Clinical/Translational Research

Inhibition of Four-and-a-Half LIM Domain Protein-2 Increases Survival, Migratory Capacity, and Paracrine Function of Human Early Outgrowth Cells Through Activation of the Sphingosine Kinase-1 Pathway Implications for Endothelial Regeneration

Talin Ebrahimian, Omar Arfa, Stefania Simeone, Catherine A. Lemarié, Stéphanie Lehoux,* Sven Wassmann*

Rationale: Inhibition of four-and-a-half LIM domain protein-2 (FHL2) attenuates atherosclerotic lesion formation and increases endothelial cell migration. Early outgrowth cells (EOCs) contribute substantially to endothelial repair. Objective: We investigated the role of FHL2 in the regulation of EOCs.

Methods and Results: Human EOCs were cultured from peripheral blood. FHL2 knockdown in EOCs by siRNA resulted in increased EOC numbers and reduced apoptosis, as indicated by decreased cleaved caspase-III and reduced Bax/Bcl-2 expression ratio. This was mediated through increased phosphorylation and membrane translocation of sphingosine kinase-1, increased sphingosine-1-phosphate levels, and Akt phosphorylation. FHL2 knockdown increased stromal cell–derived factor-1–induced EOC migration through upregulation of αv/β3, αv/β5, and β2 integrins, associated with increased cortactin expression. Reduced apoptosis, increased EOC migration, and cortactin upregulation by FHL2 siRNA were prevented by CAY10621, the sphingosine kinase-1 inhibitor, and the sphingosine-1-phosphate receptor-1/-3 antagonist VPC23019. These findings were confirmed using spleen-derived EOCs from FHL2−/− mice. Apoptosis was decreased and migration increased in endothelial cells exposed to the conditioned medium of FHL2−/− versus wild-type (WT) EOCs. These paracrine effects were abolished by VPC23019. Importantly, reendothelialization after focal carotid endothelial injury in WT mice was significantly increased after intravenous injection of FHL2−/− versus WT EOCs.

Conclusions: Our findings suggest that FHL2 negatively regulates EOC survival, migration, and paracrine function. FHL2 inhibition in EOCs reduces apoptosis and enhances survival and migratory capacity of both EOCs and surrounding endothelial cells by activation of the sphingosine kinase-1/sphingosine-1-phosphate pathway, resulting in improvement of endothelial regeneration. (Circ Res. 2014;114:114-123.)

Key Words: apoptosis ■ endothelium ■ physiology ■ stem cells ■ signal transduction

Endothelial damage, the initial and essential step in atherogenesis, can result in apoptosis or inflammatory conditions that stimulate endothelial dysfunction. Diminished endothelial regeneration is considered to play an important role in the pathogenesis of atherosclerosis.1 Endothelial regeneration is largely accomplished by resident endothelial cells (ECs), but it can be significantly bolstered by early outgrowth cells (EOCs; formerly referred to as early endothelial progenitor cells). EOCs are premature circulating cells that are involved in postnatal vasculogenesis and reendothelialization after endothelial damage.2,3 Several animal models have demonstrated that endogenous mobilization (eg, by physical activity or statins) or systemic application of EOCs leads to enhancement of reendothelialization, improvement of angiogenesis and endothelial function, decreased neointima formation, and reduced development of atherosclerosis.3–7 In humans, low numbers and impaired function of circulating EOCs are associated with endothelial dysfunction and poor cardiovascular outcomes.8

Four-and-a-half LIM domain protein-2 (FHL2; SLIM3) is the best studied member of the LIM-only subclass of the LIM protein superfamily. LIM proteins are defined by the presence of one or more LIM domains composed of a conserved cysteine-rich module that mediates protein–protein interactions.9 The functions of LIM domain proteins in the nucleus

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Nonstandard Abbreviations and Acronyms

<table>
<thead>
<tr>
<th>Acronym</th>
<th>Definition</th>
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<tr>
<td>EOC</td>
<td>early outgrowth cells</td>
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<tr>
<td>FHL2</td>
<td>four-and-a-half LIM domain protein-2</td>
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<tr>
<td>S1P</td>
<td>sphingosine-1-phosphate</td>
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<tr>
<td>S1PR</td>
<td>S1P receptor</td>
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<tr>
<td>SDF-1</td>
<td>stromal-cell-derived factor-1</td>
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<td>SK-1</td>
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are mainly in tissue-specific gene regulation and determination of cell fate, whereas cytoplasmic LIM domain proteins are mainly involved in cytoskeleton organization.10,11 Multiple functions have been ascribed to FHL2. FHL2 acts as a transcriptional cofactor through interaction with a broad range of transcription factors, and it forms diverse protein complexes in many cell types. It, therefore, modulates the activity and expression of several proteins, including, but not limited to, androgen receptor, cyclic adenosine monophosphate response element-binding protein, integrins, β-catenin, presenilin-2, extracellular signal-regulated kinase-2, activator protein-1, and sphingosine kinase-1 (SK-1). In so doing, FHL2 modulates cellular processes such as signal transduction, cell proliferation, and survival.18

FHL2 is strongly expressed in early cardiac precursor cells, in the adult heart, in skeletal muscle, and to a lower extent in many epithelial tissues.19,20 Recently, FHL2 expression has been demonstrated in ECs and vascular smooth muscle cells of the vessel wall.21,22 Overexpression of FHL2 inhibits vascular endothelial growth factor–induced EC migration via suppression of the SK-1/sphingosine-1-phosphate (S1P) pathway.23 FHL2 interacts with and inhibits the activity of SK-1, which is the enzyme responsible for S1P production.24–26 Deletion of FHL2 in mice is associated with reduced atherogenesis27 and changes in vascular smooth muscle phenotype and endothelial function.28 However, the role of FHL2 in ECs is presently unknown. In this study, we investigated the role of FHL2 in the regulation of EOC number and function in vitro and their reendothelialization capacity in vivo.

Methods

An expanded Methods section describing all procedures and protocols is available in the Online Data Supplement.

Preparation of EOCs

Mononuclear cells were isolated from peripheral blood of healthy donors (age 20–45 years; men and women; no known disease or medication) using a Ficoll density gradient (Sigma) according to standard protocols as previously described.28 Blood cell donation was approved by the research review committee of the Lady Davis Institute for Medical Research. For mouse mononuclear cells, male 8- to 10-week-old, wild-type (WT) and FHL2 knockout (FHL2 −/−) mice with C57BL/6J background, initially provided by Dr Büttner, University of Bonn, Germany, were used. Spleens were explanted, mechanically minced, and mononuclear cells isolated using a Ficoll gradient, as previously described.29 We have previously characterized cultured human EOCs by immunocytochemistry.26

Transfection With siRNA

Human EOCs were transfected at day 6 of cell culture with a specific FHL2 siRNA (siFHL2; 5 nmol/L; Qiagen) or luciferase siRNA (siLuc), used as transfection control siRNA (Qiagen), for 24 hours in endothelial basal medium with supplements (Cedarlane) by using HiPerFect transfection reagent (Qiagen).

Apoptosis

Cleaved (active) caspase-III immunocytochemistry was performed as previously described.28 Fluorescence was visualized with a DM2000 microscope (Leica Microsystems). As an additional approach, the Bax/Bcl-2 expression ratio was assessed by Western blot, as previously described.24

Migration

Migratory capacity was evaluated by using modified Boyden chambers (8 μm pore size; Becton Dickinson), as previously described.28 EC migratory capacity was evaluated by using the scratch assay.

Carotid Endothelial Injury

All procedures were approved by the Animal Care Committees of McGill University and have followed recommendations of the Canadian Council on Animal Care.

Statistical Analysis

Results are expressed as means±SEM. All data were evaluated by ANOVA. Statistical significance for comparisons between 2 groups was calculated using the 2-tailed Student t test. To assess comparisons between multiple groups, data were analyzed using the Newman–Keuls test. A probability value <0.05 was considered statistically significant. Data that were significant (P<0.05) were further assessed by Student t test using GraphPad Prism software (GraphPad Software).

Results

FHL2 Knockdown Increases EOC Numbers Through Decreased Apoptosis

FHL2 is expressed in the cytosol as well as in the nucleus of human ECs, as assessed by immunocytochemistry (Figure 1A). To determine the role of FHL2 in EOCs in vitro, we used specific siRNA against FHL2 that inhibited FHL2 protein expression levels by 40% compared with luciferase siRNA (used as control siRNA; Figure 1B). The transfection efficiency, determined by FITC-coupled control RNA, was 57.5% (data not shown). FHL2 knockdown significantly increased the number of cultured human ECs, as determined by immunostaining of fibronectin-deranged adherent cells with Dil-acetylated low density lipoprotein (acLDL) and lectin (Figure 1C). Because the rate of proliferating cells, as determined by Ki67 immunocytochemistry, was low (<1%) in EOCs (Figure 2A), and because the FHL2 siRNA did not influence adhesion of EOCs to the substrate (Online Figure I), we assessed apoptosis. We found that there was a significant decrease of apoptotic EOCs, as determined by cleaved (active) caspase-III expression in Dil-acLDL/lectin double-positive cells (Figure 2B), by Bax/Bcl-2 expression ratio (Figure 2C), and by Annexin V staining (Online Figure IIIB), all of which are molecular indices of apoptosis.

FHL2 Knockdown Increases EOC Migration Through Upregulation of αv/β3, αv/β5, and β2 Integrins

Boydchen chamber assays revealed that FHL2 knockdown significantly increased stromal cell–derived factor-1 (SDF-1)-induced migration of human ECs (Figure 3A; Online Figure III). This was associated with a significant increase in the number of cells expressing αv/β3 and αv/β5 integrins at their surface, as determined by fluorescence-activated cell sorting.
There were no differences in the number of cells expressing the active forms of β1 or β2 integrins (Figure 3B). In contrast, no difference was observed in the mean fluorescence intensities of the integrin signals (Online Figure IV), indicating that surface expression of integrins was an all-or-none phenomenon in EOCs. To evaluate if increased EOC migration by FHL2 inhibition was mediated through this upregulation of αvβ3 and αvβ5, which are known to participate in cell migration, we used integrin-neutralizing antibodies. Neither anti-β1 antibody nor IgG control prevented the enhanced migration in siFHL EOCs compared with siLuci EOCs (Figure 3C). However, the difference between siFHL- and siLuci-transfected EOCs was abolished in the presence of αvβ3, αvβ5, or β2-neutralizing antibodies. Furthermore, we found a significant increase in cortactin expression in EOCs transfected with siFHL2 versus siLuci, as determined by immunocytochemistry (Figure 3D).

Cortactin is an F-actin–binding protein that plays an important role in actin filament assembly and cell motility. These results suggest that knockdown of FHL2 increases SDF-1–induced migration through upregulation of αvβ3, αvβ5, and β2 integrins as well as cortactin.

**FHL2 Knockdown Decreases Apoptosis and Increases Migration of EOCs Through Activation of the SK-1/S1P Pathway**

To determine the molecular pathway involved in the effects of FHL2 knockdown on human EOC apoptosis and migration, we investigated the SK-1 pathway. FHL2 was shown to bind to and suppress the activity of SK-1, the enzyme producing S1P, in cardiomyocytes.24–26 We observed a significant increase in SK-1 phosphorylation (Figure 4A) and SK-1 translocation from the cytosol to the cell membrane (Figure 4B), which is important for its activation, in siFHL EOCs compared with siLuci EOCs. This was associated with a significant increase in the intracellular levels of S1P (0.16±0.02 versus 0.10±0.01 µmol/L; Figure 4C) as well as phosphorylation of Akt (Figure 4D), which is a
downstream effector of S1P. We also investigated the effect of exogenous S1P on EOC function; apoptosis was reduced and SDF-1–induced migration increased in S1P-stimulated EOCs compared with vehicle-stimulated EOCs (Figures 4E and 4F), similar to what we found in siFHL EOCs (Figures 2 and 3). To further test the causal relationship between siFHL, SK-1 upregulation, and EOC function, we used the specific pharmacological SK-1 inhibitor CAY10621. We found that the reduced apoptosis, the increased SDF-1–induced migration, and the increased cortactin expression in EOCs with FHL2 knockdown were completely abolished by CAY10621 (Figure 5A–5C). Because S1P could be secreted in the extracellular space and bind to its specific receptors by autocrine effects, we investigated if the antiapoptotic and promigratory effects of FHL2 silencing are mediated through activation of S1P receptor-1 and -3 (S1PR-1/-3). The S1PR-1/-3 antagonist VPC23019 increased basal apoptosis and completely abolished the reduction in apoptosis observed with siFHL (Figure 5A). Moreover, VPC23019 decreased basal SDF-1–induced migration and partly inhibited the promigratory effect of FHL2 silencing (Figure 5B). Taken together, these data suggest that the antiapoptotic effect observed with...
siFHL2 is mediated through activation of SK-1, increased production of S1P, and activation of S1PR-1/-3. The promigratory effect of FHL2 silencing is also mediated through activation of SK-1 and increased S1P production, but is only partly mediated by S1PR-1/-3.

FHL2−/− EOCs Show Increased Cell Numbers, Reduced Apoptosis, and Enhanced Migration

The human cell data were verified by using spleen-derived cultured EOCs from FHL2−/− and WT mice. Interestingly, FHL2−/− mice contained more spleen-derived EOCs compared with their WT counterpart (Online Figure V). The number, apoptosis, and migratory capacity of these cells were assessed. Confirming the human EOC results, the number of Dil-acLDL/lectin double-positive mouse EOCs was significantly increased (Figure 6A), cleaved caspase-III expression in Dil-acLDL/lectin double-positive EOCs was significantly reduced (Figure 6B), and migration was significantly enhanced (Figure 6C) in FHL2−/− compared with WT EOCs.

FHL2−/− EOCs Have Enhanced Paracrine Effects on ECs Through Increased S1P Secretion and Activation of S1PRs

Because we demonstrated that FHL2 knockdown activates SK-1 and increases S1P production in EOCs, we tested the hypothesis that increased S1P production and release into the extracellular space would lead to subsequent regulation of EC function. First, we verified the effect of exogenous S1P on ECs; S1P significantly increased EC migratory capacity and decreased EC cleaved caspase-III expression (Figure 7A and 7B). Second, to determine whether absence of FHL2 regulates secretion of S1P by EOCs, we measured S1P levels in the conditioned medium (cm) of EOCs from WT (cmWT) and FHL2−/− mice (cmFHL). S1P was significantly enhanced in cmFHL compared with cmWT (0.12±0.02 versus 0.08±0.01 µmol/L, respectively; Figure 7C). Third, we exposed murine ECs to cmFHL or cmWT. We observed a significant increase in migration and decrease in cleaved caspase-III expression after stimulation with cmFHL compared with cmWT (Figure 7D and 7E). Finally, ECs were pretreated with S1PR-1/-3 antagonist VPC23019 or vehicle before being exposed to cmWT or cmFHL. Increased migration and decreased cleaved caspase-III expression after stimulation with cmFHL were completely abolished by the S1PR-1/-3 antagonist (Figure 7D and 7E). These results demonstrate...
that absence of FHL2 in EOCs regulates production and secretion of S1P by these cells, which in turn regulates EC function through activation of S1PRs.

**FHL2−/− EOCs Have an Increased Reendothelialization Capacity**

To investigate the functional significance of FHL2 deletion in EOCs in vivo, we assessed reendothelialization capacity of these cells after focal carotid endothelial injury in mice. Carotid artery electric injury was induced in splenectomized WT mice, and spleen-derived cultured EOCs from WT or FHL2−/− donor mice, or saline, were injected intravenously. Subsequently, carotid artery reendothelialization was assessed after staining with Evans blue dye. Figure 8A demonstrates that injection of EOCs from WT mice enhanced reendothelialization by 28±4% compared with saline injection. Importantly, injection of EOCs from FHL2−/− mice significantly enhanced reendothelialization by an additional 30±2% as compared with WT EOC injection. Moreover, although the total number of EOCs attached to the arterial wall was low, we observed a 2-fold increase in vascular wall attachment of labeled FHL2−/− EOCs compared with WT EOCs (cell number/injured area ratio, 16±1.9 versus 8.6±1.7; Figure 8C). These findings clearly indicate the functional relevance of FHL2 knockout for enhanced EOC function in vivo.

**Discussion**

The present study demonstrates that FHL2 is a negative regulator of EOC number and function. We showed, for the first time to our knowledge, that FHL2 is expressed in EOCs. Inhibition of FHL2 increases EOC survival and migratory capacity by activating the SK-1/S1P pathway and upregulating αv/β3 and αv/β5 integrins. Moreover, inhibition of FHL2 regulates paracrine functions of EOCs, which leads to enhanced EC survival and migration through activation of S1PRs on ECs. Ultimately, inhibition of FHL2 results in improvement of endothelial regeneration by EOCs (Online Figure VI).

FHL2 is expressed by vascular smooth muscle cells and ECs,21,22 suggesting a potential role of FHL2 in the vasculature.9 However, the expression and function of FHL2 in EOCs was previously unknown. We demonstrated in vitro that inhibition of FHL2 in both human and murine EOCs increased their numbers. We observed a low rate of proliferation in EOCs in this and previous studies,28 and there was no effect of FHL2 inhibition on adhesion of EOCs to several matrix proteins. However, the apoptosis rate in human and murine EOCs with low FHL2 was significantly reduced. These data suggest that FHL2 inhibition supports prosurvival signaling in EOCs. Contradictory data are described in the literature regarding the effect of FHL2 on apoptosis in cell lines. Ectopic expression of FHL2 triggered apoptosis in COS1 cells, NIH 3T3 cells, and rhabdomyosarcoma cells.30 However, FHL2 failed to show any proapoptotic role in HeLa and human embryonic kidney 293 cells.31 Similarly, FHL-2 has divergent trophic effects, supporting proliferation in cancer cells32 but abating cardiac hypertrophic growth.33 These studies suggest that FHL2 may exert opposing biological functions in different cell types, possibly related to its interaction with diverse protein partners.

**Figure 7.** Conditioned medium from FHL2−/− early outgrowth cells increases migration and decreases apoptosis in endothelial cells (ECs) through sphingosine-1-phosphate (S1P) receptor-1 and -3 activation. A and B, Mouse ECs were stimulated with exogenous S1P (1 μmol/L) or vehicle (Veh) for 15 hours. A, EC migration was assessed by scratch assay. Representative images and corresponding bar graphs representing means±SEM of n=3; *P<0.05 vs Veh; scale bar, 50 μm. B, EC apoptosis was determined by cleaved caspase III expression. Means±SEM of n=3; **P<0.01 vs Veh. C, Mononuclear cells were isolated from the spleen of wild-type (WT) and FHL2−/− mice. Cells were cultured for 7 days; conditioned medium (cm) was collected at day 6 for S1P quantification. Means±SEM of n=4; *P<0.05 vs cmWT. D and E, Mouse ECs were pretreated with Veh or VPC23019 (20 nmol/L), an antagonist of S1P receptor-1 and -3 (S1PR-1/-3), and then exposed to cm for 15 hours. D, Migration was assessed by scratch assay. Bar graphs are means±SEM of n=4 to 6; *P<0.05 vs cmWT plus Veh; †P<0.05 vs cmFHL plus Veh; scale bar, 50 μm. E, Apoptosis was determined by cleaved caspase-III expression. Means±SEM of n=4 to 6; **P<0.01 vs cmWT plus Veh; †P<0.05 vs cmWT plus Veh; †P<0.05 vs cmFHL plus Veh; scale bar, 25 μm.
An important functional property of EOCs, essential for EOC-mediated reendothelialization, is their ability to migrate. We observed a significant increase in the migratory capacity of EOCs after inhibition or in the absence of FHL2. Our results are in agreement with the literature; it was reported that overexpression of FHL2 inhibits vascular endothelial growth factor–induced EC migration. Moreover, another recent study showed that FHL2 is an important regulator of chemotactic dendritic cell migration. Integrins play a key role in cell motility, and we found a significant increase in αvβ3 and αvβ3 subunit expression in human EOCs after FHL2 knockdown. In contrast, active β1 and β2 subunits were unchanged. Interestingly, specific blocking antibodies against αvβ5, αvβ3, and β2 integrins abolished the increase in migration associated with siFHL2. αvβ3 and αvβ5 integrins were shown to be involved in restenosis after angioplasty and constrictive vascular remodeling after injury through stimulation of smooth muscle cell migration. They have been shown to stimulate EC migration as well. Integrins β1 are well known to mediate transendothelial migration of human peripheral blood–derived EOCs. Our data indicate that FHL2 inhibition enhances SDF-1–induced EC migration through αvβ3, αvβ3, and β2 integrins. In contrast, β2 integrin does not seem to be involved in this process.

Previous studies reported that FHL2 binds to SK-1 and that this interaction inhibits SK-1 activity. Activation of SK-1 involves its phosphorylation and translocation to the cell membrane where its substrate, sphingosine, is located. SK-1 produces S1P, a bioactive sphingolipid that is known to act extracellularly via binding to 5 receptors of the lysophospholipid receptor family (S1PR-1–5). S1P has intracellular effects as well, acting as a signaling molecule and promoting cell survival, proliferation, and differentiation. S1P acts as a potent angiogenic mediator in ECs and cultured EOCs and stimulates the neovascularization capacity of EOCs and bone marrow cells. Likewise, SK-1 is involved in NO-mediated EC migration and tube formation. Recently, it was reported that FHL2 regulates phosphatidylinositol-3-kinase/Akt via direct suppression of the SK-1/S1P pathway in ECs. Moreover, regulation of dendritic cell migration by FHL2 involves activation of S1PR-1. Therefore, we hypothesized that FHL2 inhibition in ECs could activate the SK-1/S1P system, leading to the activation of prosurvival and promigratory pathways. We observed less apoptosis and enhanced SDF-1–induced migration in EOCs stimulated with exogenous S1P. We also showed that FHL2 knockdown increased SK-1 phosphorylation and translocation to the membrane and increased intracellular S1P levels and Akt phosphorylation. It has been reported that SK-1 enhances resistance to apoptosis through activation of the phosphatidylinositol-3-kinase/Akt/ NF-κB pathway in different cell types. Furthermore, S1P has antiapoptotic effects in human embryonic stem cells through extracellular signal-regulated kinase-1/2 and phosphatidylinositol-3-kinase/Akt signaling pathways. Importantly, we observed that SK-1 inhibition completely prevented the reduction of apoptosis and the increase of migration associated with siFHL2, suggesting that inhibition of FHL2 regulates survival and migration of EOCs through activation of SK-1. Our results are in agreement with a report showing that inhibition of FHL2 in
cardiomyocytes prevents myocardial apoptosis through activation of SK-1.  

To verify if S1P produced by EOCs acts intracellularly or is secreted and binds to its receptors present on the surface of EOCs by an autocrine effect, we used an antagonist of S1PR-1/-3. We found that the antagonist increased basal apoptosis and reduced basal migration. Moreover, the decrease in apoptosis induced by FHL2 siRNA was abolished by the antagonist. However, the antagonist only partially inhibited the increase in migration induced by FHL2 siRNA. These data suggest that S1P acts through S1PR-1/-3 to regulate EOC survival, and that the effect of FHL2 siRNA on EOC migration may involve intracellular S1P signaling or other S1PRs. The role of S1P in SDF-1–induced migration and bone marrow homing of human CD34+ progenitor cells has been reported.  

S1P-induced cell migration is dependent on the translocation of the cortactin–actin-related protein-2/3 complex (Arp2/3) to the lamellipodia region. Cortactin is an actin-binding protein. It is a substrate of Src kinase and is activated by tyrosine phosphorylation, leading to membrane ruffling, cortical actin assembly, lamellipodia formation, and cell mobility, suggesting a critical role of cortactin in cellular migration. Because LIM domain proteins are involved in cytoskeleton organization and our results show that FHL2 regulates EOC migration, we investigated the effect of FHL2 inhibition on the expression of cortactin in EOCs. We found a significant increase in cortactin with FHL2 siRNA, which was abolished with the SK-1 inhibitor. Similarly, S1P was shown to induce membrane ruffling and cortical actin assembly in human umbilical vein ECs, which ultimately resulted in endothelial chemotactic responses. Taken together, our results show that inhibition of FHL2 activates the SK-1/S1P/Akt pathway, which in turn could upregulate αvβ3 and αvβ5 integrins as well as cortactin, leading to actin organization and increased EOC migration. However, the exact mechanisms involved in the activation of integrins or cortactin by S1P need further investigation.  

EOCs have been shown to participate in endothelial repair either by differentiating into mature ECs or by producing soluble factors that act in a paracrine manner to mobilize tissue-resident progenitor cells or to regulate EC functionality. Considering this, we tested the hypothesis that FHL2 inhibition in EOCs regulates the secretion of paracrine factors that could in turn influence EC function. Having shown that FHL2 inhibition activates the SK-1/S1P pathway in EOCs, we first confirmed that this leads to S1P secretion into the extracellular space. S1P levels were enhanced in the conditioned media of FHL2−/− EOCs (cmFHL) compared with WT EOCs (cmWT). Because S1PRs are also present on the surface of ECs, S1P secreted by EOCs could affect ECs in a paracrine manner. We found that cmFHL, but not cmWT, reduced EC apoptosis and increased EC migration. Moreover, pretreatment of ECs with a S1PR-1/-3 antagonist not only completely abolished these effects induced by cmFHL, but also further increased the apoptosis rate in ECs. Several studies reported the strong EC cytoprotective properties of factors released by EOCs, such as vascular endothelial growth factor, SDF-1, insulin-like growth factor-1, and thymidine phosphorylase. Our data add S1P to this list of factors and reveal a critical role for FHL2 in regulating its release. Our results also support the notion that signaling pathways regulated by S1PR-1/-3 are required for EC chemotaxis, adherence junction assembly, EC morphogenesis, and angiogenesis in vitro and in vivo. Hence, we showed, for the first time to our knowledge, that inhibition or absence of FHL2 in EOCs improves EOC paracrine functions through activation of the SK-1/S1P pathway, which in turn activates S1PR-1/-3 on ECs, leading to an increase of EC survival and migratory capacity.  

Circulating EOCs contribute to endothelial regeneration by homing to and regulating ECs via paracrine signals. To assess the functional in vivo relevance of FHL2 inhibition in EOCs, we investigated carotid artery reendothelialization after injury. Importantly, we observed that reendothelialization was enhanced after intravenous injection of EOCs from FHL2−/− mice compared with EOCs from WT mice. In conjunction, FHL2−/− EOC incorporation at the site of injury was 2-fold higher compared with WT EOC. These results could be explained by an increased survival of FHL2−/− cells, in line with our in vitro data. The recruited EOCs were scattered among the new cells lining the site of injury, which supports the notion that the EOCs act mostly by paracrine functions. Our findings, therefore, underline the pathophysiological relevance of FHL2 expression in circulating cells.  

In summary, our data show, for the first time to our knowledge, that FHL2 is a strong negative regulator of EOC number, survival, and migratory capacity through regulation of the SK-1/S1P pathway. On the one hand, FHL2 knockdown in EOCs improves EOC functions by activating SK-1 and Akt, leading to a reduction of apoptosis, upregulation of integrins, and enhancement of migration. On the other hand, FHL2 knockdown improves EC function by paracrine effects, through regulation of S1P secretion by EOCs and S1PR-1 or S1PR-3 activation on ECs, leading to a reduction in EC apoptosis and increased EC migration. This study, with a considerable clinical relevance, provides mechanistic insight into the regulation of EOC survival and function and identifies a novel factor that may constitute a target with therapeutic potential.

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Disclosures

None.

References


**Novelty and Significance**

**What Is Known?**

- Early outgrowth cells (EOCs) participate in endothelial regeneration.
- Inhibition of four-and-a-half LIM domain protein-2 (FHL2) decreases atherosclerotic lesion formation.
- Inhibition of FHL2 increases endothelial cell migration.

**What New Information Does This Article Contribute?**

- Knockdown of FHL2 increases EOC number and viability through upregulation of sphingosine kinase-1 (SK-1).
- Knockdown of FHL2 increases EOC migration through upregulation of SK-1 and αβ/αβ integrin subunits.
- FHL2 knockdown increases secretion of sphingosine-1-phosphate (S1P) by EOCs leading to an increase in endothelial cell migration and viability through activation of S1P receptor-1/-3 (S1PR-1/-3).
- Absence of FHL2 in EOCs increases their capacity to repair the endothelium.

FHL2 is highly expressed in vascular cells. It can regulate cell function and structure by interacting with multiple proteins at specific sites in the cell. However, the role of FHL2 in EOCs is not known. Here, we report that knockdown of FHL2 in human EOCs significantly increases their number, viability, and migration through activation of SK-1, the enzyme that produces S1P, and upregulation of integrins. We also found that knockdown of FHL2 regulates the secretion of S1P by EOCs that improves endothelial cell function via activation of its receptor, S1PR-1/-3, expressed on the surface of endothelial cells. Importantly, absence of FHL2 in EOCs increases their capacity to repair the endothelium. These findings support the notion that FHL2 is a potential therapeutic target to improve EOC function to combat diminished endothelial regeneration, which is considered to play an essential role in vascular pathogenesis.
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Inhibition of Four-and-a-Half LIM domain protein 2 increases survival, migratory capacity and paracrine function of human early outgrowth cells through activation of the sphingosine kinase-1 pathway: Implications for endothelial regeneration

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**Supplemental Methods:**

**Compounds**
CAY10621 and S1P were purchased from Cayman Chemical, (R) - Phosphoric acid mono- [2-amino-2- (3-octyl-phenylcarbamoyl)- ethyl] ester (VPC 23019) from Avanti Polar Lipids, fibronectin, Evans blue, salts and other chemicals were obtained from Sigma.

**Preparation of mononuclear cells**
Mononuclear cells (MNCs) were isolated from peripheral blood of healthy donors (age 20-45 years, male and female, no known disease or medication) using a Ficoll density gradient (Sigma) according to standard protocols, as previously described. The study was approved by an institutional review committee and the subjects gave informed consent. For mouse MNCs, male 8-10 week-old wild-type (WT) and FHL2 knockout (FHL2/-) mice with C57BL/6J background, initially provided by Dr. Büttner, University of Bonn, Bonn, Germany, were used. Spleens were explanted, mechanically minced, and MNCs were isolated using a Ficoll gradient, as previously described.

**Early outgrowth cells**
Human or mouse MNCs (5x10^5) were seeded on fibronectin-coated 24-well plates in endothelial basal medium (EBM) with supplements (Cederlane), as previously described. After 7 days of culture, fibronectin-adherent cells were stained with 1,1'-dioctadecyl- 3,3,3',3' tetramethylindocarbocyanine (Dil)-labeled acetylated low-density lipoprotein (acLDL; Invitrogen) and stained with FITC-labeled lectin from *Ulex europaeus* agglutinin (Sigma) for human early outgrowth cells (EOCs), and FITC-labeled lectin 1 from *Bandeiraea simplicifolia* (Griffonia simplicifolia) (Sigma) for mouse EOCs, respectively. Cells double-positive for Dil-acLDL and lectin staining were considered to be early outgrowth cells. EOCs are defined according to surface markers and properties. One recent classification distinguishes between early outgrowth EOCs, which display paracrine actions, and late outgrowth EOCs, which are characterized by high proliferative potential, by differentiating between cellular functional capacities, time of appearance during cell culture and the cell culture protocol used.

**Transfection with siRNA**
Human EOCs were transfected at day 6 of cell culture with a specific FHL2 siRNA (siFHL2) (5nM, Qiagen) or luciferase siRNA (siLuci), used as transfection control siRNA (Qiagen), for 24 hours in endothelial basal medium (EBM) with supplements (Cederlane) by using HiPerFect transfection reagent (Qiagen).

**Proliferation**
MNCs were cultured for 7 days in EBM with supplements (Cederlane) and transfected with siRNA for 24 hours. Fibronectin-adherent cells were stained with Dil-acLDL and lectin, washed and incubated with BSA (3%), Ki67 primary antibody (Abcam) and a CY5-coupled secondary antibody (Invitrogen). Nuclei were stained with DAPI. Fluorescence was visualized with a DM2000 microscope with DAPI (358 nm), FITC (488 nm), CY3 (555 nm) and CY5 (647nm) filters, respectively (Leica Microsystems).
**Apoptosis**

MNCs were cultured for 7 days in EBM with supplements (Cederlane) and transfected with siRNA for 24 hours. In some experiments cells were preincubated for 30 minutes with CAY10621, a specific inhibitor of sphingosine kinase-1 (SK-1) (5 µM) or with VPC23019 (20nM), an antagonist of sphingosine-1-phosphate receptor-1, -3 (S1PR-1/-3) or stimulated with TNF-α (25ng/ml) for 16 hours. Fibronectin-adherent cells were stained with Dil-acLDL and lectin, washed and incubated with BSA (3%), cleaved caspase-III primary antibody (Abcam) and a CY5-coupled secondary antibody (Invitrogen). Nuclei were stained with DAPI. Fluorescence was visualized with a DM2000 microscope with DAPI (358 nm), FITC (488 nm), CY3 (555 nm) and CY5 (647nm) filters, respectively (Leica Microsystems).

To determine mouse endothelial cell (EC) apoptosis, ECs were preexposed to VPC23019 (20nM), an antagonist of S1PR-1/-3 for 30 minutes, and then were exposed to the conditioned medium from WT or FHL2 knockout mouse EOCs for 15 hours. Immunostaining for cleaved caspase-III and nuclear staining with Dapi was then performed in the ECs.

As an additional approach, the Bax/Bcl-2 expression ratio was assessed by western blot (see western blot paragraph), as previously described. ¹

**Migration**

Migratory capacity of EOCs was evaluated by using modified Boyden chambers. An uncoated polycarbonate filter with 8-µm pore size (Becton Dickinson) was placed between the upper and lower chamber. Cell suspensions were placed in the upper chamber, and the lower chamber was filled with EBM containing human recombinant stromal cell-derived factor-1 (SDF-1, Chemicon). Migration was evaluated by the mean number of Dil-acLDL/lectin positive cells counted in at least 3 high-power fields per filter.

After 5 days of initial cell culture and 30 min pre-exposure to blocking antibodies against αv/β3 (10 µg/ml, mouse IgG1- clone 23C6), αv/β5 (10 µg/ml, mouse IgG1- clone P5H9), β1 (10 µg/ml, clone P5D2- R&D), β2 (10 µg/ml, clone 24- Hycult) or control IgG (10 µg/ml, clone EM-07), for experiments that involved integrin inhibition, or CAY10621, a specific inhibitor of SK-1 (5 µM) or with VPC23019 (20nM), an antagonist of S1PR-1/-3, for experiments that involved SK-1/S1P pathway inhibition, followed by 24 hours transfection with siFHL2 or siLuci (for human EOCs), cell suspensions (5x10⁴ cells per well) were placed in the upper chamber, and the lower chamber was filled with EBM containing 50 ng/ml of human recombinant stromal cell-derived factor-1 (SDF-1, Chemicon). Cells were then incubated for additional 15h. The migrated cells on the lower side of the filter were fixed and stained for Dil-acLDL/lectin. Migration capacity was evaluated by the mean number of Dil-acLDL/lectin positive cells counted in at least 3 high-power fields per filter.

Endothelial cell migratory capacity was evaluated by using the scratch assay. Cells were seeded into 24-well plates and grown to 100% confluence. The cells were then rendered quiescent by changing the medium to basal EBM without FBS for 16 h and were wounded once with a small tip by scratching across the maximum diameter of each well. The cells were then washed twice, and conditioned medium from WT or FHL2-/- EOCs was added. For experiments with the inhibitor, cells were pretreated 30 min before wounding with VPC23019, for experiments that involved S1PR-1/-3 inhibition. Cells were wounded once with a small tip by scratching across the maximum diameter of each well. The cells were then washed twice, and conditioned medium from WT or FHL2 knockout EOCs was added. Pictures were taken using a Leica digital camera of an inverted microscope with 4x or 10x objective lenses. Pictures were taken immediately after scratching as well as 24 h after. Images were analyzed using Image J.
software (NIH) and the size of the denuded area was measured using the polygon selection tool.
Additionally, cortactin immunocytochemistry was performed. MNCs were cultured for 7 days as described above. Fibronectin-adherent cells were washed and incubated with BSA (3%), primary antibodies against cortactin (Abcam) and a CY3-coupled secondary antibody (Invitrogen). Nuclei were stained with DAPI (Sigma). Fluorescence was visualized with a DM2000 microscope with DAPI (358 nm) and CY3 (555 nm) filters (Leica Microsystems).

Western blotting
MNCs were cultured for 7 days as described above. Fibronectin-adherent cells were collected and fractioned or total proteins (15-20 μg) were extracted, separated by SDS-PAGE, transferred to nitrocellulose membranes and incubated overnight at 4°C with primary antibodies against phosphorylated and total form of SK-1 (Ecm Biosciences), Akt (Cell Signaling Technology) and total forms of Bcl-2 (Abcam) and Bax (Santa Cruz), respectively. After incubation with secondary antibodies, signals were revealed by chemiluminescence (Western Lightning Plus ECL, Perkin Elmer) with the Molecular Imager Chemidoc XRS system (Bio-Rad) and quantified by densitometry using Quantity One software (Bio-Rad). Membranes were subsequently stripped and re-probed with a β-actin antibody (Sigma) to verify equal loading.

Flow Cytometry
Flow cytometry analysis was performed as recently described.‡ Integrin expression was assessed in human EOCs. MNCs were cultured for 7 days as described above. At day 6, siFHL2 or siLuci transfected EOCs were detached and incubated with FITC-conjugated anti-human specific integrin αv/β3 (Mouse IgG1, clone 23C6), αv/β5 (Mouse IgG1, clone P1F6) antibodies (Becton Dickinson) as well as with the anti- human specific activated forms of β1 (clone HUTS-4-Millipore) and β2 (clone 24- Hycult) antibodies. For β1 and β2 integrins the incubation with the first antibodies was followed by 1 hour incubation with an alexa-fluor 488 secondary antibody (Invitrogen). Cell fluorescence was measured using a FACS Calibur instrument. Data were analyzed using CellQuestPro software (Becton Dickinson). Units of all measured components are fluorescence-positive cells obtained after measuring of 20,000 events in a pre-specified lymphocyte gate.

S1p levels in human EOCs and in mouse conditioned medium
Human or mouse MNCs were cultured for 7 days as described above. Fibronectin-adherent human EOCs were lysed and mouse EOC conditioned medium was collected. S1p levels were measured using a kit from Echelon bioscience, following the manufacturer’s instructions.

Paracrine effects
Mononuclear cells were isolated from the spleen of wild-type (WT) and FHL2 knockout (FHL2-/-) mice. Cells were cultured in supplemented endothelial basal medium for 7 days. Conditioned medium (cm) of the cells was collected at day 6, centrifuged and filtered. Mouse endothelial cells pretreated with vehicle or VPC23019 (20 nM), an antagonist of S1P receptor-1 and -3 (S1PR-1/-3), were exposed to cm for 15 hours.

Carotid injury
All procedures were approved by the Animal Care Committees of McGill University, and followed recommendations of the Canadian Council on Animal Care. For all invasive procedures, mice were anesthetized with isoflurane (0.75-1 % isoflurane, 1L/min O2). Spleens from WT and FHL2-/- donor mice were explanted, mechanically minced, MNCs
were isolated as previously described and were cultured on fibronectin in EBM with supplements (Cederlane) for 7 days to obtain early outgrowth EOCs. Splenectomy of recipient WT mice was performed as previously described.\textsuperscript{1,2} Animals were allowed to recover for 7 days before further treatment was performed. For assessment of reendothelialization, carotid artery endothelial injury was performed in splenectomized WT mice, as previously described.\textsuperscript{1} The common carotid artery was exposed and submitted to an electric injury starting at the bifurcation and continuing to the proximal artery with a total denudation length of 2mm (with 2W for 5s, 2mm-tip bipolar coagulation forceps (force FX-Cs generator), Tyco Healthcare, Pointe Claire, Canada). After induction of carotid artery electric injury, splenectomized WT mice received $1 \times 10^6$ EOCs (from WT or FHL2-/- mice) in 200 \mu l normal saline solution by intravenous tail vein injection on 2 consecutive days. The denuded area of the carotid artery was determined at day 4 after injury in an en-face preparation of the vessel after intravenous injection of 50\mu l Evans blue 5%. Non-reendothelialized lesion areas show intense blue staining. Vessels were examined under a Leica M651 dissecting microscope and pictures were taken with an Infinity-1 digital camera. Reendothelialization is expressed as Evans blue-positive (non-reendothelialized) lesion area over the total area. Lesion and total areas were measured using Image J software. For another set of experiments splenectomized WT mice received two injections of $1 \times 10^6$ green fluorescent labeled-EOCs extracted from the spleen of WT or FHL2-/- mice, on two consecutive days after carotid injury. At day 4 after injury the blue lesion area was dissected, the vessel was opened, mounted with a Dapi mounting medium and observed En face with fluorescent microscope.

**Statistical Analysis**

Results are expressed as mean±SEM. All data was evaluated by ANOVA. Statistical significance for comparisons between 2 groups was calculated using the 2-tailed Student t test. To assess comparisons between multiple groups, data were analyzed using the Newman-Keuls test. A probability value of <0.05 was considered to be statistically significant. Data that was significant (p<0.05) was further assessed by Student t test using GraphPad Prism software (GraphPad Software).
Supplemental References


Supplemental figure legends:

Online Figure I: FHL2 knockdown has no effect on EOC static adhesion.

Human mononuclear cells were isolated and cultured on fibronectin for 7 days. EOC static adhesion on Human Umbilical Vein Endothelial Cells (HUVECs) (A), fibronectin (B), collagen (C), or laminin (D) was evaluated by immunocytochemistry. EPCs were pre-stained with Dil-acLDL before the adhesion assay. A: Representative microscopic scans showing stained EPCs and HUVECs. Blue=Dapi (HUVECs), red=Dil-acLDL (EOCs). A-D: Bar graphs represent the number of adhered EOCs to different matrix proteins, mean±SEM of n=5 for each experiment.

Online Figure II: Absence of FHL2 decreases EOC apoptosis.

A: Human mononuclear cells were isolated and cultured on fibronectin for 7 days. Cells were transfected on day 5 with FHL2 siRNA (siFHL) or luciferase siRNA (siLuci) for 24 hours, and then stimulated with vehicle (Veh) or TNF-α (25ng/ml) for 16 hours. Cell apoptosis was assessed by cleaved (active) caspase-III immunostaining in Dil-acLDL/lectin double-positive EOCs. Data are mean±SEM of n=4-6, *P<0.01, **P<0.001 vs. siLuci. †P<0.05 vs. siLuci+TNF-α. B: FACS analyses for Annexin+/PI- on cultured EOCs. Mononuclear cells were isolated from the spleen of wild-type (WT) and FHL2 knockout (FHL2-/-) mice. Cells were cultured on fibronectin for 7 days. Bar graph represents percentages of fluorescent-positive cells among 20,000 gated events mean±SEM of n=6, *P<0.05 vs WT.

Online Figure III: SDF-1 induces EOC migration.

Human mononuclear cells were isolated and cultured on fibronectin for 7 days. Cell migration was assessed by Boyden chamber assays with BSA (10%) or SDF-1 (50 ng/ml) placed in the lower chamber. Bar graph represents the number of Dil-acLDL/lectin double-positive EOCs, mean±SEM of n=5, ***P<0.0001 vs BSA.

Online Figure IV: FHL2 knockdown increases αv/β3 and αv/β5 surface expression but does not have any effect on their mean fluorescence intensities.

Human mononuclear cells were isolated and cultured on fibronectin for 7 days. Cells were transfected with FHL2 siRNA (siFHL) or luciferase siRNA (siLuci) for 24 hours. FACS analysis was performed to detect αv/β3, αv/β5, active forms of β1 or β2 integrins, or isotype control. A:
Scatter plots representing percentages of positive cells for $\alpha v/\beta 3$ and $\alpha v/\beta 5$. B: Bar graphs representing mean fluorescence intensities among 20000 gated events. Mean±SEM of n=4-6.

**Online Figure V: EOC number is increased in spleen derived mononuclear cells from FHL2-/- mice.**

Mononuclear cells were isolated from the spleen (A), the bone- marrow (B), and peripheral blood (C) of wild-type (WT) and FHL2-/- mice. FACS analysis for Flk-1+/Sca-1+ was performed. A-C: Bar graphs represent percentages of fluorescent- positive cells among 20,000 gated events. Mean±SEM of n=6, *P<0.05 vs WT.

**Online Figure VI: Proposed role for FHL2 as a regulator of EOC function.**

Knockdown of FHL2 in EOCs activates sphingosine kinase-1 (SK-1) by phosphorylation (P) and translocation to the cell membrane. Activation of SK-1 leads to the phosphorylation of sphingosine (S) and increases sphingosine-1-phosphate (S1P). S1P is secreted into the extracellular space and binds to its specific S1P receptors 1 and 3 (S1PR-1/-3) on the surface of EOCs (autocrine effect) or endothelial cells (EC) (paracrine effect). Activation of S1PR-1/-3 on ECs reduces apoptosis and enhances cell migration. S1P activates pro-migratory and pro-survival pathways in EOCs, involving Akt, integrins, and cortactin/actin filaments, either directly or through the activation of S1PR-1/-3 on EOCs.
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