ARTICLE
Journal of Cellular Biochemistry 112:3103–3111 (2011)

Journal of Cellular Biochemistry

Gene Modification With Integrin-Linked Kinase Improves Function of Endothelial Progenitor Cells in Pre-Eclampsia In vitro

Fang Wang, Ying Wang, Li Zhang, and Li Zou*

Department of Gynecology and Obstetrics, Union Hospital, Huazhong University of Science and Technology, Wuhan 430022, China

ABSTRACT

Integrin-linked kinase (ILK), a multifunctional serine–threonine protein kinase, has been shown to have implications for the treatment of ischemia vascular diseases by promoting angiogenesis in various tissues. However, whether this kinase has therapeutic potential in pre-eclampsia is not well studied. In this report, we determined the changes in the production and action of ILK on endothelial progenitor cells (EPCs) isolated from patients with pre-eclampsia. The effects of ILK transfection on proliferation, migration, and angiogenesis of EPCs were investigated. We showed that EPCs transfected with the ILK gene expressed high levels of ILK protein and mRNA. Transfection with ILK also enhanced the proliferative, migratory, and angiogenic capabilities of EPCs, and promoted the production of VEGF. These results suggest that ILK gene transfection is an effective approach to augment angiogenic properties of EPCs in vitro and providing basis for clinical cell-based gene therapy in patients with pre-eclampsia. J. Cell. Biochem. 112: 3103–3111, 2011. © 2011 Wiley Periodicals, Inc.

KEY WORDS: INTEGRIN-LINKED KINASE; ENDOTHELIAL PROGENITOR CELLS; PRE-ECLAMPSIA; VASCULOGENESIS

Vasculogenesis is a process defined as the differentiation of precursor cells into the endothelial lineage and the de novo formation of a primitive vascular network [Vailhé et al., 2001]. Initially thought to occur only during embryonic developmental stages, vasculogenesis is now considered an important contributor to postnatal vascular formation and pathological vascular remodeling [Takahashi et al., 1999]. Endothelial progenitor cells (EPCs), first characterized in the 1990s by Asahara et al. [1997], are critical for maintaining endothelial function in mature blood vessels by contributing to re-endothelialization and vascularization [Reyes et al., 2002]. Also, EPCs participate in pathologic angiogenesis such as that found in retinopathy and tumor growth [Nolan et al., 2007; Gao et al., 2008]. It is therefore conceivable that any impairment of this vasculogenic element may account for the progression of endothelial dysfunction and organ ischemia. Recent studies showed a reduction of the number and impairment of biological activity of EPCs in patients with ischemic diseases including pre-eclampsia [Kwon et al., 2007; Xia et al., 2007; Zhou et al., 2008]. The important pathological feature of pre-eclampsia is defective vasculogenesis of placenta [Roberts and Cooper, 2001]. The EPCs, therefore, have been proposed as a potential therapy target for pre-eclampsia.

Integrin-linked kinase (ILK), a novel, ankyrin repeat containing serine–threonine protein kinase, was originally isolated in a yeast 2-hybrid screen by virtue of its interaction with the cytoplasmic domain of β1 integrins [Hannigan et al., 1996]. It comprises three structurally distinct regions and can interact directly with the integrin β1 and β3 subunits. ILK also interacts with critical actin-binding proteins, such as paxillin, CH-ILKBP, and PINCH1 via its C-terminal kinase and first ankyrin domain [Wu and Dedhar, 2001]. Recently, ILK was found to have direct targets for its kinase activity, glycogen synthase kinase-3b (GSK3B), phosphorylating protein kinase B (AKT), and β-parvin [Persad et al., 2001; Yamaji et al., 2001; Elustondo et al., 2006]. In addition to its function as a kinase, ILK can be involved in a range of signaling pathways, bridging extracellular matrix signals, and inside–out signals with receptor tyrosine kinases and the actin cytoskeleton [Hynes, 2002; Boulter et al., 2006]. Overexpression of active ILK in normal epithelial cells results in loss of cell–cell adhesion, suppression of apoptosis, and stimulating anchorage-independent cell cycle progression [Zhang et al., 2002; Terpstra et al., 2003; Troussard et al., 2003; Cho et al., 2005]. Moreover, ILK has certain therapeutic effect in ischemia vascular diseases, for example, myocardial infarction, attributing to its pivotal role in inflammation and blood vessel formation during embryogenesis and other physiological settings [Ding et al., 2009]. However, the potential role of ILK as an angiogenesis factor in the process of placenta ischemia/hypoxia has not been studied.

Grant sponsor: National Natural Science Foundation of China; Grant number: 30872776.
*Correspondence to: Li Zou, Department of Gynecology and Obstetrics, Union Hospital, Huazhong University of Science and Technology, Wuhan 430022, China. E-mail: zoulihust@hotmail.com
Received 17 May 2011; Accepted 10 June 2011 • DOI 10.1002/jcb.23236 • © 2011 Wiley Periodicals, Inc.
Published online 17 June 2011 in Wiley Online Library (wileyonlinelibrary.com).

Accordingly, in the present study, we explored the possibility of genetic modification with ILK of EPCs from patients with pre-eclampsia. We also investigated the effects of this gene transfer on important angiogenic characteristics of EPCs in vitro.

MATERIALS AND METHODS

CELL CULTURE AND EPCS CHARACTERIZATION

Endothelial progenitor cells were isolated from 20 pre-eclampsia patients who were defined following the criteria as development, after 20 week gestation, of blood pressure of 140/90 mmHg or higher and proteinuria greater than 300 mg/24 h in previously normotensive women. Exclusion criteria were: Rupture of membranes, tobacco use, history of cardiovascular disease or diabetes mellitus, prenatal maternal infection, fetal anomaly, or intrauterine growth restriction. Besides, five age-matched normotensive women having normal pregnancy were also included for the study. Cesarean section was carried out in all the subjects (normotensive ones undergoing cesarean section due to their own demands). Immediately after the delivery of placenta, cord blood (30–50 ml) was sampled from the umbilical vein with heparinized syringes. Ethical approval was obtained before any patient's enrolment from the ethics committee of Union hospital, Tongji Medical College, Huazhong University of Science and Technology, China. Informed consent was obtained from each patient.

The mononuclear cells (MNCs) from cord blood were isolated by employing the method described previously [Kalka et al., 2000]. Briefly, MNCs were isolated by Ficoll density gradient centrifugation (Histopaque 1077, Sigma-Aldrich). After purification with PBS, MNCs were seeded on fibronectin-coated (Sigma-Aldrich), 6-well culture dishes (Corning) at a density of 2×10^6 cells/well in M-199 (Sigma-Aldrich) supplemented with 20% fetal bovine serum (Gibco, Rockville, MD), vascular endothelial growth factor (VEGF) (10 ng/ml, Sigma-Aldrich), fibroblast growth factor-2 (b-FGF-2) (2 ng/ml, Sigma-Aldrich), penicillin (100 μg/ml), and streptomycin (100 μg/ml). After 5 days of culture, nonadherent cells were discarded and all experiments were performed with EPCs at day 7.

To assess the ability of cells to take up DiI-acetylated-low-density lipoprotein (DiI-Ac-LDL, Molecular Probes, Carlsbad, CA) and FITC-labeled Ulex europaeus agglutinin (FITC-UEA-1, Sigma-Aldrich, St. Louis, MO), attached cells were incubated with DiI-Ac-LDL (2.4 μg/ml) for 4 h at 37°C. Cells were washed three times and fixed with 2% paraformaldehyde for 15 min and then incubated with FITC-UEA-1 (10 μg/ml) for 1 h. Double-positive fluorescence cells for both DiI-Ac-LDL and FITC-UEA-1 were identified as differentiating EPCs using laser scanning confocal microscope (LSCM, Leica) [Kalka et al., 2000; Vasa et al., 2001] (Fig. 2). Also, to compare the basal expression of ILK in EPCs from healthy controls and pre-eclampsia patients, endogenous ILK expression in the cells was quantified by immunoblot and RT-PCR analysis.

TRANSFECTIONS

The adenovirus expressing ILK (Ad-ILK) was produced by SinoGenoMax Co (Beijing, China). Briefly, human wild-type ILK plasmid was subcloned into the adenoviral shuttle plasmid pShuttle–CMV (driven by cytomegalovirus promoter/enhancer). The recombinant shuttle vector, pShuttle–CMV–ILK, was cotransfected with adenoviral genome (pAdxsi)-containing green fluorescence protein (GFP) gene into 293a cells to generate the recombinant adenovirus. The complete construct of Ad-ILK was further confirmed by DNA sequencing, and the expression was confirmed by immunoblot analysis. Adenovirus-encoding GFP was used as controls. In preparation for the transfections, the cells were cultured in 6-well plates. When the cells reached 90% confluency they were transfected with indicated virus at an MOI of 50 for all experiments.

WESTERN IMMUNOBLOTTING

EPCs transfected with the indicated virus were washed in ice-cold PBS and harvested by scraping in lysis buffer Nonidet P-40 (50 mmol/L HEPES pH 7.4, 150 mmol/L NaCl, 1% Nonidet P-40, 0.5% deoxycholate, 2 mmol/L Na_3VO_4, 1 mmol/L NaF, 2 mmol/L β-glycerophosphate). Lysates were centrifuged at 12,000 rpm for 30 min at 4°C and protein concentration was determined with BCA protein assay kit (Pierce). Thirty micrograms protein was separated on 10% SDS–polyacrylamide electrophoresis gel and transferred to a polyvinylidene fluoride membrane (Millipore). After incubated in 5% non-fat milk in phosphate-buffered saline with 0.1% Tween, the blots was incubated with a rabbit monoclonal ILK antibody at 1:3,000 concentration (Epitomics) and a rabbit VEGF monoclonal antibody at 1:2,000 concentration (Epitomics) overnight at 4°C. After washing, blots were incubated with appropriate secondary antibodies for 1 h at room temperature and ECL-PLUS (Amersham Biosciences) was used for detection. β-actin served as an internal control to assess protein loading.

RNA EXTRACTION AND REVERSE TRANSCRIPTION–PCR

Total RNA was extracted from EPCs transfected with the indicated virus for 24 h using TRIZOL reagent (Invitrogen). RNA was quantified and reverse transcribed into complementary DNA (cDNA) using RevertAidTM First Strand cDNA Synthesis Kit (Fermentas). GAPDH was used as the internal control. The RT-PCR primer sequences and annealing temperatures were as follows: Integrin-linked kinase, forward primer: 5′-TTC AAA CAG CTT AAC TTC CT-3′, reverse primer: 5′-ACT CGA CAT GTC TGC TGA GC-3′, product size: 564 bp, annealing temperature: 50°C for 30 cycles. GAPDH, forward primer: 5′-ACC ACA GTC CAT GCC ATC AC-3′, reverse primer: 5′-TCC ACC ACC CTG TTG CTG TA-3′, product size: 452 bp, annealing temperature: 51°C for 32 cycles.

CELL PROLIFERATION ASSAY

The effect of ILK on EPCs proliferation was assessed by DNA synthesis, which was determined by [methyl-^{3}H]-thymidine incorporation. Briefly, after 48 h of transfection, Ad-ILK-EPCs and Ad-GFP-EPCs or untransfected EPCs were synchronized in M199 containing 2% serum for 24 h. After that cells were treated with 2% serum in absence or presence of VEGF (50 ng/ml), with [^{3}H]-thymidine (Amersham Corporation) at a final concentration of 0.5 μCi for another 24 h. Cells were harvested using a cell harvester (Packard Bioscience, Meriden, CT) and counted in a liquid scintillation counter (Wallac).

CELL MIGRATION ASSAY

EPC migration was evaluated using a modified Boyden chamber (Costar). The Ad-ILK-EPCs and Ad-GFP-EPCs or untransfected EPCs were trypsinized and resuspended in 500 µl M199. 5×10^4 EPCs were plated in the upper chamber of a modified Boyden chamber. The chamber was placed in a 24-well culture dish containing M199 and 10 ng/ml stromal cell-derived factor-1 (SDF-1, Sigma-Aldrich). After incubation at 37°C for 24 h, the lower side of the filter was washed with PBS and fixed with 2% paraformaldeyde. For quantification, cell nuclei were stained with crystal violet. Cells migrating into the lower chamber were counted manually in three random fields under 100× magnifications by two independent observers.

CELL DIFFERENTIATION ASSAY

After 24 h of transfection, Ad-ILK-EPCs and Ad-GFP-EPCs or untransfected EPCs were trypsinized and suspended in complete medium. 2×10^6 cells were re-plated onto fibronectin-coated culture dishes and incubated at 37°C for 24 h. The number of spindle-shaped elongated cells representing differentiated cells [Asahara et al., 1997] was counted in five random fields under a phase-contrast microscope.

IN VITRO ANGIOGENESIS ASSAYS

Tube formation assay was performed to assess the effect of ILK gene transfer on the ability of EPCs vasculogenesis. According to the manufacturer's instructions, matrigel (BD Biosciences, San Jose, CA) was thawed at 4°C overnight and plated in a 48-well dishes at 37°C for 1 h 2×10^4 untransfected EPCs were co-plated with 2×10^4 Ad-ILK-EPCs or Ad-GFP-EPCs on top of the matrigel. After 12 h of co-culture, the formation of a tubular network was documented by phase-contrast and fluorescence microscopy and the number of gene modified EPCs contributing to tube formation was assessed microscopically by counting the number of GFP-labeled cells. Human umbilical vein endothelial cells (HUVECs) tube formation of the identical cell numbers served as positive control.

STATISTICAL ANALYSIS

The data shown in the figures and the text are expressed as mean ± SD. Comparisons between two groups were made using Student's *t*-tests and more than two groups means were done using one-way ANOVA. Post hoc tests involved the Student–Newmann–Keuls test. A value of $P < 0.05$ was considered significant.

RESULTS

MORPHOLOGY AND CHARACTERIZATION OF EPCS

Freshly isolated MNCs were round and formed small round EPC clusters the next day of culture. After 96 h round cells started elongating and gradually assumed cobblestone-like monolayer growth pattern with nearly complete confluence (Fig. 1A). And after 7 days of culture, EPC clusters displayed the characteristic

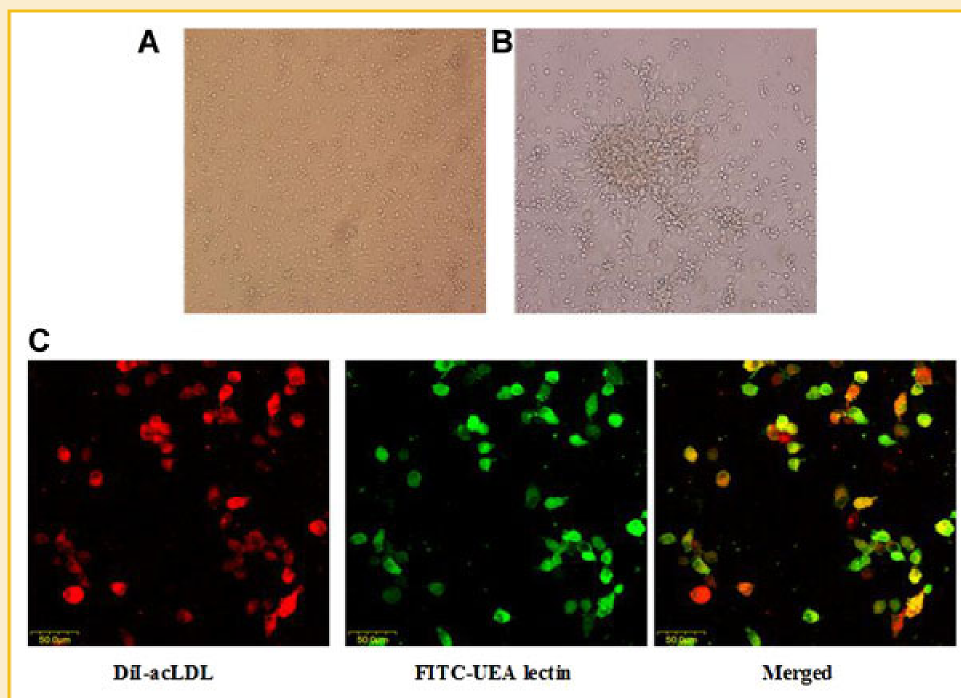

Fig. 1. Characterization of isolated EPCs. A: Photomicrograph of cobblestone-like EPCs grown to confluence on 7 day of culture (×100). B: Phase-contrast photomicrograph of a typical endothelial cell colony-forming unit (CFU-EC) that is counted as an EPC (×200). C: At 7 days of culture, the representative pictures of dual-stained EPCs for Dil-ac-LDL uptake, FITC-UEA-1 binding and merged in confocal microscopy (×400). [Color figure can be seen in the online version of this article, available at http://wileyonlinelibrary.com/journal/jcb]

phenotype consisting of central cluster of rounded cells with spindle-shaped cells sprouting at the periphery of the colony (Fig. 1B). DiI-ac-LDL uptake and FITC-UEA-1 binding were also observed in these spindle-shaped attached cells (Fig. 1C), further confirming their EPC phenotype. In addition, ILK endogenous expression was compared between EPCs from healthy subjects and patients with pre-eclampsia. ILK protein and mRNA was expressed in both the groups and showed greater decreases in pre-eclampsia patients. ($P < 0.01$) (Fig. 2).

ILK OVEREXPRESSION IN TRANSFECTED EPCS

We used adenovirus technique to transfect EPCs with vectors containing GFP, GFP-ILK, and negative control. After 8 h of transfection, GFP expression was detected by fluorescent microscopy in both the GFP and GFP–ILK groups (Fig. 3A). After 24 h of transfection, ILK protein was expressed in all the groups and showed greater increases in Ad-ILK genetically modified cells ($P < 0.01$) (Fig. 3B, C). And VEGF protein in the Ad-ILK group was also higher than that in the other two groups ($P < 0.01$) (Fig. 3B, C). Moreover, the RT-PCR detection with ILK primers revealed a 564 bp fragment in all the groups; however, the expression of ILK mRNA in the Ad-ILK group was significantly higher than that in the Ad-GFP or control groups ($P < 0.01$) (Fig. 3D, E).

CELL PROLIFERATION, MIGRATION, AND DIFFERENTIATION

The proliferation capacity was examined by thymidine uptake, a measure of de novo DNA synthesis. The thymidine uptake of Ad-ILK-EPCs was significantly enhanced in comparison to that observed in Ad-GFP-EPCs or untransfected EPCs ($P < 0.01$) (Fig. 4A). It has been reported that SDF-1 is important in EPC recruitment to ischemic tissue [Ceradini et al., 2004; Chavakis et al., 2005]. Therefore, we used a modified Boyden chamber to examine the effect of ILK gene transfection on SDF-1-mediated EPC migration. After 24 h of incubation at 37°C, the migratory capacity of Ad-ILK-EPCs was significantly higher than that of Ad-GFP-EPCs or untransfected EPCs ($P < 0.01$) (Fig. 4B). Next, we investigated the effects of ILK transfection on EPC differentiation in patients with pre-eclampsia. The Ad-ILK-transfected and Ad-GFP-transfected EPCs or untransfected EPCs were re-plated onto fibronectin-coated dishes and the number of endothelial-like spindle-shaped cells was counted. The Ad-ILK-EPCs showed a marked increase in the number of spindle-shaped attached cells in comparison to that observed in Ad-GFP-EPCs or untransfected EPCs ($P < 0.01$) (Fig. 5).

ANGIOGENESIS IN VITRO

A matrigel model was used to assess the capacity of EPCs to differentiate into tube-like structures. There were no tube-like structures formed when untransfected EPCs seeded alone on the matrigel (data not shown). However, when co-cultured with Ad-ILK-EPCs, the tendency of tube-like formation was more clear and obvious than that of Ad-GFP-EPCs (Fig. 6A, C). The number of GFP-labeled EPCs contributing to tube formation in the Ad-ILK group was significantly enhanced in comparison to that observed in Ad-GFP group ($P < 0.05$) (Fig. 6B, D, F).

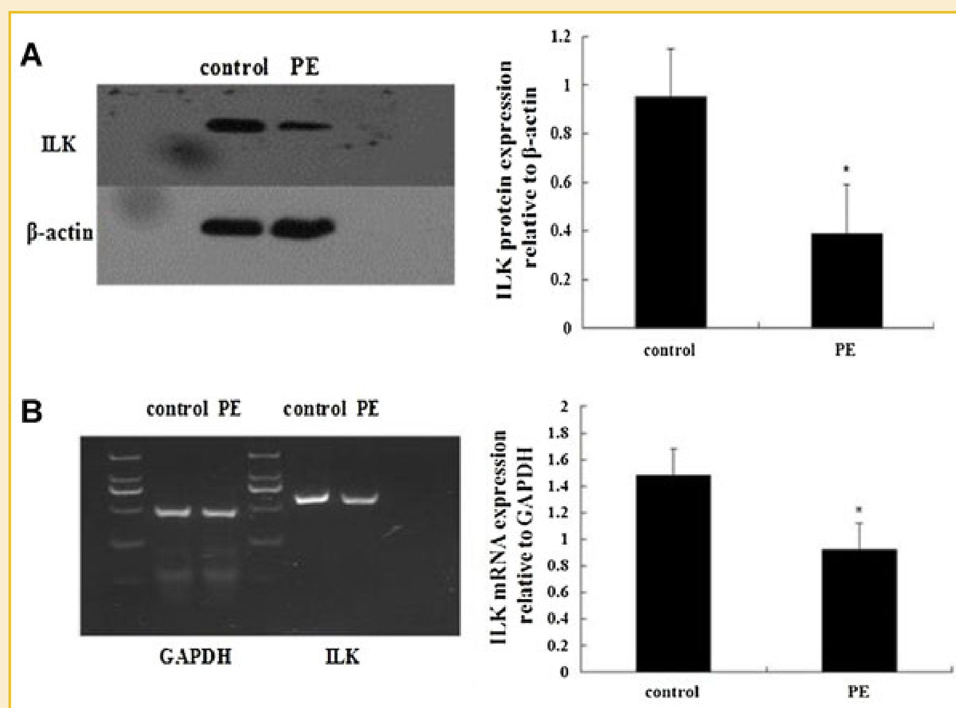

Fig. 2. Basal expression of ILK in EPCs. A: Representative Western immunoblotting showing ILK protein expression in healthy controls (control) and pre-eclampsia patients (PE). β-actin has been used as internal control. Densitometry of ILK protein relative to β-actin protein was determined using semi-quantitative analysis ($^{*}P < 0.01$ vs. control). B: Representative RT-PCR showing ILK mRNA expression in healthy controls (control) and pre-eclampsia patients (PE). GAPDH has been used as internal control. Densitometry of ILK mRNA relative to GAPDH was determined using semi-quantitative analysis ($^{*}P < 0.01$ vs. control).

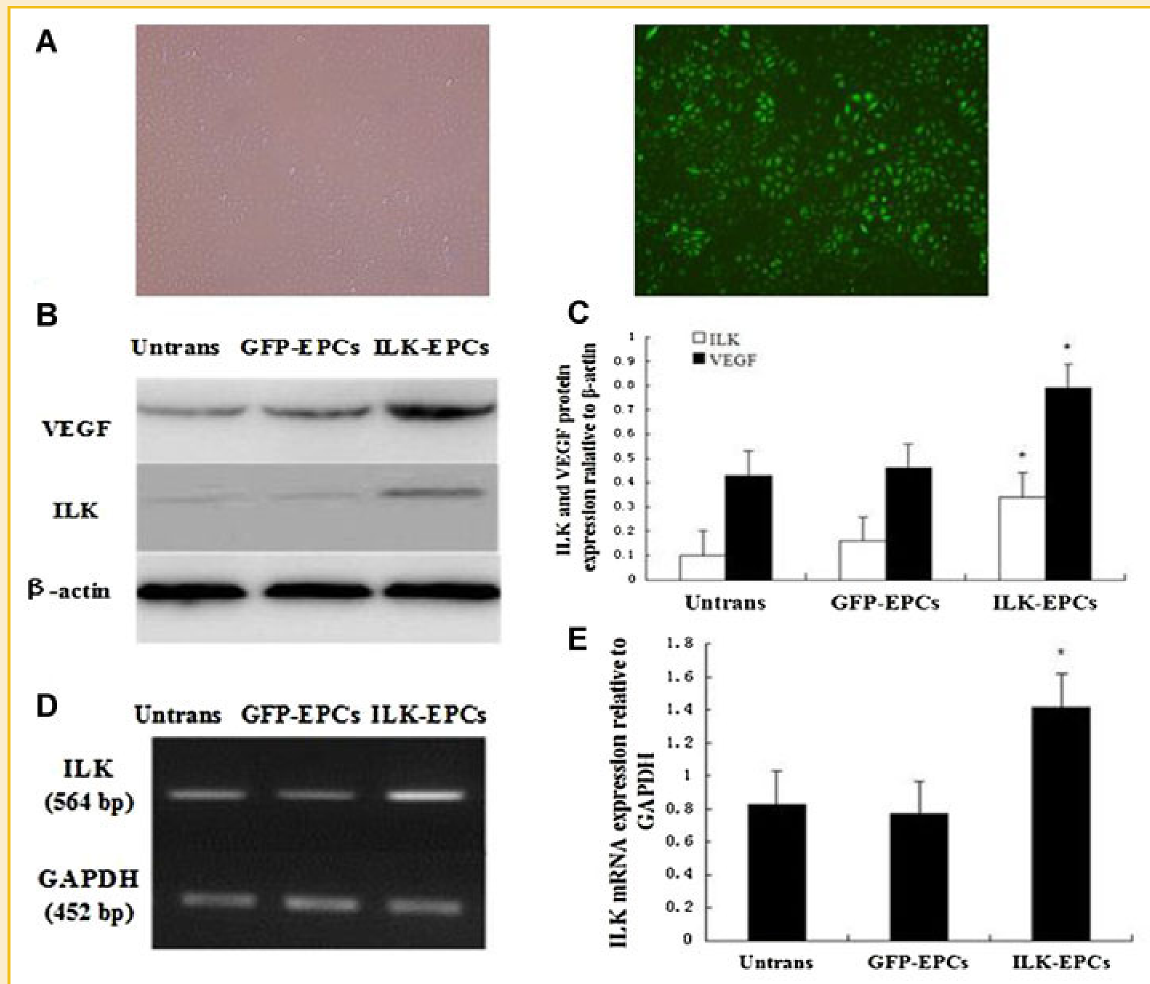

Fig. 3. ILK overexpression in EPCs in vitro. A: Adenoviral transduction of EPCs was performed and confirmed with inverted fluorescence microscope (×100). B: Representative Western immunoblotting showing ILK and VEGF protein expression in untransfected, GFP transfected and ILK transfected EPCs. β-actin has been used as internal control. C: Densitometry of ILK and VEGF protein relative to β-actin protein was determined using semi-quantitative analysis ($^{*}P < 0.01$ vs. untrans or GFP-EPCs, n = 4). D: Representative RT-PCR showing ILK mRNA expression in untransfected, GFP transfected and ILK transfected EPCs. GAPDH has been used as internal control. E: Densitometry of ILK mRNA relative to GAPDH was determined using semi-quantitative analysis ($^{*}P < 0.01$ vs. untrans or GFP-EPCs, n = 4). [Color figure can be seen in the online version of this article, available at http://wileyonlinelibrary.com/journal/jcb]

DISCUSSION

Pre-eclampsia is a pregnancy condition in which high blood pressure and protein in the urine develop after the 20 weeks' gestation. It is characterized by placental defective angiogenesis caused by widespread vascular endothelial malfunction and vasospasm [Roberts and Cooper, 2001]. The global incidence of pre-eclampsia has been estimated at 5–14% of all pregnancies, becoming the second most common obstetrical cause of stillbirths and early neonatal deaths in developing countries [Ngoc et al., 2006]. The therapy of pre-eclampsia has been the focus of medical researchers over the years. Recently, many studies have confirmed that circulating EPCs as a population of pluripotent stem cells that integrate into the endothelial layer of blood vessels, play an essential role in postnatal vasculogenesis. These cells can be mobilized from the bone marrow and enter the circulation in conditions of trauma, burns, aorto-coronary bypass, arthritis, retinal disease, atherosclerosis, and tumor growth, promoting re-endothelialization in response to the onset of a vascular lesion [Gill et al., 2001; Folkman, 2002; Carmeliet et al., 2003]. Several clinical lines of evidence have however, indicated that EPCs in patients with pre-eclampsia display a significant impairment in the number and biological functions [Kwon et al., 2007; Xia et al., 2007; Zhou et al., 2008]. Sugawara et al. [2005] suggested that the depletion and cellular senescence of maternal circulating EPC can cause pre-eclampsia. Accordingly, ex vivo expansion of EPCs or genetically modifying them to improve their biological properties was expected to be the targets of EPC-mediated therapy in pre-eclampsia.

ILK is a novel serine threonine kinase that exhibits cell type dependent on activation and inhibition [Hannigan et al., 1996]. It has recently been demonstrated an important regulator of angiogenesis [Tan et al., 2004]. A study by Friedrich et al. [2004] provides evidence that ILK can regulate endothelial cell survival and vascular development. In another study, ILK silencing inhibits

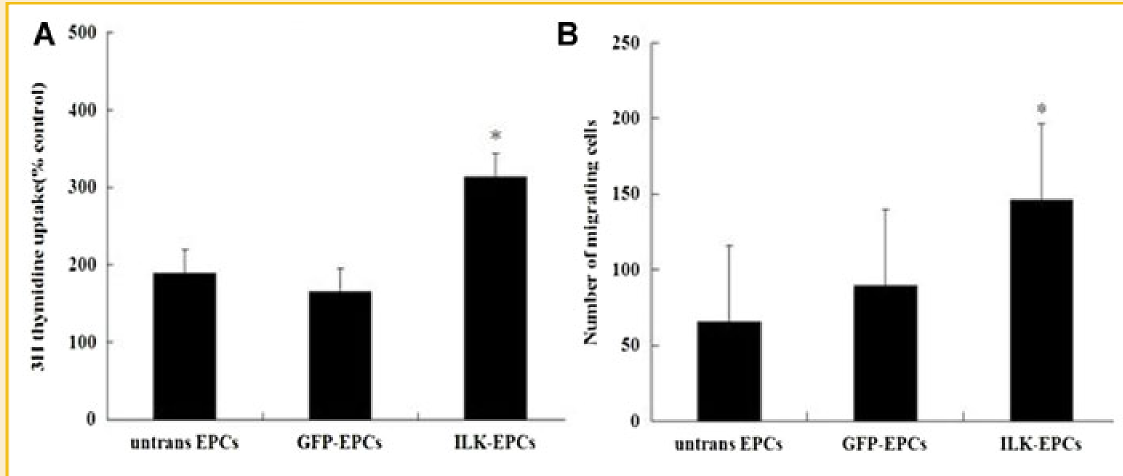

Fig. 4. Transfection with the ILK gene enhances the ability of endothelial progenitor cells to proliferate and migrate. A: Cell proliferation assay. Histogram showing [^{3}H]-thymidine incorporation in untransfected, GFP-transfected, and ILK-transfected EPCs. Results are presented as percentage of the untransfected control as mean ± SD; n = 5. $^*P < 0.01$ vs. untrans and GFP-EPCs. B: Cell migration assay. Histogram showing the average number of untransfected, GFP-EPCs, and ILK-EPCs per high power field migrated through filters in a modified Boyden chamber. Results are presented as mean ± SD; n = 5. $^*P < 0.01$ vs. untrans and GFP-EPCs.

endothelial cell migration and tube-like structure formation [Vouret-Craviari et al., 2004]. Taking into account the contribution of ILK in angiogenesis, in the present experiment, we attempted to investigate effects of this gene transfer on important angiogenic characteristics of early EPCs with pre-eclampsia. We confirm the feasibility of adenovirus-mediated ILK gene transfect in EPCs isolated from patients with pre-eclampsia. And the results of our study suggest that ILK gene modified EPCs from patients with pre-eclampsia exhibit greater superiorities in their fundamental functional attributes of angiogenesis.

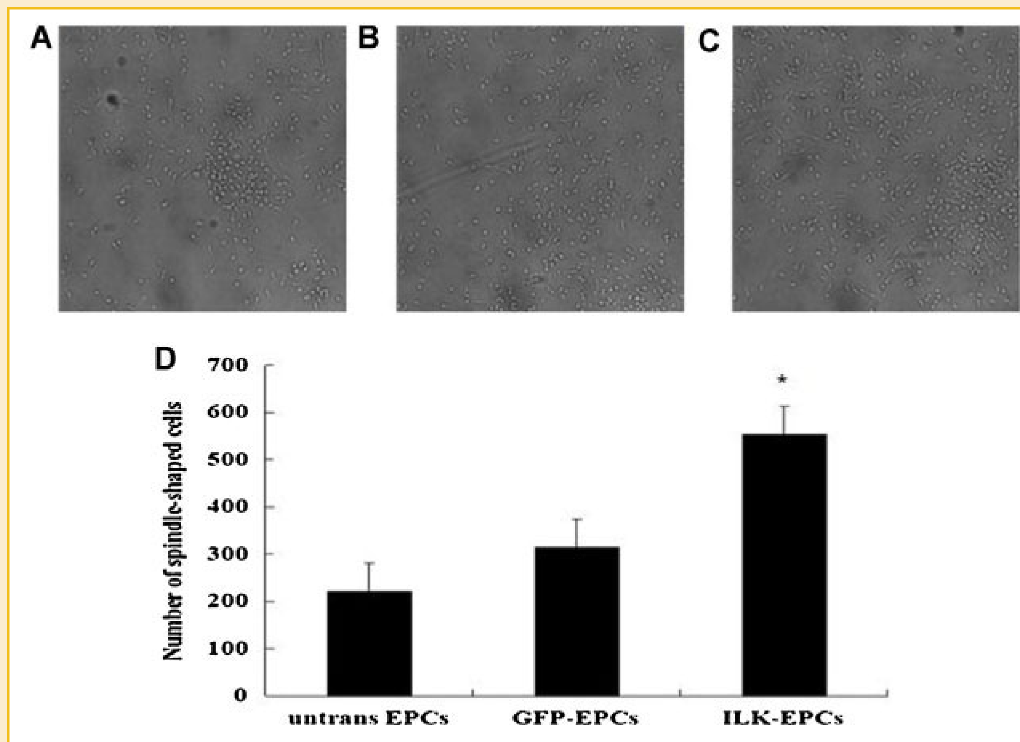

Fig. 5. Transfection with the ILK gene enhances the ability of endothelial progenitor cells to differentiate. Untransfected (×100), (B) GFP-transfected EPCs (×100), (C) ILK-transfected EPCs (×100) observed under a phase-contrast microscope. D: Cell differentiation assay. Histogram showing average number of spindle-shaped attached cells formed from untransfected, GFP-EPCs, and ILK-EPCs after re-plating. Results are presented as mean ± SD from three independent experiments. $^*P < 0.01$ vs. untrans and GFP-EPCs.

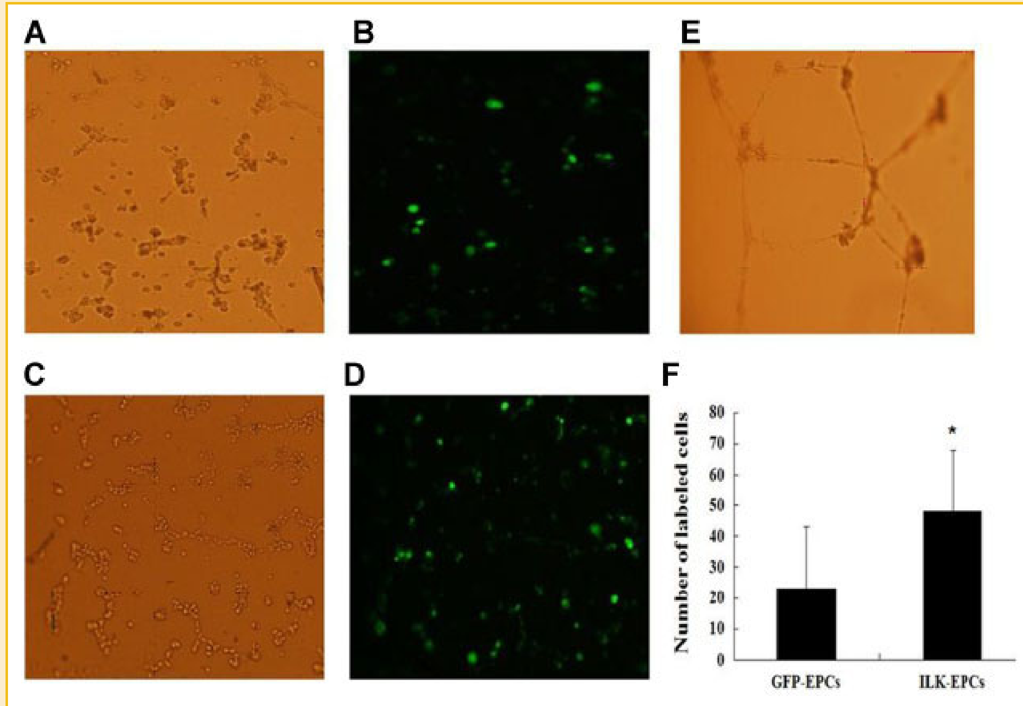

Fig. 6. Matrigel tube-forming assay assessed by phase-contrast and fluorescence microscopy after 12 h. A, B: Untransfected EPCs co-plated with GFP-transfected EPCs (×200). C, D: Untransfected EPCs co-plated with ILK-transfected EPCs (×200). E: Tube formation of HUVECs served as positive control (×200). F: Histograms showing average number of labeled EPCs per field within tube-like structures in co-culture assays of GFP-transfected EPCs and ILK-transfected EPCs with untransfected EPCs. Results are presented as mean ± SD; n = 5. $^{*}P < 0.05$ vs. GFP-EPCs. [Color figure can be seen in the online version of this article, available at http://wileyonlinelibrary.com/journal/jcb]

The ILK-overexpressing EPCs from pre-eclampsia patients showed a significant improvement in proliferation, migration, and differentiation into endothelial-like spindle-shaped cells compared with unmodified EPCs. Furthermore, untransfected EPCs alone scarcely formed tube-like structures, showing poor angiogenic capacity on the matrigel, which was consistent with the previous study of Kaur et al. [2009]. And it was partly attributed to the fact that they were early EPCs used only 7 days of culture. EPCs are a heterogeneous cell population classified into two types according to their time-dependent appearance: Spindle-shaped early EPCs with limited proliferative potential, and cobblestone-like late EPCs with high expansion capacity. But early EPCs contributed to neovasculogenesis in vivo equally as the late EPCs as a result of their stronger secretory function of angiogenic cytokines [Hur et al., 2004]. Previous studies have genetically modified EPCs with VEGF, endothelial NO synthase (eNOS), hepatocyte growth factor (HGF), or other genes to enhance their vasculogenic function [Ikeda et al., 2004; Matsumoto et al., 2004; Li et al., 2006; Kaur et al., 2009; Song et al., 2009]. Unlike these genes, the advantage of using ILK modifying EPCs would be that it mediates a diversity of cell functions. ILK is a downstream substrate of phosphoinositide 3-kinase (PI3K) and an important upstream kinase for the regulation of protein kinase B (PKB/Akt), and glycogen synthase kinase 3(GSK3) [Delcommenne et al., 1998; Yoganathan et al., 2002]. In addition, ILK has been implicated in the regulation of anchorage-dependent cell growth and survival, cell cycle progression, epithelial–mesenchymal transition, invasion and migration, cell motility and contraction, vascular development, and tumor angiogenesis [Guo et al., 2009]. In this study, an Ad-GFP-ILK was found to be an effective promoter of EPCs proliferation, migration and tube formation, all three of which are indispensable events for angiogenesis [Folkman, 1986].

Furthermore, ILK not only mediates cell functions but also growth factors to cascades of downstream signaling events. Platelet-derived growth factor (PDGF), epidermal growth factor (EGF), insulin, VEGF, and other growth factors can only be activated in the case of cell adhesion. VEGF is widely accepted the most critical mediator of angiogenesis. Guo et al. [2009] observed that silencing ILK with siRNA significantly reduced VEGF secretion in the culture medium of RF/6A cells. Some have further reported that inhibition of ILK expression and activation by siRNA regulates VEGF expression in a PKB/Akt- and mTOR/FRAP-dependent manner [Tan et al., 2004]. Our data support an important role for ILK upregulating VEGF expression in EPCs with pre-eclampsia, which was consistent with the new study of Wani et al. [2011]. Hence, a combinatorial approach using EPCs and ILK may be an effective strategy not only to enhance the functional activity of EPCs but also regulation the growth factors attributes of angiogenesis.

In summary, we demonstrate that adenovirus-mediated transfection of EPCs with ILK is an efficient strategy for improving the angiogenic properties of EPCs from pre-eclampsia patients in vitro. And an evaluation of the in vivo efficacy of ILK-EPCs transplant should also be assessed in future studies. ILK overexpression strategy may be of therapeutic benefit for the patients with pre-eclampsia.

REFERENCES

Asahara T, Murohara T, Sullivan A, Silver M, van der Zee R, Li T, Witzenbichler B, Schatteman G, Isner JM. 1997. Isolation of putative progenitor endothelial cells for angiogenesis. Science 275:964–967.

Boulter E, Grall D, Cagnol S, Obberghen-Schilling E. 2006. Regulation of cell-matrix adhesion dynamics and Rac-1 by integrin linked kinase. FASEB J 20:1489–1491.

Carmeliet P. 2003. Angiogenesis in health disease. Nat Med 9:653–660.

Ceradini DJ, Kulkarni AR, Callaghan MJ, Tepper OM, Bastidas N, Kleinman ME, Capla JM, Galiano RD, Levine JP, Gurtner GC. 2004. Progenitor cell trafficking is regulated by hypoxic gradients through HIF-1induction of SDF-1. Nat Med 10:858–864.

Chavakis E, Aicher A, Heeschen C, Sasaki K, Kaiser R, El Makhfi N, Urbich C, Peters T, Scharffetter-Kochanek K, Zeiher AM, Chavakis T, Dimmeler S. 2005. Role of beta2-integrins for homing and neovascularization capacity of endothelial progenitor cells. J Exp Med 201:63–72.

Cho HJ, Youn SW, Cheon SI, Kim TY, Hur J, Zhang SY, Lee SP, Park KW, Lee MM, Choi YS, Park YB, Kim HS. 2005. Regulation of endothelial cell and endothelial progenitor cell survival and vasculogenesis by integrin-linked kinase. Arterioscler Thromb Vasc Biol 25:1154–1160.

Delcommenne M, Tan C, Gray V, Rue L, Woodgett J, Dedhar S. 1998. Phosphoinositide- 3-OH kinase-dependent regulation of glycogen synthase kinase 3 and protein kinase B/AKT by the integrin-linked kinase. Proc Natl Acad Sci USA 95:11211–11216.

Ding L, Dong L, Chen X, Zhang L, Xu X, Ferro A, Xu B. 2009. Increased expression of integrin-linked kinase attenuates left ventricular remodeling and improves cardiac function after myocardial infarction. Circulation 120: 764–773.

Elustondo PA, Hannigan GE, Caniggia I, MacPhee DJ. 2006. Integrin-linked kinase (ILK) is highly expressed in first trimester human chorionic villi and regulates migration of a human cytotrophoblast-derived cell line. Biol Reprod 74:959–968.

Folkman J. 1986. How is blood vessel growth regulated in normal and neoplastic tissue? G.H.A. Clowes memorial award lecture. Cancer Res 46: 467–473.

Folkman J. 2002. Role of angiogenesis in tumor growth and metastasis. Semin Oncol 16:15–18.

Friedrich EB, Liu E, Sinha S, Cook S, Milstone DS, MacRae CA, Mariotti M, Kuhlencordt PJ, Force T, Rosenzweig A, St-Arnaud R, Dedhar S, Gerszten RE. 2004. Integrin-linked kinase regulates endothelial cell survival and vascular development. Mol Cell Biol 24:8134–8144.

Gao D, Nolan DJ, Mellick AS, Bambino K, McDonnell K, Mittal V. 2008. Endothelial progenitor cells control the angiogenic switch in mouse lung metastasis. Science 319:195–198.

Gill M, Dias S, Hattori K, Rivera ML, Hicklin D, Witte L, Girardi L, Yurt R, Himel H, Rafii S. 2001. Vascular trauma induces rapid but transient mobilization of VEGFR2(+) AC133(+) endothelial precursor cells. Circ Res 88:167–174.

Guo L, Yu W, Li X, Zhao G, He P. 2009. Targeting of integrin-linked kinase with a small interfering RNA inhibits endothelial cell migration, proliferation and tube formation in vitro. Ophthalmic Res 42:213–220.

Hannigan GE, Leung-Hagesteijn C, Fitz-Gibbon L, Coppolino MG, Radeva G, Filmus J, Bell JC, Dedhar S. 1996. Regulation of cell adhesion and anchorage-dependent growth by a new beta 1-integrin-linked protein kinase. Nature 379:91–96.

Hur J, Yoon CH, Kim HS, Choi JH, Kang HJ, Hwang KK, Oh BH, Lee MM, Park YB. 2004. Characterization of two types of endothelial progenitor cells and their different contributions to neovasculogenesis. Arterioscler Thromb Vasc Biol 24:288–293.

Hynes RO. 2002. Integrins: Bidirectional, allosteric signaling machines. Cell 110:673–687.

Ikeda Y, Fukuda N, Wada M, Matsumoto T, Satomi A, Yokoyama S, Saito S, Matsumoto K, Kanmatsuse K, Mugishima H. 2004. Development of angiogenic cell and gene therapy by transplantation of umbilical cord blood with vascular endothelial growth factor gene. Hypertens Res 27: 119–128.

Kalka C, Masuda H, Takahashi T, Kalka-Moll WM, Silver M, Kearney M, Li T, Isner JM, Asahara T. 2000. Transplantation of ex vivo expanded endothelial progenitor cells for therapeutic neovascularization. Proc Natl Acad Sci USA 97:3422–3427.

Kaur S, Kumar TR, Uruno A, Sugawara A, Jayakumar K, Kartha CC. 2009. Genetic engineering with endothelial nitric oxide synthase improves functional properties of endothelial progenitor cells from patients with coronary artery disease: An in vitro study. Basic Res Cardiol 104:739–749.

Kwon JY, Maeng YS, Kwon YG, Kim YH, Kang MH, Park YW. 2007. Decreased endothelial progenitor cells in umbilical cord blood in severe pre-eclampsia. Gynecol Obstet Invest 64:103–108.

Li B, Sharpe EE, Maupin AB, Teleron AA, Pyle AL, Carmeliet P, Young PP. 2006. VEGF and PlGF promote adult vasculogenesis by enhancing EPC recruitment and vessel formation at the site of tumor neovascularization. FASEB J 20:1495–1497.

Matsumoto K, Kanmatsuse K, Mugishima H. 2004. Development of angiogenic cell and gene therapy by transplantation of umbilical cord blood with vascular endothelial growth factor gene. Hypertens Res 27:119–128.

Ngoc NT, Merialdi M, Abdel-Aleem H, Carroli G, Purwar M, Zavaleta N, Campódonico L, Ali MM, Hofmeyr GJ, Mathai M, Lincetto O, Villar J. 2006. Causes of stillbirths and early neonatal deaths: Data from 7993 pregnancies in six developing countries. Bull World Health Organ 84:699–705.

Nolan DJ, Ciarrocchi A, Mellick AS, Jaggi JS, Bambino K, Gupta S, Heikamp E, McDevitt MR, Scheinberg DA, Benezra R, Mittal V. 2007. Bone marrow-derived endothelial progenitor cells are a major determinant of nascent tumor neovascularization. Genes Dev 21:1546–1558.

Persad S, Attwell S, Gray V, Mawji N, Deng JT, Leung D, Yan J, Sanghera J, Walsh MP, Dedhar S. 2001. Regulation of protein kinase B/Akt-serine 473 phosphorylation by integrin-linked kinase. J Biol Chem 276:27462–27469.

Reyes M, Dudek A, Jahagirdar B, Koodie L, Marker PH, Verfaillie CM. 2002. Origin of endothelial progenitors in human postnatal bone marrow. J Clin Invest 109:337–346.

Roberts JM, Cooper DW. 2001. Pathogenesis and genetics of pre-eclampsia. Lancet 357:53–56.

Song MB, Yu XJ, Zhu GX, Chen JF, Zhao G, Huang L. 2009. Transfection of HGF gene enhances endothelial progenitor cell (EPC) function and improves EPC transplant efficiency for balloon-induced arterial injury in hypercholesterolemic rats. Vascul Pharmacol 51:205–213.

Sugawara J, Mitsui-Saito M, Hayashi C, Hoshiai T, Senoo M, Chisaka H, Yaegashi N, Okamura K. 2005. Decrease and senescence of endothelial progenitor cells in patients with pre-eclampsia. J Clin Endocrinol Metab 90:5329–5332.

Takahashi T, Kalka C, Masuda H, Chen D, Silver M, Kearney M, Magner M, Isner JM, Asahara T. 1999. Ischemia- and cytokine-induced mobilization of bone marrow- derived endothelial progenitor cells forneovascularization. Nat Med 5:434–438.

Tan C, Cruet-Hennequart S, Troussard A, Fazli L, Costello P, Sutton K, Wheeler J, Gleave M, Sanghera J, Dedhar S. 2004. Regulation of tumor angiogenesis by integrin- linked kinase (ILK). Cancer Cell 5:79–90.

Terpstra L, Prud'homme J, Arabian A, Takeda S, Karsenty G, Dedhar S, St-Arnaud R. 2003. Reduced chondrocyte proliferation and chondrodysplasia in mice lacking the integrin-linked kinase in chondrocytes. J Cell Biol 162:139–148.

Troussard AA, Mawji NM, Ong C, Mui A, St-Arnaud R, Dedhar S. 2003. Conditional knock-out of integrin-linked kinase demonstrates an essential role in protein kinase B/Akt activation. J Biol Chem 278:22374–22378.

Vailhé B, Vittet D, Feige JJ. 2001. In vitro models of vasculogenesis and angiogenesis. Lab Invest 81:439–452.

Vasa M, Fichtlscherer S, Aicher A, Adler K, Urbich C, Martin H, Zeiher AM, Dimmeler S. 2001. Number and migratory activity of circulating endothelial progenitor cells inversely correlate with risk factors for coronary artery disease. Circ Res 89:E1–E7.

Vouret-Craviari V, Boulter E, Grall D, Matthews C, Van Obberghen-Schilling E. 2004. ILK is required for the assembly of matrix-forming adhesions and capillary morphogenesis in endothelial cells. J Cell Sci 1l7:4559–4569.

Wani AA, Jafarnejad SM, Zhou J, Li G. 2011. Integrin-linked kinase regulates melanoma angiogenesis by activating NF-κB/interleukin-6 signaling pathway. Oncogene Jan 31.

Wu C, Dedhar S. 2001. Integrin-linked kinase (ILK) and its interactors: A new paradigm for the coupling of extracellular matrix to actin cytoskeleton and signaling complexes. J Cell Biol 155:505–510.

Xia L, Zhou XP, Zhu JH, Xie XD, Zhang H, Wang XX, Chen JZ, Jian S. 2007. Decrease and dysfunction of endothelial progenitor cells in umbilical cord blood with maternal pre-eclampsia. J Obstet Gynaecol Res 33:465–474.

Yamaji S, Suzuki Y, Sugiyama Y, Koide Y, Yoshida H, Kanamori H, Mohri H, Ohno S, Ishigatsubo Y. 2001. A novel integrin-linked kinase-binding protein, affixin, is involved in the early stage of cell-substrate interaction. J Cell Biol 153:1251–1264.

Yoganathan N, Yee A, Zhang Z, Leung D, Yan J, Fazli L, Kojic DL, Costello PC, Jabali M, Dedhar S, Sanghera J. 2002. Integrin-linked kinase, a promising cancer therapeutic target: Biochemical and biological properties. Pharmacol Ther 93:233–242.

Zhang Y, Guo L, Chen K, Wu C. 2002. A critical role of the PINCH-ILK interaction in the regulation of cell shape change and migration. J Biol Chem 277:318–326.

Zhou Y, Zhu J, Zou L, Wang J. 2008. Changes in number and biological function of endothelial progenitor cells in hypertension disorder complicating pregnancy. Huazhong Univ Sci Technol Med Sci 28:670–673.