raf kinase domains, when lacking adjacent regulatory parts, are equally effective in inducing eEPC differentiation. In contrast, the C-Raf kinase domain is a more potent stimulator of eEPC proliferation than B-Raf. In a complimentary approach, we used siRNA silencing to knockdown endogenously expressed B-Raf and C-Raf in eEPCs. In this experimental setting, we found that eEPCs lacking B-Raf failed to differentiate, whereas loss-of C-Raf function primarily slowed cell growth without impairing cAMP-induced differentiation. These findings were further corroborated in B-Raf null eEPCs, isolated from the corresponding knockout embryos, which failed to differentiate *in vitro*. Thus, gain- and loss-of-function experiments point to distinct roles of B-Raf and C-Raf in regulating growth and differentiation of endothelial progenitor cells, which may harbour therapeutic implications.

Keywords: MAP kinases • signal transduction • cAMP • stem cells • EPCs

Introduction

Experimental evidence in animal models shows that administration of EPCs ameliorates the function of ischaemic organs, or improves re-endothelialization after arterial injury [1–3]. Mouse embryonic EPCs (eEPCs) are a subtype of progenitors, Tie-2^+, c-Kit^+, Sca-1^+, Flk-$1^{-/low}$, which we have initially isolated and characterized [4, 5]. The embryonic cells may harbour functional advantages over adult EPCs including reliable genetic manipulation and high proliferative capacity with practically unlimited growth potential in culture [4]. Moreover, eEPCs promote blood vessel growth in ischaemic conditions after

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transplantation making them an attractive experimental system for molecular studies [6,7].

Recent clinical trials demonstrated a benefit of bone marrow or peripheral blood-derived EPCs in tissue revascularization and recovery after ischaemic injury [8, 9]. However, it appears that long-term survival, growth and differentiation of donor cells may be limited [7,10,11]. Therefore, there is a need to identify signalling mechanisms to enhance growth and differentiation of EPCs after transplantation.

The Raf serine/threonine kinases are important components of the mitogen-activated protein kinase (MAPK) pathway. The family comprises of three members A-, B- and C-Raf (or Craf1). Raf proteins have been studied in many cell types [12–14], but remain of particular interest for their roles in a broad variety of tumours [15–17]. Their function in endothelial development has been suggested in a mouse knockout model of B-Raf, which revealed a vascular phenotype characterized by elevated numbers of eEPCs and increased apoptosis of mature endothelial cells, leading to vessel rupture, haemorrhage and death between E10-12 [18].

We have previously identified cAMP as an agent promoting eEPC differentiation. cAMP treatment leads to induction of endothelial gene markers like Flk-1 (VEGFR-2), von Willebrand Factor (vWF), Thrombomodulin (TM), endothelial Nitric Oxide Synthase (eNOS) and P-selectin [4, 5]. It is known from studies in other cell types that cAMP regulates the activity of B- and C-Raf [19–21]. For example, cAMP promotes B-Raf activation through Protein Kinase A (PKA), whereas it acts as an inhibitor of C-Raf though the same pathway [19, 21–23].

Based on these data, we postulated that Raf proteins might be engaged in the growth and/or maturation of eEPCs. To address this question, we undertook gain- and loss-of-function approaches targeting Raf family members in eEPCs. We focused on B- and C-Raf, because preliminary analysis indicated that eEPCs do not express A-Raf (unpublished data). Our results show that B-Raf and C-Raf have distinct roles in eEPCs suggesting new ways to control the proliferation or differentiation of endothelial progenitor cells before or during cell therapy.

Materials and methods

Isolation and culture of mouse embryonic EPCs

eEPCs were isolated from mouse embryos at E7.5, propagated as primary cell lines, and cAMP treated to induce differentiation as described [4]. eEPCs lacking B-Raf were derived from single B-Raf$^{-/-}$ embryos from the B-Raf knockout mouse line ([18]; kindly provided by Drs. L. Wojnowski and A. Zimmer).

Genetic manipulation of eEPCs

Expression plasmids for ΔB-Raf:ER and ΔC-Raf:ER were constructed by PCR amplification of the kinase domains using mouse embryonic cDNA as template. The hormone binding sites of the estrogen receptor (ER) or its mutated variant (tamoxifen receptor ER*) were amplified from plasmids kindly provided by Dr. B. Kempkes. To establish stable lines expressing the fusion proteins, we electroporated eEPCs and selected neomycin-resistant colonies. For transient transfections, we used lipofection.

Small inhibitory RNAs (siRNAs) against B- and C-Raf were generated using the Silencer® siRNA Construction Kit (Applied Biosystems, Darmstadt, Germany) based on unique sequences for each Raf gene.

RNA and protein analysis

We prepared RNA using Qiagen kits (Hilden, Germany) and reverse-transcribed total RNA into cDNA. cDNA (20 ng per reaction) was then used as template for Polymerase Chain Reaction (PCR) amplification with Taq DNA polymerase (Promega, Mannheim, Germany) and gene-specific primers.

For immunoprecipitation (IP) and Western blots, eEPCs were lysed in IP buffer and insoluble material was removed by centrifugation (16,000 g, 10 min.). Intact B- or C-Raf endogenous proteins or ΔB-Raf:ER and ΔC-Raf:ER fusions were immunoprecipitated with anti-B-Raf (sc-166), anti-C-Raf (sc-133) or anti-ER (sc-154) antibodies (Santa Cruz Biotechnology, Heidelberg, Germany) and Protein A-Sepharose (GE Healthcare, Munich, Germany). Pellets were washed 3x with tissue lysis buffer (TLB: 20 mM tris-HCl pH 7.4, 137 mM NaCl, 25 mM sodium β-glycerophosphate, 2mM sodium pyrophosphtate, 2 mM EDTA, 1 mM sodium varadate, 10% glycerol, 1% Triton X-100, 5 μg/ml leupeptin, 5 μg/ml aprotinin) buffer, resuspended in 2x Laemmli loading buffer, resolved by 12% SDS-PAGE and transferred to polyvinylidene fluoride (PVDF) membranes. For protein detection, we used mouse anti-B-Raf and anti-C-Raf antibodies from Upstate-Millipore (Schwalbach, Germany; 07-453 and 07-396, respectively) and horseradish peroxidase (HRP)-conjugated goat antimouse antibodies (Dianova, Hamburg, Germany). Blots were developed using enhanced chemiluminescence (GE Healthcare). To evaluate MEK1/2 phosphorylation, cells were starved in serum-free medium containing Nutridoma (Roche Applied Science, Mannheim, Germany) for 12 hrs and fusion Raf:ER kinases were activated with 0.5 μM β-estradiol or

0.1 μM 4-hydroxy-tamoxifen (estrogen and 4-HT; Sigma-Aldrich, Munich, Germany) for 1 hr. Lysates were analysed by Western blotting using anti-MEK1/2 (9122) and anti-phosphoMEK1/2 (9121) antibodies (Cell Signalling Technology, Danvers, MA, USA).

Proliferation assays

eEPCs (1 x 10^5 to 2 x 10^6 cells) were transfected with Raf expression constructs or siRNAs and activated with 4-HT, or estrogen in triplicates. We counted cells with an automated Coulter counter using mock-transfected and untreated cells as controls.

Statistical analysis

Data represent mean±SD and compared by either 2-tailed Student's t-test or 1-way anova followed by Newmann–Keuls post-test (InStat software, GraphPad). Differences with $P<0.05$ were considered statistically significant. Cloning and silencing strategies, buffer compositions, primer sequences and detailed protocols are described in the supplement.

Results

Molecular cloning of Raf: estrogen receptor fusion constructs and genetic engineering of eEPCs

To study the role of B- and C-Raf in eEPCs, we established cell lines expressing conditionally active forms of B- and C-Raf proteins. For this purpose, we constructed fusions of the Raf kinase domains with the hormone-binding part of the human ER α [24]. In this setting, Raf kinase activity can be induced by hormone addition to the culture medium, which liberates fusion proteins from bound heat shock proteins that mask their activity. For efficient expression levels, we cloned ΔB-Raf:ER and ΔC-Raf:ER behind the phosphoglycerate kinase (PGK) promoter (Fig. 1A), which is highly active in embryonic cells.

We confirmed that eEPCs do not express ERs to ensure that the observed effects described below are due to Raf kinase domain activation and not to stimulation of endogenous ERs (unpublished data). As additional control, we performed most experiments with the kinase domains of the Raf proteins fused to a mutated ER (constructs ΔB-Raf:ER* and ΔC-Raf:ER*, Fig. 1A), which can be activated by the estrogen analogue 4-hydoxy-tamoxifen or 4-HT that has no cellular receptor.

The high transfection efficiency of eEPCs (more than 90%) allowed us to work with a relatively homogeneous, transiently transfected, cell population. In parallel, we also obtained stable eEPC lines expressing B- and C-Raf fusion constructs. The integration of the expression vectors into genomic DNA was tested using Raf:ER fusion-specific genotyping primers (Fig. 1B). To show that the constructs were able to express Raf:ER, we isolated RNA from transfected and control, empty vector transfected, cells. RT-PCR analysis, using primers to amplify a unique region encompassing parts of the ER and kinase domains, detected robust levels of ΔB-Raf:ER and ΔC-Raf:ER transcripts (Fig. 1C). We then examined expression of Raf:ER fusion proteins by IP and Western blotting using antibodies against either the ER or the carboxyl terminus of Raf proteins (which are included in the Raf:ER fusion). Both antibodies detected bands of the expected size, around 61.5 kD, indicating that the fusion proteins were synthesized appropriately (Fig. 1D).

Raf:ER fusion proteins phosphorylate downstream Raf targets after hormone stimulation

To check if the kinase domains of ΔB-Raf:ER and ΔC-Raf:ER proteins are functional, we investigated the phosphorylation of Raf downstream targets after estrogen activation. Immediate partners in the signalling pathway of Raf proteins are the MAP/extracellular signal-regulated kinases (ERK) kinases MEK1 and MEK2, which in turn phosphorylate the ERKs [25]. To test MEK phosphorylation after estrogen stimulation, ΔB-Raf:ER or ΔC-Raf:ER transfected stable eEPC lines were grown in serum-free medium to reduce basal levels of MEK phosphorylation and then induced with estrogen (Fig. 2). Using MEK-recognizing antibodies, we found comparable MEK protein levels in all samples (Fig. 2A and B upper panels). In contrast, using antibodies recognizing specifically phospho-MEK, we detected phosphorylation only in estrogen-induced cells transfected with the ΔB-Raf:ER and ΔC-Raf:ER constructs (Fig. 2A and B lower panels). Starved, or estrogen-induced mock-transfected cells showed no phosphorylated MEK, demonstrating that the Raf:ER proteins were responsible for MEK activation after estrogen treatment

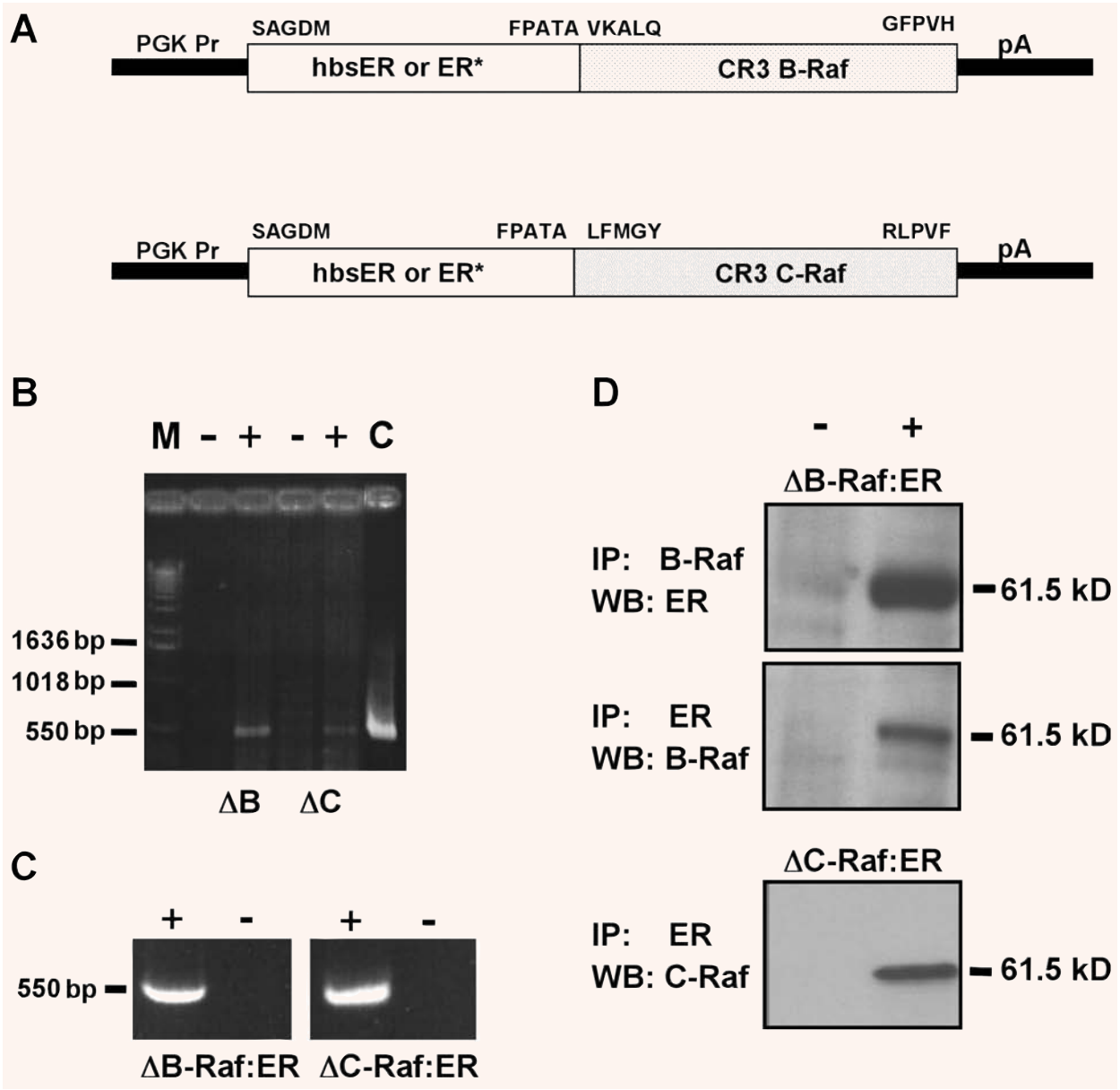

Fig. 1 Genetic engineering of eEPCs with inducible Raf kinases. (**A**) Structure of DNA constructs carrying the kinase domains of B- and C-Raf fused to the hormone binding site (hbs) domain of the estrogen (ER) and tamoxifen (ER*) receptors under the PGK promoter (PGK Pr); pA: polyadenylation region of the bovine growth hormone gene. The first and last five amino acids of the B- and C-Raf kinase domains and the hormone binding sites of the estrogen/tamoxifen receptors are provided on top of the diagrams. (**B**). PCR genotyping of G418-selected eEPC clones transfected with the expression constructs depicted in **A**. (–) mock-transfected cells; (+) cells transfected with ΔB- or ΔC-Raf:ER expression vectors (ΔB and ΔC). M: DNA size marker; sizes given in base pairs (bp). Lane **C** represents a positive control using ΔC-Raf:ER plasmid as template. (**C**). Genetically engineered eEPCs express high levels of ΔB- and ΔC-Raf:ER RNAs. (**D**). Engineered eEPCs express high levels of ΔB- and ΔC-Raf:ER proteins. The ΔB- and ΔC-Raf:ER fusion proteins were detected by immunoprecipitation (IP) with an anti-ER antibody followed by Western blotting (WB) with antibodies that recognize specifically the kinase domain of B- or C-Raf. In complementary fashion, the same proteins were detected by IP with the B- or C-Raf antibodies followed by Western blotting with anti-ER. Protein sizes are indicated in kDaltons (kD). In C,D: (–) mock transfected cells; (+) cells transfected with ΔB- and ΔC-Raf:ER expression vectors.

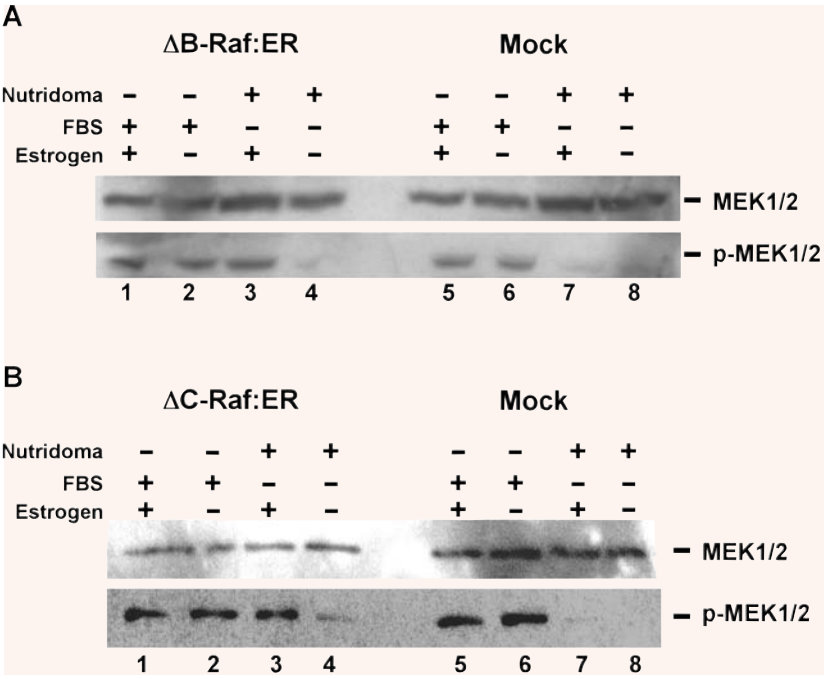

Fig. 2 ΔB- and ΔC-Raf:ER phosphorylate MEK after estrogen stimulation. (**A, B**) Western blotting with antibodies recognizing MEK1/2 (top panels) and antibodies recognizing phosphorylated MEK1/2 (p-MEK1/2) in serines 217 and 221 (lower panels). eEPCs were grown in starvation medium for 12 hrs with Nutridoma as nutritional supplement, and then induced for 1 hr with either estrogen, or serum as positive control. MEK phosphorylation is evident in ΔB-Raf:ER (**A**) and ΔC-Raf:ER (**B**) expressing cells, but not in mock-transfected cells after estrogen treatment (compare lanes 3–7). Serum addition leads to MEK phosphorylation in both mock- and ΔRaf:ER- expressing cells (lanes 1,2 and 5,6).

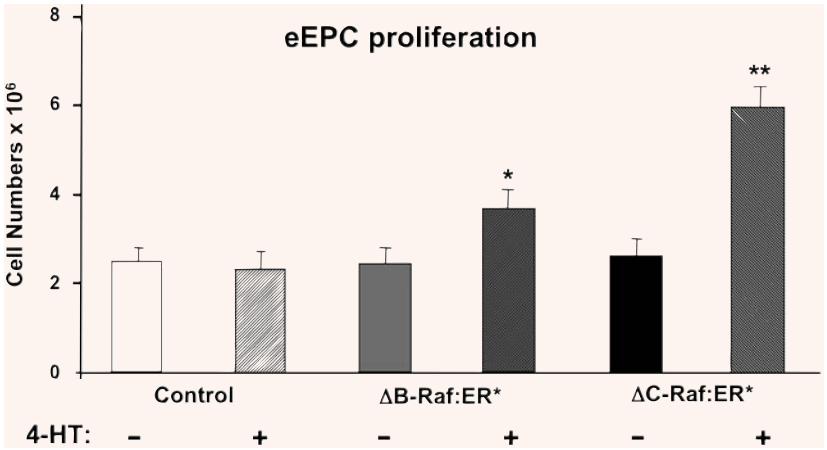

Fig. 3 Active C-Raf kinase domain stimulates eEPC growth. eEPCs were transiently transfected with ΔB-Raf:ER*, ΔC-Raf:ER* or an EGFP reporter construct as a control. The cells were left untreated (–) or stimulated (+) with 100 nmol/l tamoxifen (4-HT) and counted 48 hrs later. Activation of the C-Raf kinase domain strongly induces eEPC growth, whereas activation of the corresponding B-Raf domain has only a modest effect (*P<0.01 and **P<0.001 versus control, respectively).

(Fig. 2A and B comparing lanes 3 and 7). We obtained comparable results using ΔB-Raf:ER* and ΔC-Raf:ER* after 4-HT stimulation (not shown).

B- and C-Raf kinase domains stimulate eEPC growth to variable degree

Raf proteins have been implicated in the regulation of proliferation in different cell types [26, 27]. To identify possible effects of activated Raf members on eEPC growth, we compared ΔB-Raf:ER* and ΔC-Raf:ER* transiently transfected eEPC with or without 4-HT induction of kinase activity. Cells transfected in parallel with an enhanced green fluorescent protein (EGFP) expression construct served as negative control. We induced eEPCs with 4-HT for 8 hrs and counted cell numbers 48 hrs later. As shown in Figure 3, 4-HT treatment led to 2.5-fold higher cell numbers in ΔC-Raf:ER* engineered eEPCs compared to controls, whereas there was only a slight increase in ΔB-Raf:ER* expressing cells. We obtained similar results with stably transfected eEPCs (not shown). Although both B- and C-Raf:ER

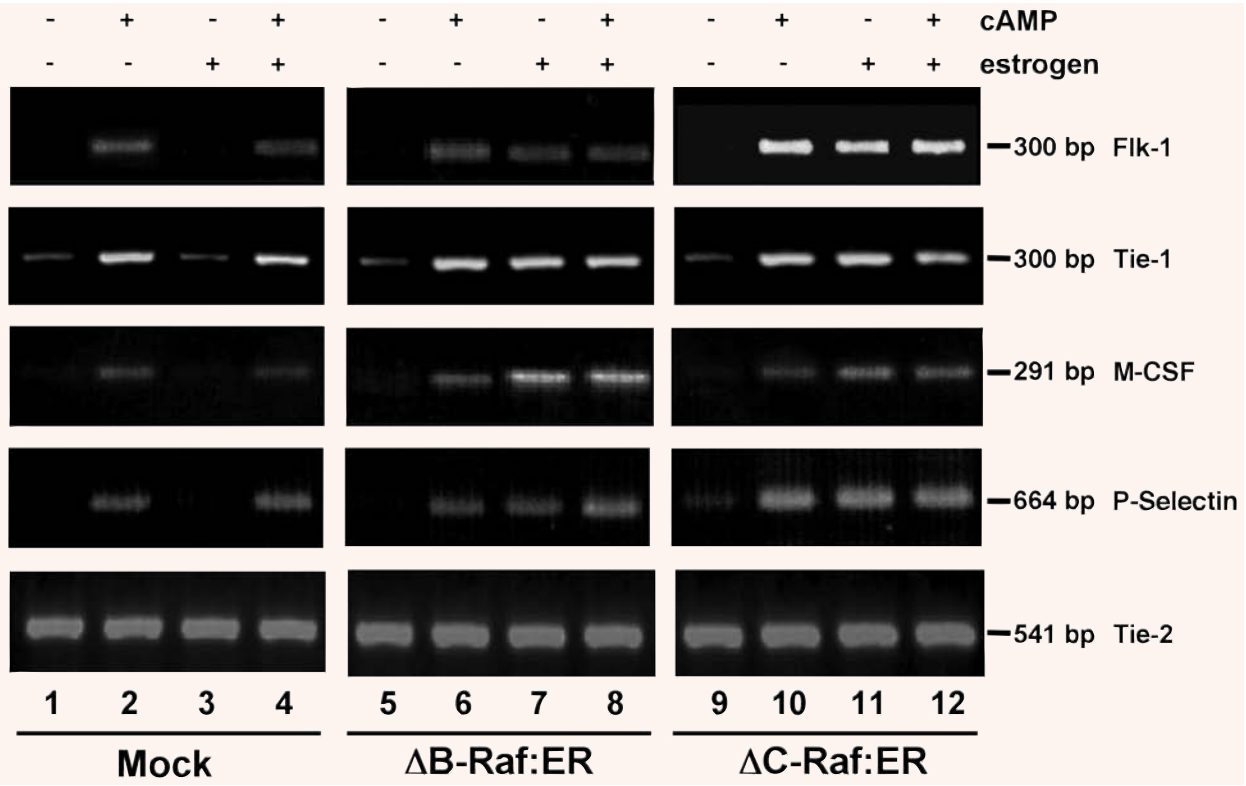

Fig. 4 Activation of Raf kinase domains induces eEPC differentiation. RT-PCR analysis using gene-specific primers of mock-, ΔB-Raf:ER- and ΔC-Raf:ER- transfected cells. eEPCs were left untreated or stimulated for 8 hrs with either cAMP, estrogen or cAMP and estrogen together. A number of genes including *Flk-1, Tie-1,* M-CSF and *P-selectin* are up-regulated after B- and C-Raf activation with estrogen to similar levels as with cAMP treatment. There is no additive effect between estrogen and cAMP activation. *Tie-2,* which is not induced upon eEPC differentiation, is shown as control. The expected band sizes for the various PCR products are indicated on the right.

proteins are capable of phosphorylating MEKs (Fig. 2), it appears that the C-Raf kinase domain is a more potent stimulator of eEPC proliferation than the corresponding B-Raf domain.

B- and C-Raf kinase domains stimulate eEPC differentiation

We showed previously that eEPCs differentiate after cAMP treatment leading to induction of genes like *Flk-1, vWF, eNOS, Alk-1, Tie-1, M-CSF, TM* and *P-selectin* [4,5]. To investigate a possible link between B- or C-Raf and cAMP-induced differentiation, we tested gene expression in ΔB-Raf:ER and ΔC-Raf:ER expressing eEPCs following activation with estrogen for 8 hrs. We observed induction of *Flk-1, Tie-1, P-selectin* and *M-CSF,* which were also stimulated upon cAMP-treatment (Fig. 4). Estrogen-induced gene up-regulation (Fig. 4, lanes 7,11) to a similar extent as cAMP (Fig. 4, lanes 6,10). Mock-transfected cells did not respond to estrogen, but showed a robust induction with cAMP as expected (Fig. 7, lanes 3,4). Of note, we did not observe additive effects between cAMP and estrogen activation (Fig. 4, lane 8,12). Thus, it is likely that the cAMP effects on eEPC differentiation are mediated mostly by activation of Rafs. We tested this idea in cAMP-treated eEPCs after knockdown of Raf proteins as described below.

Moreover, it appears that although the truncated kinase domains of B- and C-Raf stimulate eEPC proliferation at different levels, they are equally potent in inducing differentiation of eEPCs. This could be

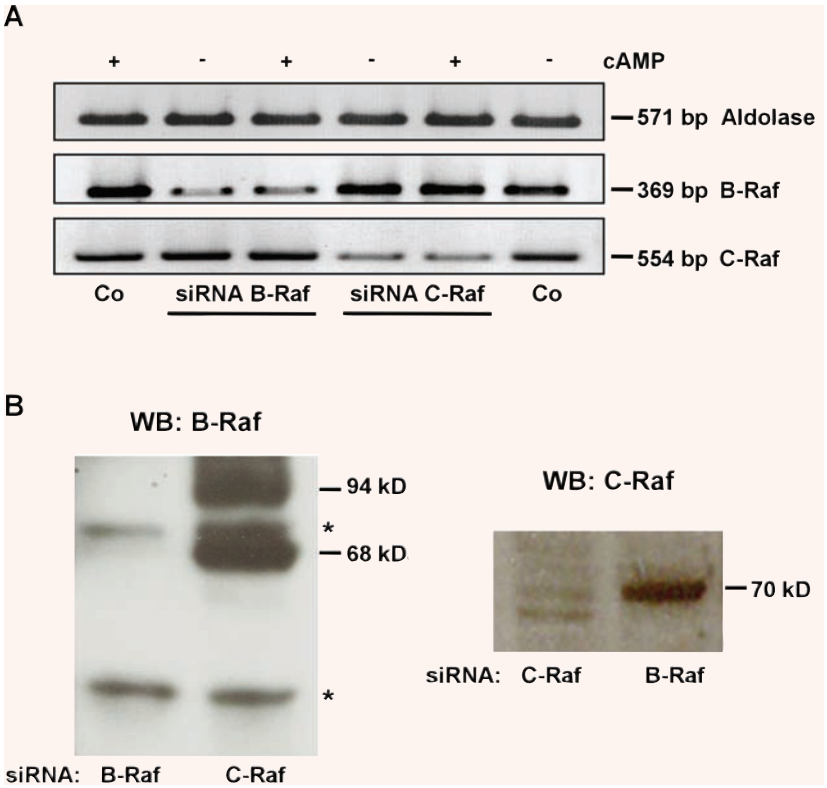

Fig. 5 RNA interference-mediated silencing of B- and C-Raf in eEPCs. (**A**) eEPCs were transfected with 20 nM siRNAs against B- or C-Raf using oligofectamine alone as control (Co). Total RNA was isolated 48 hrs later and assayed for levels of endogenous *B*- and *C*-Raf mRNAs using RT-PCR. cAMP treatment did not interfere with the silencing process. siRNAs against B-raf do not affect C-Raf mRNA levels, and vice versa, indicating that the RNAi tools are specific for the corresponding targeted Raf isoform. (**B**) eEPCs were transfected for 72 hrs with 20 nM siRNAs against B-Raf or C-Raf. Western blotting shows that siRNA treatment effectively abolishes endogenous B- and C-Raf proteins. As for mRNA in A, there is no cross reactivity between the knockdown tools since anti-B-Raf siRNAs do not affect C-Raf protein and vice versa. The unspecific bands in the B-Raf blot (asterisks) served as loading controls.

because either B-Raf or C-Raf can substitute for each other, or adjacent regulatory domains, missing in the truncated Raf forms, modulate kinase activity. To distinguish between these two possibilities, we analysed the role of Raf isoforms in eEPCs in a loss-of-function approach using siRNAs.

RNA interference-mediated silencing of B- and C-Raf in eEPCs

For the silencing experiments, we transfected eEPCs with siRNA cocktails against B- and C-Raf for 48 hrs. The siRNAs were first tested for correct and efficient targeting by RT-PCR using gene-specific primer pairs that distinguish between B- and C-Raf transcripts. The results showed that siRNAs against either Raf isoform reduced the corresponding mRNA levels in a specific manner, that is, without affecting the expression levels of the other Raf gene (Fig. 5A). We also observed that cAMP treatment did not interfere with the silencing process (Fig. 5A). Quantification of expression levels demonstrated a reduction of 3-fold in B-Raf and around 2-fold in C-Raf mRNAs after siRNA-mediated knockdown.

We then investigated the effects of the RNAi-induced gene silencing on Raf proteins. For this purpose, eEPCs were transfected with siRNA against B- and C-Raf mRNAs and protein levels were evaluated by Western blotting. The results in Figure 5B show that both B-Raf splice variants, with molecular weights of 94 kD and 68 kD, are significantly reduced after anti-B-Raf siRNA treatment. Importantly, the C-Raf-specific siRNAs had no effect on the B-Raf protein levels. In complementary fashion, we observed a dramatic loss of C-Raf protein, only with the anti-C-Raf siRNAs (Fig. 5B). The more marked effects on protein compared to mRNAs levels may be because siRNAs, next to transcript degradation, concurrently block mRNA translation.

RNA interference-mediated silencing of C-Raf impairs eEPCs proliferation

Following the specific and efficient knockdown of B- and C-Raf proteins, we analysed the effects of Raf

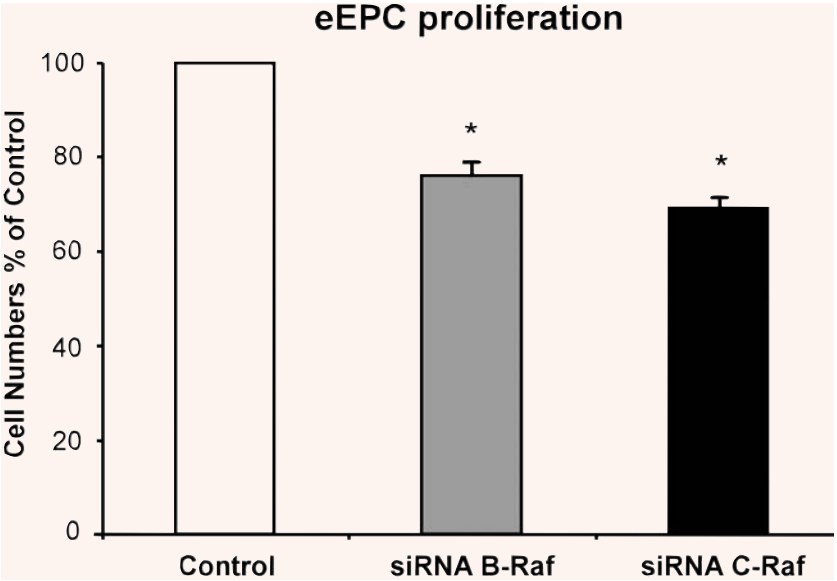

Fig. 6 Loss of C-Raf function impairs proliferation of eEPCs. eEPCs were transfected with 20 nM siRNA against B-Raf or C-Raf for 72 hrs. Oligofectamine alone treatment served as a negative control. Each transfection was done in triplicates. At the end of the 72-hrs period, cells were counted twice using an automatic Coulter counter (*P<0.001 *versus* control).

loss-of-function on eEPC proliferation and differentiation. We found that silencing of B-Raf or C-Raf impaired the growth of eEPCs at variable degrees. Specifically, the effect was more pronounced in cells transfected with siRNAs against C-Raf than B-Raf (Fig. 6). This result is in accordance with the gain-of-function experiments, where ΔC-Raf:ER* was more efficient in inducing eEPC proliferation than ΔB-Raf:ER* after 4-HT stimulation (Fig. 3).

RNA interference-mediated silencing of B-Raf impairs eEPCs differentiation

We next tested whether silencing of endogenous B-Raf or C-Raf influences the differentiation of eEPCs. eEPCs were transfected with siRNAs against B-Raf and C-Raf, or treated with oligofectamine as control for 72 hrs. In the last 8 hrs of siRNA treatment, cells were induced with cAMP, or left untreated as control. We then isolated RNA and performed RT-PCR analysis (Fig. 7A). The results showed that cAMP-induced differentiation proceeds normally in oligofectamine, mock-treated controls, and in C-Raf siRNA transfected eEPCs (Fig.7A, lanes 1,2 and 5,6). On the contrary, differentiation was totally abrogated in B-Raf siRNA transfected cells (Fig. 7A, lanes 3,4). These data indicate that B-Raf, but not C-Raf, is required for cAMP-mediated eEPC-differentiation.

eEPCs isolated from B-Raf null mouse embryos fail to differentiate *in vitro*

When combined, the gain- and loss-of-function experiments demonstrate that both B- and C-Raf kinase domains can engage downstream targets to induce eEPC differentiation when separated from adjacent regulatory domains and fused to the ER. However, only the intact B-Raf appears to regulate the differentiation process. To corroborate these conclusions, we isolated eEPCs from single embryos generated from mating of *B-Raf*$^{+/-}$ heterozygote mice. B-Raf knockout mice die around E10-12 [18], so isolation of eEPCs at E7.5 was feasible. eEPC lines were genotyped to identify clones derived from B-Raf null embryos; eEPCs isolated in parallel from wild-type siblings served as controls. We then added cAMP to induce differentiation and compared wild-type and *B-Raf*$^{-/-}$ cells (Fig. 7B). RNA analysis showed that *B-Raf*$^{-/-}$ eEPCs failed to respond to cAMP, whereas control wild-type eEPCs showed the expected pattern of induced genes. These results confirmed the key role of B-Raf in eEPC differentiation.

Discussion

Endothelial progenitor cells hold promise as diagnostic, prognostic and therapeutic tools for cardiovascular disease, regenerative medicine and cancer treat-

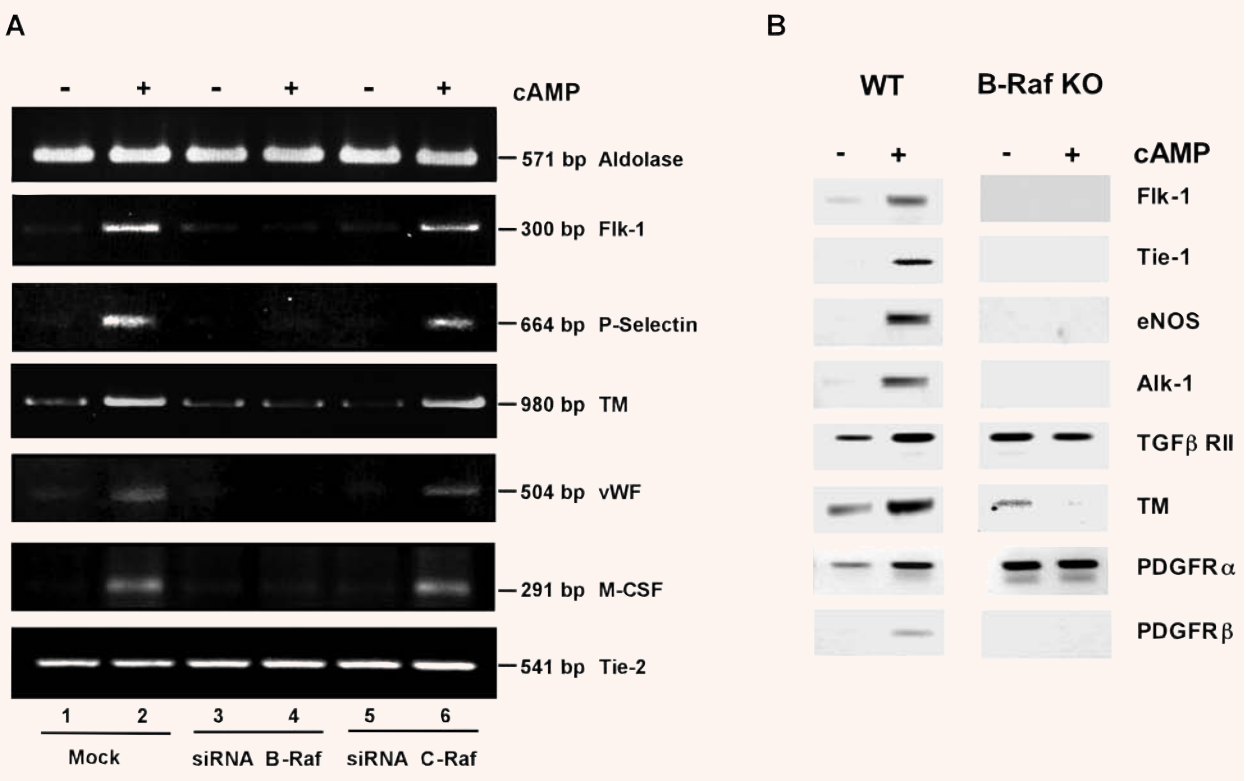

Fig. 7 B-Raf is essential for eEPC differentiation. (**A**) eEPCs were transfected for 72 hrs with 20 nM siRNAs against B-Raf (lanes 3,4), C-Raf (lanes 5,6), or mock-transfected with oligofectamine as control (lanes 1,2). Duplicates were treated with no (–) or 0.5 μmol/l cAMP (+) for the last 8 hrs of the siRNA treatment. RNA was then isolated and served as template for RT-PCR analysis using gene-specific primers. Silencing of B-Raf completely abolishes the cAMP-induced differentiation of eEPCs, whereas knockdown of C-Raf has no effect—as monitored by *Flk-1, P-selectin*, *Thrombomodulin (TM), vWF and M-CSF* expression levels. *Aldolase* and *Tie-2*, whose expression does not change during the differentiation process, are not effected by knockdown of B- and C-Raf. The expected band sizes for the various PCR products are indicated on the right. (**B**) B-Raf eEPCs isolated from *B-Raf*$^{-/-}$ embryos (KO) were left untreated (–) or induced to differentiate with cAMP (+) for 12 hrs. eEPCs isolated from wild-type siblings (WT) served as controls. The *B-Raf* null eEPCs do not differentiate after cAMP treatment. The analysed genes are indicated on the right.

ment [3, 28–30]. For this reason, there is interest in elucidating the mechanisms of mobilization, homing, survival and differentiation of EPCs [3, 31]. Similarly to adult cells, we found that transplantation of embryonic EPCs stimulated tumor growth and enhanced capillary density and tissue recovery after ischaemic injury [6, 7, 11]. However, we also observed that association of eEPCs with vascular structures was transient with only 1–7% of the new vasculature containing eEPCs [5–7, 11]. Our data, and the results of others, indicate that efficient future therapies would require optimization of long-term survival and differentiation of stem cells after transplantation [32, 33]. This outcome hinges on a better understanding of the signalling pathways that control proliferation and differentiation of progenitor cells.

Embryonic EPCs are immature cells expressing a subset of endothelial-specific markers such as TM and the Angiopoietin receptor Tie-2. cAMP treatment *in vitro* induces differentiation, activating genes specific to the endothelial lineage like *Flk-1,* eNOS or vWF [4, 5]. Here, we present evidence that the B-Raf kinase might be a key component in the regulation of the eEPC maturation process. In contrast, we found that C-Raf is a more potent stimulator of eEPC proliferation than B-Raf.

The effects of B-Raf on eEPC biology *in vitro* might reflect its critical role in vascular development,

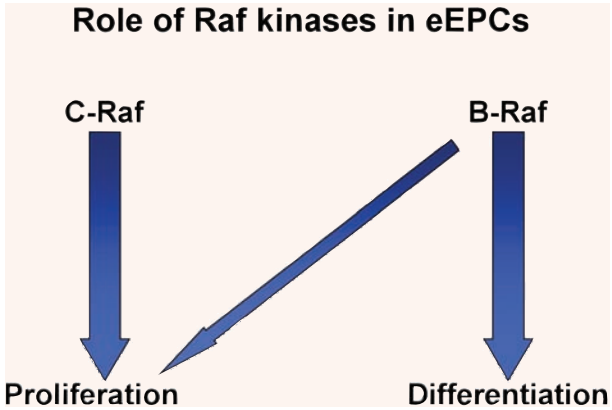

Fig. 8 B-Raf and C-Raf have partially overlapping, but distinct roles in eEPCs. The schematic model illustrates the different roles of B- and C-Raf in eEPCs. It appears that B-Raf is primarily involved in cAMP-induced differentiation with a modest effect on eEPC growth. In contrast, C-Raf is a strong inducer of proliferation, but has no role in the cAMP-mediated differentiation process.

which has been addressed in B-Raf deficient mice [18]. B-Raf null embryos have leaky vessels that lead to embryonic oedemas, suggesting a defect in endothelial differentiation and blood vessel maturation. Moreover, B-Raf null embryos have higher numbers of endothelial progenitor cells compared to wild types. The extra eEPCs do not form vessels, instead remain embedded within surrounding tissues [18]. These observations implied that B-Raf might be involved in the regulation of endothelial differentiation. Our data are in support of this notion since knockdown of B-Raf in embryonic EPCs blocked their differentiation. The same was true for eEPCs isolated from B-Raf deficient mice, which failed to properly differentiate *in vitro*.

C-Raf is also essential for development; C-Raf deficient mice displayed embryonic growth retardation, advocating a role of C-Raf in regulating cell proliferation or survival [34, 35]. Our results are consistent with this idea, since we observed that activation of the C-Raf kinase domain promotes higher eEPC growth rates compared to B-Raf. In similar fashion, we found that knockdown of C-Raf impairs eEPC growth to a greater extent than the corresponding knockdown of B-Raf. The divergent B- and C- Raf functions in eEPCs might be due to activation of isoform-specific targets other than MEK or the action of B- and C- Raf-specific effector proteins [36]. Of note, the different roles of B-Raf and C-Raf in EPCs are in line with data in PC12 cells, where EGF-activation of C-Raf increases mitotic rates, whereas B-Raf activation by NGF leads to neuronal differentiation [27]. It would be interesting to test if such divergent roles of B- and C-Raf are a general feature of other progenitor cell types.

Raf kinases are essential for normal cell functions and even single amino acid substitutions can transform cells. For instance, B-Raf mutations have been identified in various human cancers including lung adenocarcinoma, small cell carcinoma, melanoma or colorectal cancer [37]. At present, Raf kinase inhibitors are considered in anti-tumor drug therapies [38]. It would be important to test if genetic mutations in Raf sequences or inhibitory compounds of Raf kinase activities also affect adult EPCs.

Our previous work has shown that cAMP was unique, among a panel of tested agents and growth factors, to promote eEPC differentiation [4, 5]. Here, we show that this activation requires B-Raf and that, all cAMP signalling target genes analysed so far, are also induced after Raf activation. Thus, it is likely that most, if not all, of the cAMP effects on eEPC differentiation are mediated through B-Raf. In support of this proposition, we observed no additive effects after combined cAMP/Raf activation.

It is known that cAMP has diverse effects on C-Raf and B-Raf in a PKA-dependent manner. For example, PKA phosphorylates the small G protein Rap1, which blocks C-Raf and acts as a sustained activator of B-Raf [21, 26, 39]. In parallel, PKA phosphorylates B-Raf, boosting its activity [40]. Our data are consistent with this scenario, since we found that B-Raf is necessary and sufficient to transmit the cAMP-mediated differentiation signal, while C-Raf is dispensable. Conversely, cAMP treatment slows eEPC growth [4, and data not shown), consistent with the cAMP role in blocking C-Raf.

Although the B-Raf, but not the C-Raf, deficiency specifically impaired eEPC differentiation, both the truncated B- and C-Raf kinase domains were able to up-regulate target genes like *Flk-1, Tie-1, M-CSF* and *P-selectin.* It is thus likely that the regulatory domains of the Raf proteins are needed to confer functional specificity in eEPCs. In their absence, B- and C-Raf kinase domains can substitute for each other, as shown in other cell types [41].

The findings in eEPCs might have broader implications for vascular biology. The genes induced by B-Raf

have important functions under pathophysiological conditions. For example, TM and vWF are involved in coagulation, *P-selectin* in leukocyte homing during inflammation and *Flk-1* in angiogenesis. Hence, controlling the expression of these genes via modulation of B-Raf could open new ways to manipulate their function in progenitor and mature endothelial cells. In similar fashion, the preferential role of C-Raf in stimulating eEPC proliferation could be taken into account for modulation of EPCs growth *in vivo.*

In summary, our results provide a first insight into the molecular mechanisms that drive growth and differentiation of eEPCs. As depicted in Figure 8, differentiation *versus* proliferation may be promoted by B- or C-Raf respectively, depending probably on the type of extracellular stimuli. Thus, targeting either B- or C-Raf may selectively influence the fate of EPCs in therapeutic interventions. In this light, our findings could be helpful for developing new strategies in the future to enhance EPC growth and differentiation during angiogenesis and tissue repair after injury.

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