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CREG promotes vasculogenesis by activation of VEGF/PI3K/Akt pathway

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1. ABSTRACT

Knowledge about factors regulating vasculogenesis remains limited. The cellular repressor of E1A-stimulated gene (CREG) has been reported to be involved in maintaining cellular differentiation and endothelial homeostasis, thus we hypothesize that CREG may be a novel factor regulating vasculogenesis. By using mouse embryonic stem cells (ESC) derived embryoid body (EB) model, we confirmed expression of CREG was significantly up-regulated during EB differentiation. Overexpression of CREG in ESC led to accelerated cystic EB formation, increased endothelial differentiation and vasculogenesis, whereas knockdown of CREG produced opposite phenotypes. Moreover, we found expression of vascular endothelial growth factor (VEGF) was up-regulated and PI3K/Akt pathway was activated in CREG-overexpressing EB. Administration of VEGF neutralizing antibody or PI3K/Akt pharmacological inhibitor LY294002 blocked the vasculogenesis in CREG over-expressing EB, while supplement of VEGF rescued vasculogenesis deficiency in CREG knocked down EB. Further study by Western blot determined that PI3K/Akt was a downstream effector of VEGF. We identify CREG as a novel factor in regulating endothelial differentiation and vasculogenesis via VEGF/PI3K/Akt pathway.

2. INTRODUCTION

Vasculogenesis refers to the *in situ* differentiation of endothelial cells (EC) from angiogenic progenitors to form blood vessels before onset of circulation (1). It is an essential event for both normal development and pathogenesis of many postnatal conditions including cancer and cardiovascular diseases (2-4). A better understanding of factors and the cellular mechanisms regulating vasculogenesis is of great importance for developmental biology and therapeutic strategy for cancer or ischemic diseases (5, 6). Although several factors have been identified to be critical in vasculogenesis, including VEGF, Flk-1, HIF1-alpha, FGF-2 and BMP-4, knowledge in this field is still limited (7-11).

The cellular repressor of E1A-stimulated genes (CREG) is a small, 220 amino acid, secreted glycoprotein that represses transcription of E1A-responsive adenovirus E2 and cellular HSP70 promoters (12). Growing body of evidence has suggested that CREG is involved in regulation of cell growth and differentiation. CREG inhibits cell growth of human teratocarcinoma NTERA-2 cells and NIH3T3 fibroblasts by binding to the mannose 6-phosphate/insulin growth factor II receptor (M6P/IGF2R) (13-15). CREG inhibits growth of cardiomyocytes to

Table 1. Sequence of 29-mer shRNA

Number	Sequence
1	GCCACTATCTCCACAATAAAGGAGGTGCG
2	CCGCGCTGGTGGCACTACTGGTGTCGCCG
3	AGGTGAACAAGACAGAAGAGGACTATGCA
4	GGACCTAAAGTAGTGACACCTGAAGAATA

alleviate cardiac hypertrophy and fibrosis (16, 17). CREG also mediates glucocorticoid induced proliferation of EC. Our previous studies in vascular smooth muscle cells (VSMC) show that overexpression of CREG inhibits proliferation, induces apoptosis and promotes differentiation phenotype in cultured VSMC (18, 19). Moreover, CREG knockdown abrogates serum starvation induced VSMC maturation and growth arrest (18). In balloon injured rat or rabbit carotid arteries, CREG expression is significantly down regulated in the intima media in parallel with the decreased expression of differentiation markers of VSMC. Forced expression of CREG in the injured arteries inhibits VSMC proliferation, attenuates VSMC dedifferentiation and reduces neointimal formation (18, 20). Other study show that overexpression of CREG antagonizes E1A-mediated transformation of primary cultured rat kidney cells (12). CREG also promotes neuronal differentiation of NTERA-2 cells even in the absence of an inducer such as retinoic acid (21). In addition, induction of CREG mRNA transcription has been reported during differentiation of diverse cell types such as pluripotent mouse embryonic stem cells (ESC), monocytes, and myeloid cells (21-23). These results indicate that CREG might play essential roles in regulating cell growth and differentiation.

Moreover, our recent studies in EC also show that CREG might act as a key factor in regulating endothelial homeostasis. CREG expression is significantly decreased in atherogenesis-prone endothelium in apolipoprotein E knockout mice. Silencing CREG expression in EC markedly enhances staurosporine induced apoptosis, whereas CREG overexpression abrogates this effect (24). CREG also promotes migration (25), proliferation (26) and monolayer integrity of human umbilical vein EC (27).

Given the fact that CREG is involved in regulation of both cell differentiation and EC homeostasis, we hypothesize that CREG promotes endothelial differentiation and vasculogenesis. In this study, we used an ESC derived embryoid body (EB) model to investigate vasculogenesis because endothelial differentiation occurs spontaneously *in vitro* in EB, and the formation of vascular channels in EB closely mimics vasculogenesis *in vivo* (28, 29). We first confirmed that expression of CREG is significantly up-regulated during EB differentiation. Overexpression of CREG in ESCs led to accelerated cystic EB formation, increased endothelial differentiation and vasculogenesis, whereas knockdown of CREG in ESC produces undifferentiated EB, failure of endothelial differentiation and vasculogenesis. Moreover, we found the role of CREG is mediated through activation of VEGF/PI3K/Akt pathway. Our results identify CREG as a novel factor in regulating endothelial differentiation and vasculogenesis via VEGF/PI3K/Akt pathway, which may ultimately lead to valuable insight into developmental biology and targets of cell replacement therapy for vascular diseases.

3. MATERIALS AND METHODS

3.1. Plasmids

For overexpression construct, full length mouse CREG cDNA (NM_011804) in pUC57 was purchased from Sangon Biotech (Shanghai, China). The cDNA was cut from pUC57 with EcoRI/BamHI (Takara, Dalian, Liaoning, China), and subcloned into pIRES2-EGFP (Clontech, Mountain view, CA) to construct pCREG-IRES2-EGFP. Positive clones were verified by restriction enzyme digestion and sequencing. For CREG knockdown vectors, a mouse CREG shRNA kit was ordered from Origene (Catalog number TG510293; Rockville, MD). The Kit contains 4 unique 29-mer shRNA cassettes, 1 non-effective 29-mer scrambled shRNA cassette in pGFP-V-RS and 1 empty pGFP-V-RS vector. The sequence of 29-mer shRNA cassettes are shown in Table 1.

3.2. Cell culture

Mouse ESC cell line R1 (ATCC, Manassas, VA) was routinely cultured on mitomycin C (Sigma-Aldrich, St. Louis, MO) treated STO feeder cells (ATCC) in 0.1. percent gelatin (Sigma) coated plates. The ESC culture medium is high glucose Dulbecco's modified Eagle's medium (DMEM) supplemented with 15 percent ESC qualified fetal bovine serum (FBS), 0.1. mM beta-mercaptoethanol (all from Invitrogen, Carlsbad, CA), 2 mM L-glutamine, 1 mM sodium pyruvate, 0.1. mM non-essential amino acids, 100 U/mL penicillin/streptomycin (all from Sigma) and 1,000 U/mL leukemia inhibitory factor (LIF, Chemicon International, Temecula, CA). ESC were fed daily and passaged every two days.

3.3. Stable transfection of ESC

For generation of CREG overexpression (wtCREG-ESC) and knockdown (shCREG-ESC) ESC lines, ESC R1 was transfected with either pCREG-IRES2-EGFP vector or constructs in the CREG shRNA kit using Lipofectamine 2000 reagent (Invitrogen) according to the manufacturer's instruction. Stable ESC clones were selected based on G418 (500 microg/ml) resistance together with GFP fluorescence, and finally verified by Western blotting. ESC stably transfected with empty pIRES2-EGFP was established as a control (GFP-ESC).

3.4. EB formation and 2-dimensional vasculogenesis model

ESC were trypsinized and gently pipetted into aggregates with about 20 cells. ESC aggregates were then cultured in suspension to form EB in ultra-low-attachment dishes (Corning, NY) with differentiation medium (ESC medium without LIF). For 2-dimensional vasculogenesis model, day 7 EB were cultured on 10 microg/ml fibronectin coated cover-slips for additional 7 days (D7 plus 7) in differentiation medium. For blocking assay, day 4 EB derived from wtCREG-ESC were treated with either VEGF neutralizing antibody (150 ng/ml; R&D) or general PI3K/Akt inhibitor LY294002 (50 microM; Sigma) continually until D7 plus 7 with medium change every day. For rescuing assay, day 4 EB derived from shCREG-ESC were treated with VEGF (50 ng/ml) until D7 plus 7 with medium change every day.

Table 2. List of primers

Name	Sequence
VEGF-F	TACTGCTGTACCTCCACCTCCACCATG
VEGF-R	TCACTTCATGGGACTTCTGCTCT
Flk1-F	GCCCTGCCTGTGGTCTCACTAC
Flk1-R	CAAAGCATTGCCCATTCGAT
CD31-F	AGGCTTGCATAGAGCTCCAG
CD31-R	TTCTTGGTTTCCAGCTATGG
VEcadherin-F	TCAACGCATCTGTGCCAGAGAT
VEcadherin-R	CACGATTTGGTACAAGACAGTG
GAPDH-F	TGTGTCCGTCGTGGATCTGA
GAPDH-R	CCTGCTTCACCACCTTCTTGA

F: forward, R: reverse

3.5. EB histological analysis

EB were fixed in 4 percent paraformaldehyde, embedded in paraffin, and cut into 5 microm sections. Embedded paraffin EB sections were placed onto microscope slides, deparaffinized and rehydrated. Thereafter, a regular hematoxylin and eosin (HE) staining was performed in EB sections for visualization of microscopic structures.

3.6. Immunofluorescence

D7 plus 7 EB cultured on coverslips were fixed in 4 percent paraformaldehyde at room temperature for 10 minutes. Fixed cells were permeabilized with 0.1. percent Triton X-100 and 5 percent goat serum in PBS (blocking buffer) for 1 hour at room temperature. Rat anti-mouse PECAM-1 primary antibody (1:100; BD Biosciences, San Jose, CA) was diluted in blocking buffer and applied overnight at 4 °C. Alexa Fluor 594 conjugated donkey anti-rat secondary antibody (1:100; Jackson Immunoresearch, West Grove, PA) was diluted in blocking buffer and applied for 1 hour at room temperature. Cell nuclei were visualized by DAPI (1:400; Sigma). Images were captured on a Leica DFC420 C microscope controlled by Leica LAS software (Leica Microsystems, Beijing, China).

3.7. Western blotting

D7 plus 7 EB were harvested and lysed using RIPA buffer (Sigma) containing protease inhibitor cocktail and phosphatase inhibitor cocktail (Thermo Scientific, Rockford, IL). The lysate was incubated on ice for 30 minutes, centrifuged at 4 °C, and boiled in reducing conditions. Proteins were then separated by SDS-PAGE and electro-blotted onto a polyvinylidene difluoride membrane (Millipore, Billerica, MA). The membrane was incubated in 5 percent blocking buffer (non-fat dry milk in TBS-T (10 mM Tris, 15 mM NaCl containing 0.1. percent Tween 20)) with the exception of the phospho-Westerns, which were blocked in 5 percent bovine serum albumin in TBS at room temperature for 30 minutes. The membrane was then incubated in primary antibody including VEGF (1:500; Life Technologies, Beijing, China), Flk-1 (1:1,000; Millipore), IGF2R (1:2,000; Novus Biologicals, Littleton, CO), PI3K (p110 alpha), phospho-Akt (pAkt; Ser473), Akt (all 1:1,000; all from Cell Signaling Technology, Beverly, MA) and beta-actin (1:2,000; Abcam, Cambridge, MA) in 1 percent blocking buffer at 4 °C overnight. The membrane was washed three times with TBS-T before being incubated in the appropriate HRP-conjugated secondary antibody (all 1:2,000; Jackson Immunoresearch) in 1 percent blocking buffer for 1 hour at room temperature. The membrane was then developed using ECL (Amersham Biosciences, Piscataway, NJ), scanned and quantified by Quantity One analysis software (Bio-rad, Hercules, CA).

3.8. Quantitative real-time polymerase chain reaction (qRT-PCR)

Total RNA of EB at indicated days was extracted with Trizol (Invitrogen) and cDNA was generated using 1 microg DNase-treated RNA with oligo-dT primers and PrimeScript® RT Enzyme (Takara). SYBR Green qRT-PCRs were performed using SYBR® Premix Ex Taq™ (Takara) on a 7300 Fast Real-Time PCR System from Applied Biosystems. All reactions were performed at least in triplicate and the data were analyzed by 7300 System Software (Applied Biosystems, Foster City, CA). To ensure specificity of PCR, melt curve analyses were performed at the end of all PCRs. Gene expression levels were normalized to GAPDH and then analyzed using the $2\Delta\Delta Ct$ method. Primers used in qRT-PCR are listed Table 2.

3.9. VEGF enzyme-linked immunosorbent assay (ELISA)

Samples of cell culture medium taken from EB were collected at indicated days, stored at -80 °C and analyzed to detect endogenous VEGF production by using a Quantikine ELISA Mouse VEGF Immunoassay Kit (R & D Systems, Shanghai, China) according to the manufacturer's instruction. Absorption was measured at 450 nm and corrected at 570 nm by using a Bio-rad Model 550 microplate reader. Data analysis was performed with Microplate Manager Software (Bio-rad).

3.10. Statistical analysis

Statistical comparisons were made by using Student's t test for unpaired data. For experiments with more than two groups, ANOVA was performed with Tukey's post hoc. Results are expressed as the mean plus or minus SD for n given samples. A value of p less than 0.0.5 was considered to be significantly different.

4. RESULTS

4.1. CREG was up-regulated during ESC differentiation

In order to gain insight of CREG's function during ESC differentiation, we first determined the expression of CREG in EB derived from ESC. EB at day 1, 3, 5, 7 and day 7 EB cultured on fibronectin coated coverslips for additional 7 days (D7 plus 7) were harvested and CREG protein was detected by Western Blot. As shown in Figure 1A, level of CREG expression was low in day 1 EB, but significantly upregulated in day 3 EB and maintained at a high level after day 5. This result implies that CREG may be involved in regulating ESC differentiation.

To further investigate the role of CREG during ESC differentiation, we generated stable ESC lines with CREG overexpressed or knocked down. For knockdown of CREG, ESC was transfected with either plasmids harboring

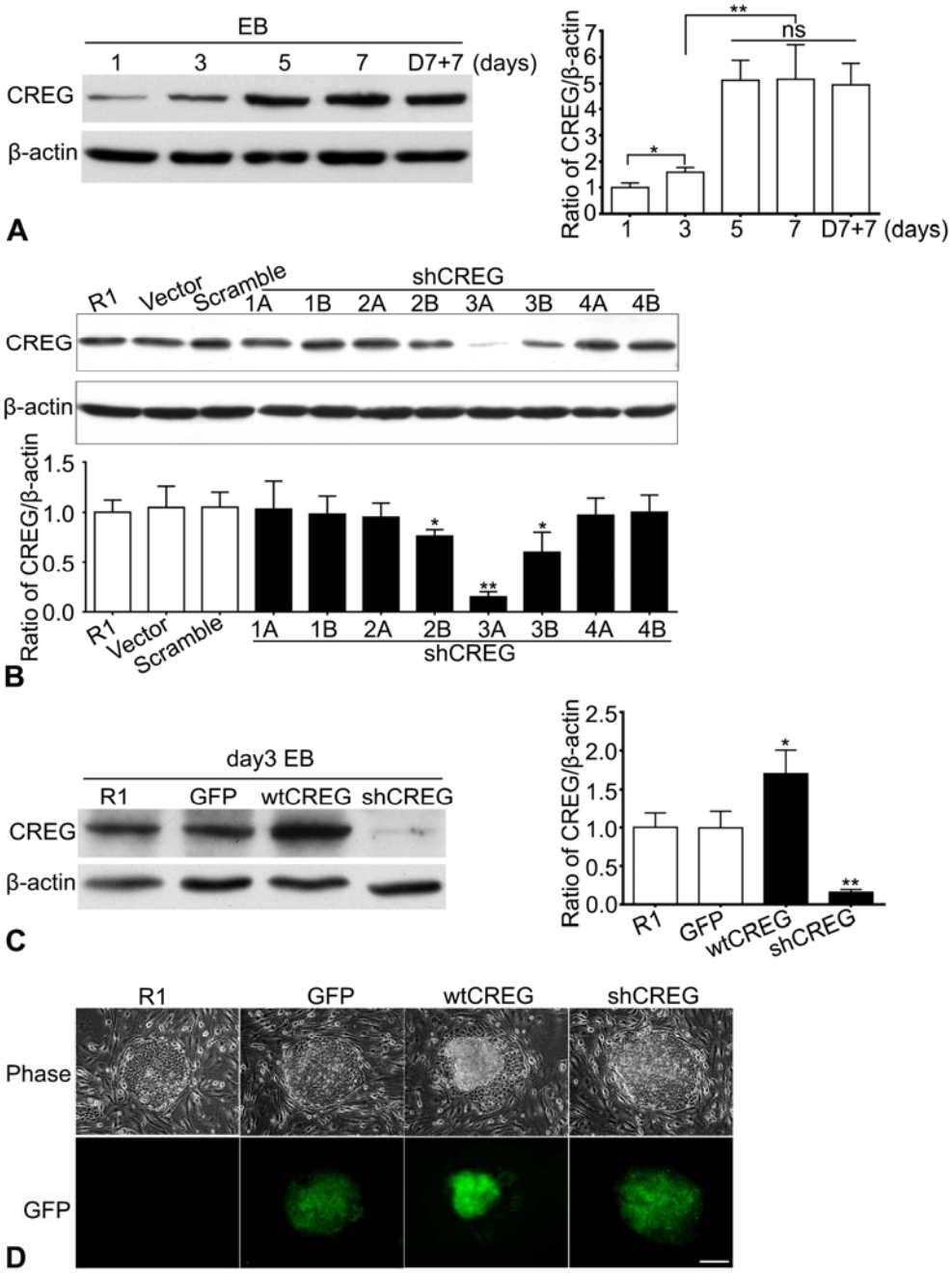

Figure 1. CREG was upregulated upon differentiation of ESC. A): Day 1, 3, 5, 7 and D7 plus 7 EB were harvested and CREG expression was assessed by Western blot and quantified relative to day 1 EB. **p* less than 0.0.5, ***p* less than 0.0.1 compared with day 1 EB. (B): Verification of CREG knockdown efficiency. ESC R1 was transfected with 4 different CREG shRNA constructs. A total of eight clones (two for each shRNA, named 1A, 1B, 2A, 2B, 3A, 3B, 4A and 4B) were selected. Day 3 EB derived from these clones were detected for CREG expression by Western blot and quantified relative to R1 control. CREG in Clone 3A was effectively knocked down to about 15 percent of R1 control, in which CREG expression was normalized to be 1.0. (C): Verification of CREG overexpression and knockdown in day 3 EB. CREG expression was quantified relative to R1 control and beta-actin served as an internal control. Data are represented as means plus or minus SD of three independent experiments. **p* less than 0.0.5, ***p* less than 0.0.1 compared with R1 control. (D): Morphology of different ESCs. R1, GFP-ESC and shCREG-ESC colonies had similar undifferentiated morphology, while some of wtCREG-ESC showed differentiated morphology with an EB like center surrounded by cell outgrowth. Scale bar: 100 μm. Abbreviations: ESC, embryonic stem cell; EB, embryoid body; D7 plus 7 EB, day 7 EB plated on coverslips and cultured for additional 7 days; GFP, green fluorescence protein; wtCREG, CREG overexpression; shCREG, CREG knockdown.

4 different shRNA targeted at various exons of CREG or control constructs (empty vector or vector containing scramble shRNA sequence). A total of eight clones (two for each shRNA, named 1A, 1B, 2A, 2B, 3A, 3B, 4A and 4B) were selected and verified by detection of CREG by Western blotting in day 3 EB derived from these clones. As shown in Figure 1B, CREG was effectively knocked down to about 15 percent in clone 3A compared with control group, in which CREG expression was normalized to be 1.0. We also obtained a stable ESC with CREG overexpressed about 1.7. fold (Figure 1C) by transfection of a pCREG-IRES2-EGFP construct.

During maintenance of ESC, we found GFP-ESC and shCREG-ESC showed typical undifferentiated morphology similar to R1, while some of wtCREG-ESC presented with a different morphology, which was more like an adhered EB with differentiated outgrowth, suggesting that CREG may promote ESC differentiation even in the presence of STO feeders and LIF (Figure 1D).

4.2. CREG promoted development of cystic EB with yolk sac like structure

EB models derived from ESC were employed to investigate the role of CREG during ESC differentiation. Morphology of day 3, day 5 and day 7 EB derived from GFP-ESC, wtCREG-ESC and shCREG-ESC were observed and assessed by histological analysis. In EB derived from GFP-ESC, endoderm (en) and basement membrane (BM) firstly appeared on day 3, then columnar epiblast epithelium (CCE) formed on day 5 and cystic EB with central cavity (CV) appeared on day 7 (Figure 2A-GFP). In wtCREG-ESC group, EB differentiation was markedly accelerated by nearly 2 days. EB developed well organized CCE as early as on day 3, cystic EB with large cavity on day 5 and fully cavitied cystic EB similar to yolk sac of embryo *in vivo* on day 7 (Figure 2A-wtCREG). In contrast, EB derived from shCREG-ESC showed few signs of differentiation (Figure 2A-shCREG).

We also quantified percentage of fully cavitied EB during differentiation. As shown in Figure 2B, on day 5, GFP and shCREG groups had no formation of fully cavitied EB, whereas wtCREG group showed appearance of fully cavitied EB with a percentage of about 7 percent. On day 7, 10 and 14, wtCREG group exhibited nearly 2 fold higher rate of fully cavitied EB than that of GFP control, while shCREG presented with significantly lower rate (less than 10 percent) at all the time points. It has been well established that the fully cavitied EB is similar to yolk sac *in vivo* and contains blood islands with EC and blood cells (30), so it is reasonable to think that CREG might promote vasculogenesis.

4.3. CREG promoted vasculogenesis in ESC

To determine whether CREG regulates vasculogenesis, transcription level of endothelial markers in day 1, day 3, day 5, and day 7 EB derived from GFP-ESC, wtCREG-ESC and shCREG-ESC were analyzed by qRT-PCR. As shown in Figure 3A, expression level of endothelial markers including PECAM-1, Flk-1 and VE-cadherin was upregulated with increasing time of differentiation in all three groups. In shCREG group, all the markers were expressed at significantly lower level compared to GFP control after day 5, indicating poor differentiation of shCREG-ESC to EC. In wtCREG group, all the markers were expressed at significantly higher level compared to GFP control on day 5 and day 7. In addition, expression of Flk-1 and VE-cadherin in this group increased on as early as day 3 compared to GFP control. Western blot analysis of Flk-1 in D7 plus 7 EB also showed that CREG overexpression upregulated Flk-1 expression, while CREG knockdown had a reverse effect (Figure 3B). We further performed immunostaining of PECAM-1 on D7 plus 7 EB. As shown in Figure 3C, GFP control developed well organized endothelial network in this *in vitro* model. CREG overexpression markedly promoted the formation of endothelial network. In shCREG group, only small clusters of EC or rod like structures were seen without endothelial network formation. These results revealed that CREG plays an essential role in regulating endothelial differentiation and endothelial network formation. Knockdown of CREG may lead to deficiency of the above developmental events.

4.4. CREG promoted vasculogenesis by activation of VEGF/PI3K/Akt pathway

Given the fact that CREG knockdown ESC showed a similar phenotype to VEGF deficiency (Figure 3C), we first detected VEGF expression at different level. The qRT-PCR results reveled overexpression of CREG significantly upregulated VEGF expression on as early as day 3 in EB and continued to day 7 (Figure 4A). This is parallel to the expression tendency of endothelial markers shown in Figure 3A. Western blotting of VEGF in D7 plus 7 EB showed that CREG overexpression upregulated VEGF expression while CREG knockdown blocked this effect (Figure 4B). ELISA assay of day 5, day 7 and D7 plus 7 EB showed almost 2 fold increases in VEGF secretion in wtCREG group at all the time points compared with GFP control (Figure 4C).

IGF2R has been reported to bind to CREG and mediate its endocytosis, and PI3K/Akt to be downstream effector of both CREG and VEGF in other systems (13, 24, 31, 32). So we further detected these candidate molecules by Western blotting analysis of D7 plus 7 EB. As shown in Figure 4D, PI3K/Akt is activated in wtCREG group and inactivated in shCREG group compared to GFP control, but no difference was found among 3 groups in expression of IGF2R.

To determine the relationship between VEGF and PI3K/Akt, VEGF neutralizing antibody and PI3K inhibitor LY294002 were used for blocking analysis in D7 plus 7 EB of wtCREG group, and VEGF was used for rescuing assay in D7 plus 7 EB of shCREG group (Figure 4E). Immunofluorescence of PECAM-1 showed that VEGF neutralizing antibody and LY294002 effectively blocked vasculogenesis promoted by CREG overexpression, whereas VEGF supplementation rescued vasculogenesis deficiency in EB with CREG knockdown. In addition, Western blotting showed that VEGF neutralizing antibody blunted activation of PI3K/Akt in EB with CREG

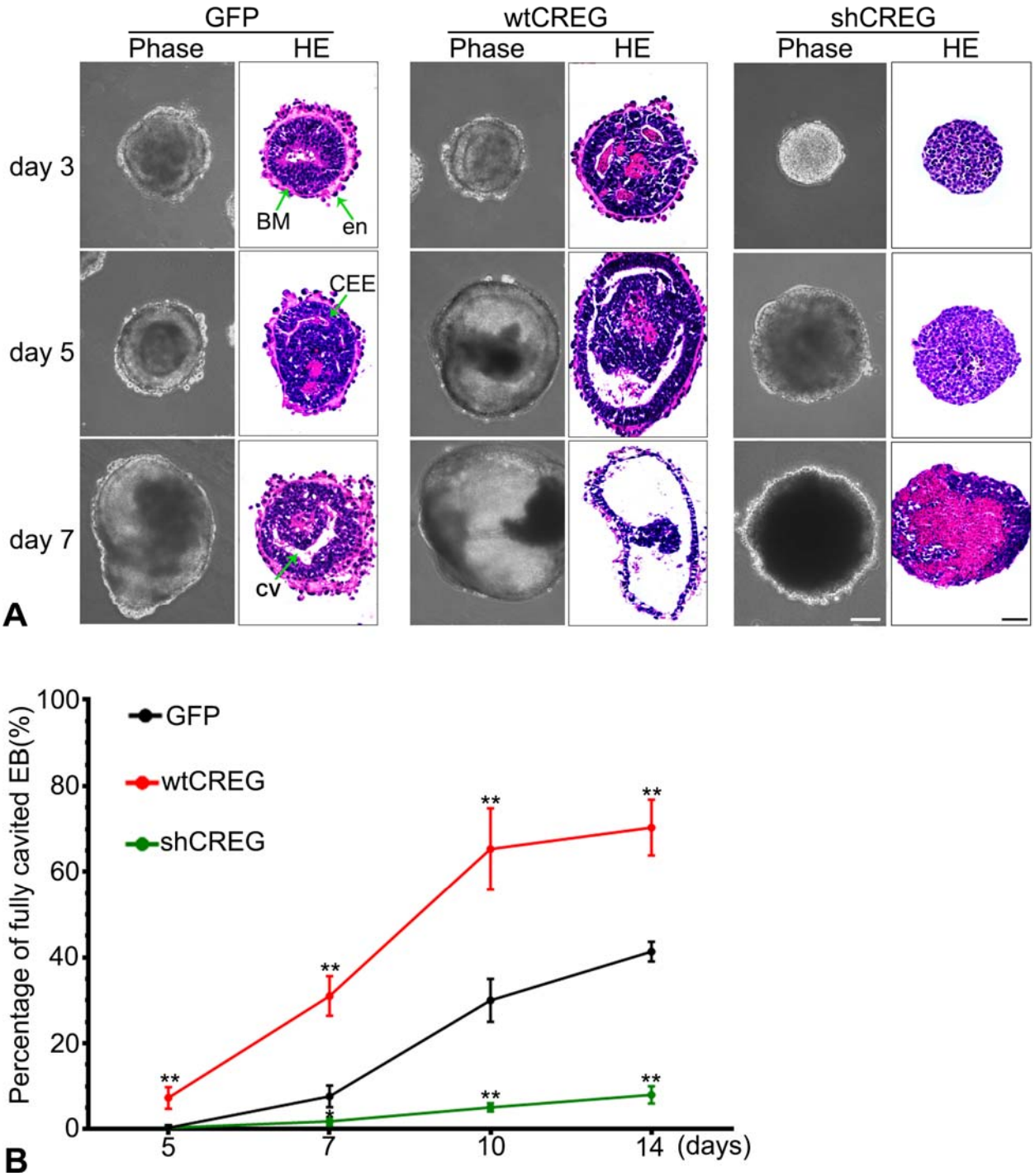

Figure 2. CREG promoted EB differentiation. (A): Representative phase contrast microscopic and corresponding histological morphology of EB derived from GFP-ESC, wtCREG-ESC and shCREG-ESC on day 3, day 5 and day 7. En, endoderm; BM: basement membrane; CEE, columnar epiblast epithelium; CV, center cavity. Scale bar: 100 μm (B): Quantification of percent of fully cavitied EB derived from GFP-ESC, wtCREG-ESC and shCREG-ESC on day 5, day 7, day 10 and day 14. Data are represented as means plus or minus SD of three independent experiments with a total of 300 EB counted for each group. *p less than 0.0.5, **p less than 0.0.1 compared with GFP control group. Abbreviations: ESC, embryonic stem cell; EB, embryoid body; GFP, green fluorescence protein; wtCREG, CREG overexpression; shCREG, CREG knockdown; HE, hematoxylin and eosin staining.

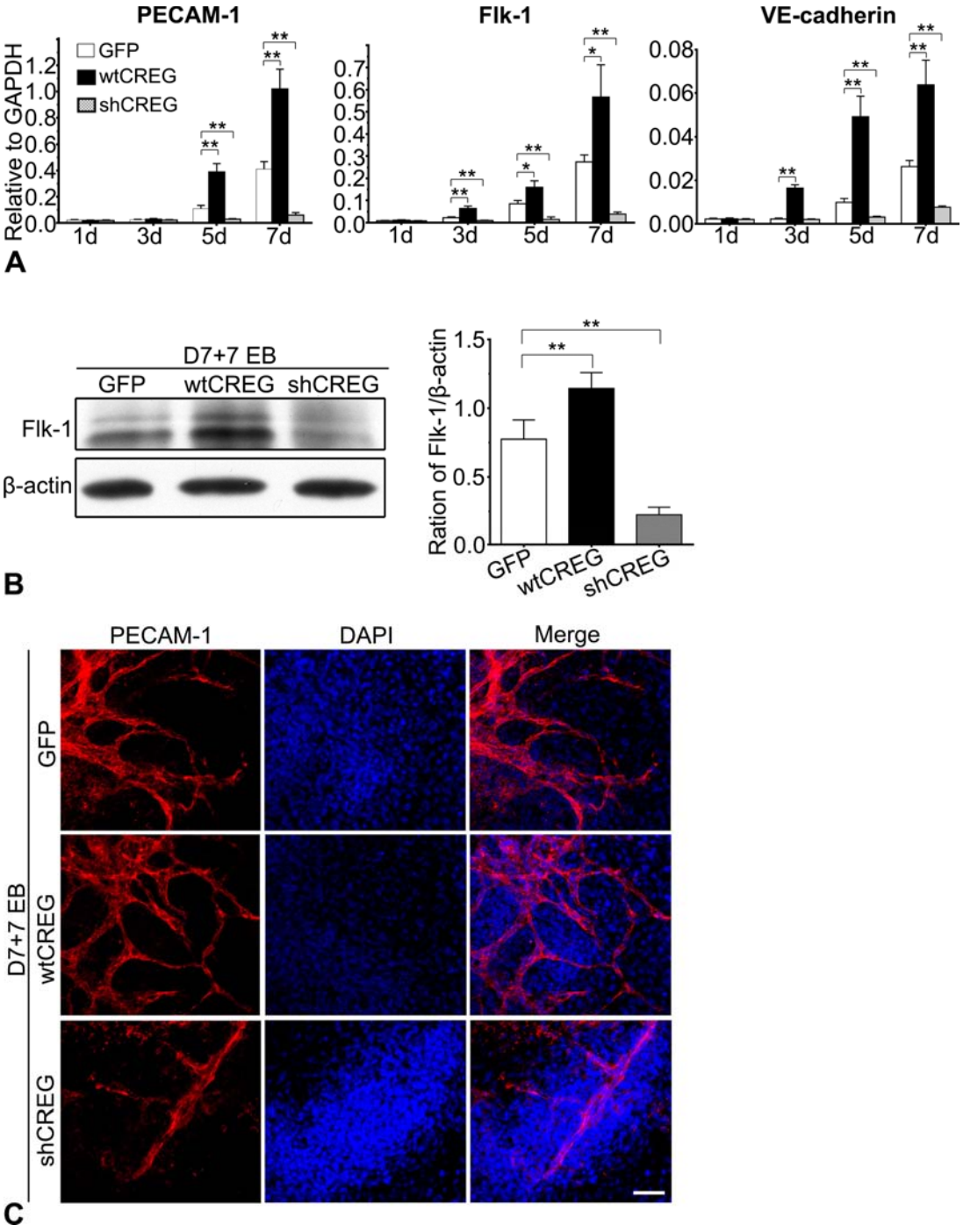

Figure 3. CREG promoted vasculogenesis in ESC. (A): CREG promoted transcription of endothelial differentiation markers. Transcription level of endothelial markers including PECAM-1, Flk-1 and VE-cadherin in day 1, day 3, day 5, and day 7 EB derived from GFP-ESC, wtCREG-ESC and shCREG-ESC were analyzed by qRT-PCR. Changes in gene expression levels are normalized to GAPDH. (n equals 4; **p* less than 0.0.5, ***p* less than 0.0.1 compared with GFP control group). (B): CREG promoted Flk-1 expression. Expression of Flk-1 in day 7 plus 7 EB was detected by Western blot. Data are represented as mean plus or minus SD of three independent experiments. **p* less than 0.0.5, ***p* less than 0.0.1 compared with GFP control group, and beta-actin served as an internal control. (C): CREG promoted formation of endothelial networks. D7 plus 7 EB were immunostained by PECAM-1 antibody and representative immunofluorescence images were shown. Scale bar: 50 microm. Abbreviations: GFP, green fluorescence protein; wtCREG, CREG overexpression; shCREG, CREG knockdown; D7 plus 7 EB, day 7 EB plated on coverslips and cultured for additional 7 days.

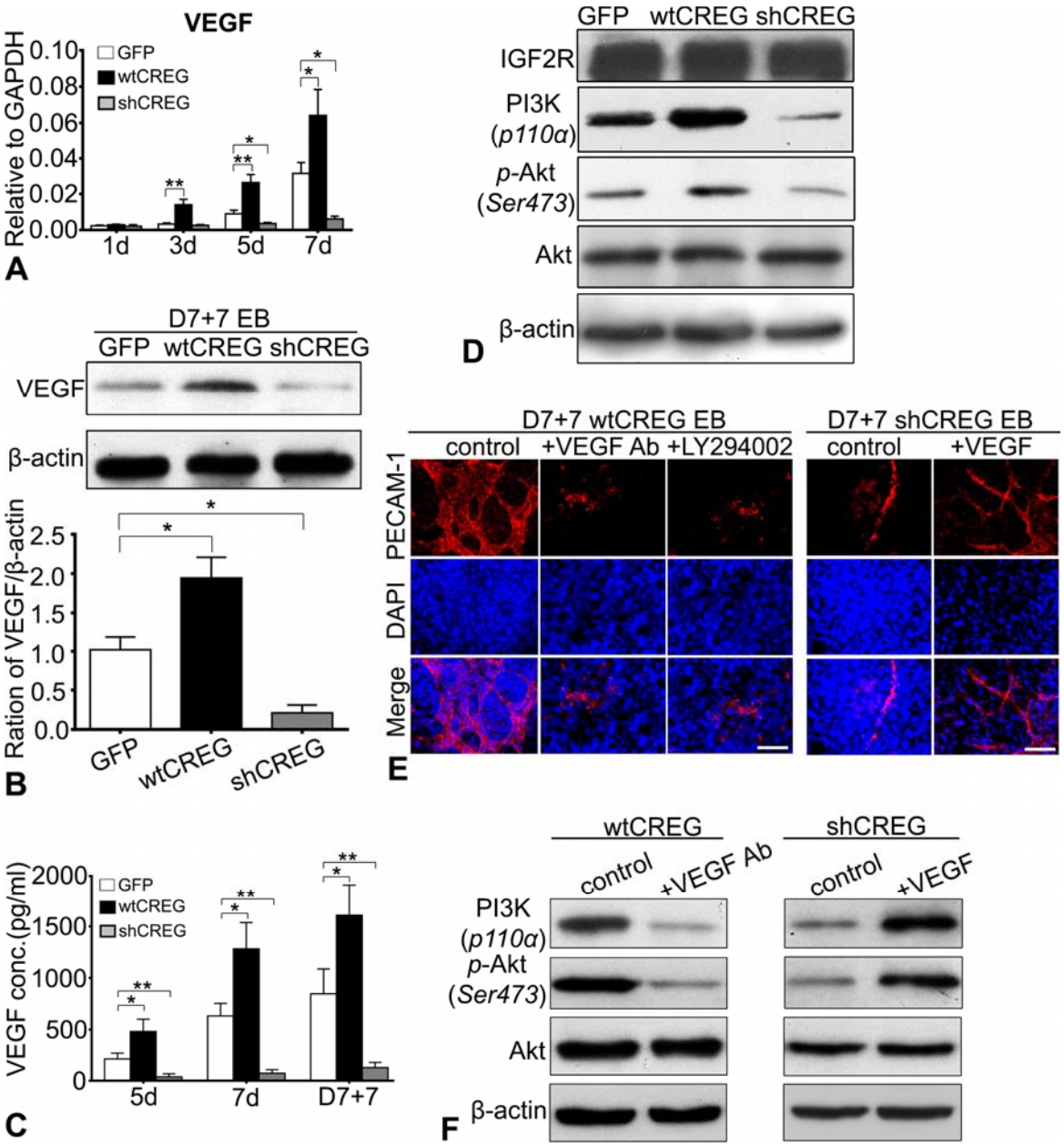

Figure 4. CREG promoted vasculogenesis via activation of VEGF/PI3K/Akt pathway. (A): CREG promoted transcription of VEGF identified by qRT-PCR analysis. Changes in gene expression levels are normalized to GAPDH (n equals 4; *p less than 0.0.5, **p less than 0.0.1 compared with GFP control group). (B): CREG promoted expression of VEGF in D7 plus 7 EB identified by Western Blot. Data are represented as mean plus or minus SD of three independent experiments. *p less than 0.0.5, **p less than 0.0.1 compared with GFP control group, and beta-actin served as an internal control. (C): CREG promoted VEGF secretion in day 5, 7, and D7 plus 7 EB identified by ELISA. Data represent mean plus or minus SD of three independent experiments, and each performed as triplicate. (D): CREG activates PI3K/Akt in D7 plus 7 EB. IGF2R showed no change in 3 groups. Beta-actin served as an internal control. (E): Blocking and rescuing assay were performed and endothelial network formation was assessed by PECAM-1 immunostaining. Administration of 150 ng/ml VEGF neutralizing antibody and 50 microM LY294002 in wtCREG group resulted in blocking of endothelial network formation promoted by CREG overexpression. Supplement of 50 ng/ml VEGF in shCREG group could rescue its deficiency in vasculogenesis. Scale bar: 50 microm. (F): In wtCREG group, administration of 150 ng/ml VEGF neutralizing antibody significantly blunted PI3K/Akt phosphorylation activated by CREG overexpression. In shCREG group, supplement of VEGF reactivated PI3K/Akt pathway. Abbreviations: GFP, green fluorescence protein; wtCREG, CREG overexpression; shCREG, CREG knockdown; D7+7 EB, day 7 EB plated on coverslips and cultured for additional 7 days.

overexpression (Figure 4F), whereas VEGF supplementation reactivated PI3K/Akt pathway attenuated by CREG knockdown. These results revealed that PI3K/Akt is downstream to VEGF, and function of CREG was mediated by activation of VEGF/PI3K/Akt.

5. DISCUSSION

Previous studies have shown that CREG is involved in differentiation of various cell types including embryonal carcinoma cells, immortal fibroblasts, and gastric cancer cells, etc. (33-35). Our recent researches also reveal that CREG plays important roles in controlling survival and migration of EC (24, 25), suggesting the possibility that CREG participates in regulating endothelial differentiation and vasculogenesis. Hence the present study was undertaken to evaluate the role of CREG and its downstream effectors during vasculogenesis in differentiating ESC.

We first explored the dynamics of CREG protein expression during ESC differentiation. If CREG is indeed pivotally involved in this process, a coordinated change in its expression would be expected over the course of ESC differentiation. Western blot showed that expression level of CREG was low in day 1 EB, but was significantly upregulated along the course of differentiation. This result is consistent with the previous study conducted by Veal *et al* (21), who found that CREG mRNA was extremely low in undifferentiated ESC and significantly induced after 3 days of differentiation. The expression pattern of both CREG mRNA and protein implies that CREG may act to promote ESC differentiation.

To elucidate this issue, we successfully established stable ESC lines with CREG knockdown (ShCREG-ESC) or overexpression (wtCREG-ESC). During culture of ESC, we found R1, GFP control and shCREG ESC maintained undifferentiated morphology, but some of the wtCREG ESC displayed differentiated tendency with an EB like center and epithelial like outgrowth, indicating forced expression of CREG in ESC may exert a potent pro-differentiation effect even in the presence of feeder cells and LIF.

By making EB in suspension, we found CREG significantly promoted EB differentiation. CREG overexpression markedly accelerated cystic EB formation both in time and percentage. Besides, CREG overexpression resulted in more fully cavitied EB with yolk sac like structure similar to embryo *in vivo*, which has been reported to contain EC and blood island (30, 36), thus raising the possibility of CREG promoting vasculogenesis. Moreover, significantly impaired EB differentiation and less fully cavitied EB formation were found in CREG knock down group, which was not surprising given the function of CREG in differentiation described previously in other systems.

Further study on transcripts of endothelial markers disclosed that CREG promoted transcription of PECAM-1, Flk-1 and VE-cadherin. Flk-1 is an early marker for EC, although it is also transiently expressed by multiple potent mesodermal lineages during early development (37). As a VEGF receptor, Flk-1 is activated by VEGF to regulate endothelial differentiation and endothelial function. In our study, Flk-1 was significantly upregulated as early as on day 3 in EB by CREG overexpression, which may lead to early activation of this receptor and thus encourage endothelial differentiation. VE-cadherin is a marker for mature EC (38). It is also upregulated by CREG overexpression in day 3 EB, suggesting that CREG may promote endothelial maturation. Western blotting of Flk-1 expression was consistent with those of qRT-PCR.

Immunostaining of PECAM in D7 plus 7 EB revealed that CREG overexpression increased endothelial network formation. CREG knocked down EB failed to form endothelial network and produced only small cluster or rod like structure of EC. The phenotype of vasculogenesis deficiency in shCREG group is similar to that of VEGF knock out *in vivo* (7) , pointing to the possible involvement of VEGF modulation in this CREG-related effect. In support of this notion, we have previously reported that CREG could promote VEGF expression in EC and bone marrow mesenchymal stem cells (24, 25, 39). VEGF signaling has shown to be the decisive factor for mesoderm cells to commit to the endothelial lineage differentiation (40). It is also a major stimulus for differentiation of endothelial progenitor cells into mature endothelium (41, 42). So we next tested the expression of VEGF in our EB system. Evidence from qRT-PCR, Western blot and ELISA clearly showed that CREG could promote expression and secretion of VEGF. Thus CREG may exert its role by VEGF signaling pathway.

To further elucidate the molecular mechanisms mediating the CREG effects on vasculogenesis, we detected candidate signaling molecules. Previous study reported that CREG exerts its effect by binding to M6P/IGF2R and promoting its endocytosis (13-15, 31), but we failed to detect any difference in IGF2R, indicating that role of CREG is not mediated by it in our system. However, we identified that PI3K slash Akt was activated in CREG overexpression group in our system. This is not surprising given the fact that PI3K signaling pathways are known to be regulated by VEGF in many studies and are required for differentiation (32, 40). By using VEGF antibody and PI3K slash Akt inhibitor LY294002, we found both could effectively block vasculogenesis promoted by CREG overexpression, whereas VEGF supplementation rescued vasculogenesis deficiency in EB with CREG knockdown. These results confirmed that the function of CREG is mediated by VEGF and PI3K slash Akt. In an attempt to further decipher the sequential order of these two molecular events, we found that VEGF neutralizing antibody blunted activation of PI3K slash Akt in EB with CREG overexpression, while VEGF supplementation reactivated PI3K slash Akt pathway in EB with CREG knockdown. Thus we determined that PI3K slash Akt is the downstream effector to VEGF.

Although VEGF slash PI3K slash Akt is a classic pathway for vasculogenesis, other molecular mechanisms

may be involved in CREG mediated vasculogenesis. Recently we performed a preliminary mRNA microarray assay to examine gene expression profiling of day7 EB derived from CREG modified ESC. Compared to day 7 EB derived from GFP modified ESC (GFP control), a total of 729 genes including VEGF, Flk-1 and VE-cadherin showed fold change larger than 2. This is not surprising and consistent with results in our study. In addition, other genes proved to play essential roles in vasculogenesis including β hyphen catenin, bone morphogenetic protein 4 (BMP hyphen 4), BMP receptor type 1a (BMPR hyphen1a), Smad5, Tbx3, fibroblast growth factor 2(FGF hyphen 2), FGF receptor 1 (FGFR hyphen 1), myocyte enhancer factor 2 (Mef hyphen 2) and angiopoietin 1 also showed significant increase in EB with CREG overexpression (data not shown). The relationship between CREG overexpression and increase of the above mentioned genes is unclear, which needs further investigation and may be elucidated in our future studies.

Taken together, we established a convincing role for CREG in promoting vasculogenesis in ESC and confirmed that one of the underlying mechanisms is VEGF mediated activation of PI3K slash Akt pathway. More detailed mechanism of CREG regulating VEGF expression and effect of CREG on differentiation of cells from mesoderm or the other two germ layers still needs further studies. To the best of our knowledge, this is the first time that CREG is identified as a novel factor in regulating vasculogenesis, which may ultimately lead to valuable insight into developmental biology of cardiovascular system and provide candidate targets for vascular regeneration therapies.

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