Response gene to complement 32 deficiency causes impaired placental angiogenesis in mice

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Aims The objectives of this study are to determine the role of response gene to complement 32 (RGC-32) in the placental angiogenesis during pregnancy and explore the underlying mechanisms.

Methods and results RGC-32-deficient (RGC32−/−) mice were generated from C57BL/6 embryonic stem cells with deletion of exon 2 and 3 of the RGC-32 gene. Most of the RGC32−/− mice can survive. However, their body sizes were much smaller compared with their wild-type littermates when they were born. By examining the embryo development and placentas at 16.5 days post-coitum, we found that RGC32−/− embryos and foetal placentas were significantly smaller than the wild-type. Further analysis showed that the labyrinth zone of RGC32−/− placenta was smaller with defective angiogenesis. Mechanistically, vascular endothelial growth factor (VEGF) receptor 2 (VEGFR2) and placental growth factor (PlGF) were significantly down-regulated in RGC32−/− placentas, suggesting that VEGFR2 and PlGF may mediate RGC-32 function in placental angiogenesis. Indeed, knockdown of RGC-32 by shRNA inhibited VEGF-induced endothelial cell proliferation, migration, and tube formation while blocking VEGFR2 expression. RGC-32 appeared to regulate VEGFR2 expression via activation of NF-kB. Moreover, RGC-32 regulated trophoblasts proliferation via control of PlGF expression.

Conclusion Absence of RGC-32 caused foetal growth restriction through interrupting the placental angiogenesis, which was due to the decrease in VEGFR2 expression through the NF-kB-dependent pathway in endothelial cells and PlGF expression in trophoblasts.

Keywords Response gene to complement 32 • Angiogenesis • Foetal growth restriction • Vascular endothelial growth factor receptor • Placental growth factor

1. Introduction

Foetal growth restriction is defined as failure of the foetus to achieve its genetically determined growth potential.1 The prevalence of foetal growth restriction is estimated to occur in 10% of the pregnancies in general population.2 Foetal growth restriction increases perinatal mortality and morbidity, and predisposes survivors to greater risk of the metabolic syndrome such as diabetes, stroke, and heart disease.3–5 Placental dysfunction is known to be one of the major factors causing foetal growth restriction. The placenta forms the interface between the maternal and foetal circulation, facilitating metabolic and gas exchange as well as foetal waste disposal. Thus, placenta is essential for sustaining the normal growth of the foetus during gestation. Defects in placenta function results in foetal growth restriction or, if more severe, foetal death.6 One of the most important determinants for the normal placental development is the formation of an adequate vascular system. Although the exact mechanisms controlling the abnormal vascularization during foetal growth restriction are not well-established, deficiencies in angiogenic growth factors including vascular endothelial growth factor (VEGF) family of proteins, fibroblast growth factor (FGF), and angiopoietins (ANGPT)-1 and -2 are known to be involved in the development of this disorder.7–9 The protein factors causing the angiogenic factor deficiency, however, remain largely unknown.

Response gene to complement 32 (RGC-32) was first cloned by Badea et al.10 and was expressed in numerous organs or tissues including placenta, kidney, liver, heart, and brain. Functionally, RGC-32 plays an important role in cell proliferation and differentiation.11–13 Recently, An et al.14 reported that RGC-32 had an antiangiogenic activity under hypoxia condition, which was dependent on hypoxia-inducible factor-1α (HIF-1α) and VEGF through down-regulating endothelial cyclin E via the FGF2 pathway. However, several other studies revealed that RGC-32 promoted the proliferation of aortic endothelial cells (ECs)
and smooth muscle cells (SMCs), suggesting that RGC-32 has different functions in different physiological and pathological conditions.

In the present study, we generated RGC-32-deficient (RGC32\(^{-/-}\)) mice and found for the first time that RGC-32 is critical for angiogenesis in foetal placentas. RGC32\(^{-/-}\) mice were born smaller than their wild-type littermates. Importantly, the growth of the embryos and foetal placentas of RGC32\(^{-/-}\) mice was restricted, and the angiogenesis of the RGC32\(^{-/-}\) placentas and yolk sacs was impaired. In vitro mechanistic studies revealed that knockdown of RGC-32 inhibited VEGF receptor 2 (VEGFR2) expression, resulting in reduced EC proliferation, migration, and tube formation. Pyrrolidine dithiocarbamate (PDTC), an inhibitor of nuclear factor kappa B (NF-kB), blocked RGC-32-induced VEGFR2 expression. RGC-32 knockdown also inhibited placental growth factor (PIGF) expression and proliferation of trophoblasts. Taken together, our results demonstrate that RGC-32 regulates foetal placental angiogenesis through induction of VEGFR2 in EC and PIGF in trophoblasts.

2. Methods

2.1 Animal care

All the animals were housed under conventional conditions in the animal care facilities and received humane care in compliance with the Principles of Laboratory Animal Care formulated by the National Society for Medical Research and the Guide for the Care and Use of Laboratory Animals. All surgical procedures were approved by Institutional Animal Care and Use Committee (IACUC) of The University of Georgia.

2.2 Generation of RGC32-deficient mice and genotyping

RGC32\(^{-/-}\) embryonic stem (ES) cells in C57BL/6 background were purchased from the Knockout Mouse Project (KOMP) Repository (UC Davis, CA, USA). RGC32\(^{-/-}\) ES cell-mouse chimeras were produced by University of Michigan Transgenic Facility. Germ line transmission was obtained by breeding the male chimeras with C57BL/6 female mice. The mice were genotyped by PCR using primers supplied in Supplementary material online.

2.3 Mating and tissue collection

The RGC32\(^{-/-}\) male mice were mated with the RGC32\(^{+/+}\) female mice. Gestational age was determined based on the presence of a vaginal plug, with the morning of detection designated as Day 0.5. Plugged females were removed from the males and housed in separate cages. The pregnant females were euthanized at 11.5 or 16.5 days post-coitum (DPC) by CO2 inhalation (≏80 L/min with mouse cage) followed by cervical dislocation to ensure the mice were dead and would not regain consciousness. The uteri were dissected and the numbers of conceptuses and resorptions were recorded. The vasculatures on yolk sacs were captured using a Cannon camera. Foetuses and placentas were removed and wet weights were recorded. Embryonic tissue was used for genotyping. Half of the placental placentas was performed using Mouse Angiogenesis RT\(^{2}\) Profiler\(^{TM}\) PCR Array (Qiagen, PAMM-024Z) according to the manufacturer’s instruction.

2.4 Histomorphometric analysis and immunohistochemistry staining

After fixing in 4% paraformaldehyde overnight, the half placenta was cut into three portions evenly, dehydrated in a graded series of ethanol and xylene solutions, and embedded in paraffin. Sections (5 μm) were cut with a microtome. Sections were deparaffinized in xylene, rehydrated to water, and stained with H&E. For immunohistochemistry, the hydrated sections underwent microwave antigen retrieval in 10 mM citrate buffer, pH 6.0. Sections were allowed to cool down to room temperature, washed with PBS, and the endogenous peroxidase activity was quenched by the addition of 3% H\(_2\)O\(_2\). Subsequently, slides were washed with PBS and non-specific antibody binding was blocked by incubating the sections in 10% normal goat serum for 30 min. The sections were then incubated with CD31 primary antibody (1:200, Abcam) at 4°C overnight followed by incubation with horseradish peroxidase-conjugated secondary antibody. The sections were counterstained with Haematoxylin. Images were captured with a CCD camera mounted on a Nikon microscope (Nikon America, Inc.).

2.5 Quantitative reverse transcription-PCR

Total RNA of placenta tissues and cultured cells was extracted using Trizol Reagent (Invitrogen) according to the manufacturer’s instruction. Reverse transcription was performed using an iScript cDNA Synthesis kit (Bio-Rad). Quantitative reverse transcription-PCR (qPCR) was performed in the Mx3005P qPCR machine using SYBR Green master mix (Agilent Technologies). Each sample was amplified in triplicate. The primer sequences for qPCR were supplied in Supplementary material online.

2.6 PCR array

Expression profiling of genes modulating the processes of angiogenesis in wild-type and RGC32\(^{-/-}\) placentas was performed using Mouse Angiogenesis RT\(^{2}\) Profiler\(^{TM}\) PCR Array (Qiagen, PAMM-024Z) according to the manufacturer’s instruction.

2.7 Western blot analysis

Western blotting was performed as described previously. Cells treated as indicated were washed twice with PBS, followed by protein extraction using RIPA buffer (50 mM Tris–HCl, pH 7.4, 1% Triton X-100, 0.25% w/v sodium deoxycholate, 150 mM NaCl, 1 mM EGTA, 0.1% SDS, protease inhibitor cocktail). Protein concentration was measured using BCA Protein Assay Reagent (Thermo Scientific). Equal amounts of protein (10 μg) were resolved in 8% SDS–PAGE and were transferred to polyvinylidene difluoride membranes (Bio-Rad). Antibodies against VEGFR2, α-tubulin, phospho- and total NF-kB p65, and cAMP response element-binding protein (CREB) were used for immunoblotting. All antibodies were purchased from Cell Signaling Technology.

2.8 Cell proliferation assay

MTT assays were performed as described previously. Briefly, 5 × 10\(^3\) cells/well were cultured in 96-well plates and treated as indicated. Ten microlitres of MTT reagent (R&D) was added to the medium and incubated in a CO2 incubator for 3 h, and then 100 μL of detergent (R&D) was added and incubated at 37°C for additional 2 h. Absorbance at 570 nm wavelength was measured in a plate reader (Bio-Tek). The medium containing no cells was used as the blank control.

2.9 Wound healing assay

Cell migration was evaluated by the wound healing assay using the CytoSelect Wound Healing Assay Kit (Cell Biolabs) as described previously. Briefly, wound healing inserts were put into 24-well cell culture plates. C166 cells (250 μL) transduced with Ad-GFP or Ad-shRGC32 were added to either side of the insert and incubated overnight to form a monolayer. Then the inserts were removed, and the cells treated with vehicle or VEGF (20 ng/mL) for 24 h to allow the cells to migrate. Images of wound healing were captured using a dissection microscope at a magnification of ×40. Cell migration was quantified by blindly measuring the migration distance.

2.10 EC tube formation

C166 cells were transduced with Ad-GFP or Ad-shRGC32 for 24 h, and then plated in a 24-well plate, which was pre-coated with 200 μL of Matrigel (Becton Dickinson, Bedford, MA, USA) at a concentration of 5 × 10\(^4\) cells/well in 500 μL DMEM medium containing 10% foetal bovine serum. The cells were incubated at 37°C for 24 h. Tube formation was defined as
2.11 Immunofluorescent staining
C166 cells were cultured on glass coverslips and treated as indicated. Then the cells were fixed with 4% paraformaldehyde, permeabilized with 0.2% Triton X-100 in PBS and incubated with 10% normal goat serum for 30 min. The cells were incubated overnight at 4 °C with anti-RGC-32 (1:100), anti-VEGFR2 (1:50), or anti-NF-κB p65 (1:100), and then FITC- (1:100) or TRITC-labelled (1:50) secondary antibody for 1 h. Nuclei were stained with 4, 6-diamidino-2-phenylindole (DAPI, Vector Laboratories, Inc.). Fluorescence images were captured by a fluorescent microscope (Nikon).

2.12 Statistical analysis
Data are presented as means ± SD. Differences between groups were evaluated by Student’s t test or one-way ANOVA followed by Tukey’s multiple comparison post hoc tests using the GraphPad Prism 5.0 software. P < 0.05 was considered statistically significant.

3. Results
3.1 Restricted growth of RGC32−/− mice
RGC32−/− mice were generated using ES cells with deletion of exon 2 and 3 of RGC-32 gene (Figure 1A). The mice were genotyped by PCR using two pairs of primers amplifying a region covering exon 1 and a trapped DNA sequences (LAR3) and a region covering intron 2 and exon 3 sequences (Figure 1B). The absence of RGC-32 in RGC32−/− mice was confirmed by qPCR using total RNA extracted from the
embryos at 16.5 DPC (Figure 1C). RGC-32 transcript was present in both heterozygous and wild-type mice, but no RGC-32 expression was detected in RGC32−/− mice.

The most striking phenotype of RGC32−/− mice was their smaller body size compared with the wild-type littermates (Figure 1D). To quantify their body weight, we monitored the growth kinetics up to 4 weeks after birth and found that RGC32−/− mice were 27% (P < 0.01, Figure 1E) lighter than their wild-type littermates when they were born. This difference remained stable until 4 weeks old (22%, P < 0.01, Figure 1E). The difference in the body size was not dramatic between the wild-type and RGC32−/− mice after 5 weeks old (data not shown), probably due to the rapid growth in the pubertal stage. Interestingly, RGC32−/− mice exhibited a growth index similar to their wild-type littermates during the first 4 weeks (Figure 1E). These data indicate that the small body size of the RGC32−/− mouse may be caused by a growth restriction during embryonic development.

3.2 Absence of RGC-32 caused foetal growth restriction and death

To determine what has caused the small body size of neonatal RGC32−/− mouse, we observed the development of embryos and placentas. Wild-type and RGC32−/− embryos and placentas were collected at 16.5 DPC. Consistent with the small body size, RGC32−/− embryos and placentas were evidently smaller than the wild-type littermates (Figure 2A). Quantification of the embryo and placenta weights revealed that both RGC32−/− embryos (80%, P < 0.01, Figure 2B), and placentas (82%, P < 0.01, Figure 2C) were significantly smaller than the wild-type. In addition, we found a small portion of stillbirths. When crossing the male heterozygous (RGC32+/−) with female heterozygous mice, the expected ratio of the embryonic genotypes for RGC32+/−:RGC32+/−:RGC32−/− was 25:50:25. However, the actual percentage of RGC32−/− embryos survived was significantly reduced (15%), while the ratios of RGC32+/− (49%) and RGC32+/− (26%) embryos were as expected (Figure 2D). Based on the Mendelian genetic principal, the stillbirths (10%) were most likely to be the RGC32−/− embryos although we were unable to determine the genotype due to the early absorption of these embryos. Nevertheless, these data demonstrate that RGC-32 deletion causes foetal growth restriction and even death.

3.3 Defective angiogenesis was evident in RGC32−/− placenta labyrinth zone

Placenta is an organ that connects the maternal and foetal circulation, facilitating metabolic and gas exchange as well as foetal waste disposal.

Figure 3 Impaired vascularity in the RGC32−/− placenta labyrinth zone and yolk sac. (A) Half of the placenta was divided into three portions evenly as indicated and paraffin embedded. The placenta sections were stained with H&E, and the labyrinth zones were circulated with yellow line. (B) Quantitative analysis of the areas of different labyrinth zones. The areas were normalized to the first portion of wild-type labyrinth zones. (C) Representative photomicrographs of CD31 immunohistochemistry staining of the wild-type and RGC32−/− placenta labyrinth zones. (D) Quantification of foetal capillary numbers in the labyrinth zones. (E and F) Gross vasculatures of two comparable locations of the wild-type and RGC32−/− yolk sacs at 11.5 DPC. *P < 0.05, **P < 0.01, $P > 0.05 compared with the corresponding wild-type group as indicated in (B) or (D) (n ≥ 3). MS, maternal sinusoid; Tr, trophoblast cell; FE, foetal endothelial cell; FCp, foetal capillary.
Defective placenta function results in foetal growth restriction during gestation. To determine whether the small size of RGC32^−/−_embryo and placenta is caused by a structural defect, we divided half of the placenta (16.5 DPC) into three portions evenly (Figure 3A). Each portion was fixed with 4% paraformaldehyde followed by paraffin-embedding and sectioning. H&E staining showed no differences in the first (edge of the placenta) and the second portions of the placenta labyrinth. However, in the midline of the placenta labyrinth area (circulated by yellow line) between the RGC32^−/−_of the placenta) and the second portions of the placenta labyrinth and sectioning. H&E staining showed no differences in the first (edge nucleated red blood cells inside the lumen (pillaries were characterized by the presence of ECs and the bigger defect, we stained the placenta with CD31, an EC marker. The foetal cation not significantly affected by RGC32 deletion. However, the expression of VEGF2 (53.8%, P < 0.05, Figure 4A) and PlGF (43.3%, P < 0.05, Figure 4B) was significantly inhibited in RGC32^−/−_placenta. A systematic analysis of angiogenesis-related genes using PCR array revealed that coagulation factor II, plasminogen, and thrombin phospholipase were decreased, whereas interleukin 6 (I66) was increased (Supplementary material online, Figure S1) in RGC32^−/−_placenta compared with the wild-type. Although these genes are important for angiogenesis, their expression levels in foetal placenta are much lower compared with VEGF2 and PlGF. Our data suggest that VEGF2 and PlGF play major roles in RGC-32-mediated placental angiogenesis.

3.5 Knockdown of RGC-32 inhibited EC function, PlGF expression, and trophoblasts proliferation

The placenta labyrinth consists of EC and trophoblasts, without SMCs. The proliferation and migration of EC are important for angiogenesis.26 To determine the role of RGC-32 in EC function, we knocked down RGC-32 expression using Ad-shRGC32 in C166 ECs, and then detected VEGF2 and PlGF expression. As shown in Figure 5A, VEGF2, but not PlGF, was dramatically suppressed. RGC-32 knockdown also blocked VEGF2 protein expression (Figure 5B).

VEGF regulates angiogenesis by stimulating EC proliferation and migration.27 VEGF2 is the major receptor responsible for mediating VEGF functions.28 Since VEGF2 is located on cell membrane, but RGC-32 is located in the EC nuclei (Supplementary material online, Figure S2), RGC-32 is unlikely to regulate VEGF2 signalling transduction or modulating VEGF2 activity. Therefore, we hypothesized that RGC-32 induces VEGF-stimulated EC proliferation and migration via the regulation of VEGF2 expression. To test this hypothesis, we transduced C166 cells with Ad-GFP or Ad-shRGC32, and then treated the cells with serum-free medium in the presence or absence of VEGF. The MTT proliferation assay showed that VEGF significantly induced the proliferation of C166 cells treated with Ad-GFP. However, knockdown of RGC-32 blocked VEGF-induced EC proliferation (Figure 5C). Likewise, the wound healing assay showed that VEGF promoted C166 cell migration, but knockdown of RGC-32 by Ad-shRGC32 blocked VEGF activity (Figure 5D and E).

In addition to EC proliferation and migration, VEGF2 is also responsible for EC tube formation.29 To further determine whether RGC-32 plays a role in VEGF/VEGF2-mediated angiogenesis, we observed the EC tube formation of Ad-GFP or Ad-shRGC32-transduced C166 cells were affected by RGC32^−/−_, we observed the vasculatures in the yolk sacs at 11.5 DPC and found that RGC32^−/−_yolk sacs had fewer and smaller vessels compared with wild-type yolk sacs (Figure 3E and F). These data demonstrate that loss of RGC-32 interrupts the angiogenesis in placenta and other vascular systems.

3.4 PlGF and VEGFR2 were down-regulated in RGC32^−/−_placenta

VEGF family,19,20 ANGPT proteins,21 FGFR,22 HIF-1α,23 endothelial nitric oxide synthase (NOS) 39,24,25 and glial cells missing (GCM) 125 are all involved in angiogenesis. To determine the mechanisms underlying the abnormal angiogenesis observed in the RGC32^−/−_placenta, we tested if RGC32^−/−_alters the expression of these genes in placenta. Interestingly, most of the angiogenic factors including VEGF, VEGFR1, ANGPT1, ANGPT2, ANGPT receptor (TEK), FGFR, HIF-1α, NOS3, NOS3 receptor (GUCY1B3), and GCM1 (Figure 4C) were not significantly affected by RGC32 deletion. To determine whether RGC32^−/−_placenta is related to an angiogenesis 2/2_/foetal placenta is caused by a structural defect, we divided half of the placenta (16.5 DPC) into three portions evenly (Figure 3A). Each portion was fixed with 4% paraformaldehyde followed by paraffin-embedding and sectioning. H&E staining showed no differences in the first (edge of the placenta) and the second portions of the placenta labyrinth zone area (circulated by yellow line) between the RGC32^−/−_and wild-type placenta. However, the third part, i.e. the midline of the placenta labyrinth area, of the RGC32^−/−_placenta was significantly smaller than the wild-type (1.69 ± 0.20 vs. 2.05 ± 0.04, P < 0.05, Figure 3A and B).

Placental angiogenesis plays a critical role in the placental development and foetal growth. To determine whether the reduction of the labyrinth area in the RGC32^−/−_foetal placenta is related to an angiogenesis defect, we stained the placenta with CD31, an EC marker. The foetal capillaries were characterized by the presence of ECs and the bigger nucleated red blood cells inside the lumen (Figure 3C). We found that RGC32^−/−_did not affect the angiogenesis in the first and second portions of placenta labyrinth. However, in the midline of the placenta labyrinth area, RGC32^−/−_foetal placenta labyrinth had much less foetal capillaries with bigger vessel lumens compared with the wild-type placenta (Figure 3C). Quantitation of foetal capillary numbers showed that foetal capillaries in the midline of RGC32^−/−_placentas were significantly less than the wild-type placentas (9.5 ± 2.3 vs. 16.2 ± 3.7, P < 0.01, Figure 3D). To determine whether other vascular systems were affected by RGC32^−/−_, we observed the vasculatures in the yolk sacs at 11.5 DPC and found that RGC32^−/−_yolk sacs had fewer and smaller vessels compared with wild-type yolk sacs (Figure 3E and F). These data demonstrate that loss of RGC-32 interrupts the angiogenesis in placenta and other vascular systems.

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were transfected with control (Ctrl) or shRGC32 plasmid for 48 h. MTT assay was performed to evaluate the trophoblasts proliferation after the cells knockdown blocked trophoblasts proliferation. The MTT assay was performed to evaluate the trophoblasts proliferation after the cells knockdown blocked trophoblasts proliferation.

The MTT assay was performed to evaluate the trophoblasts proliferation after the cells knockdown blocked trophoblasts proliferation. Consistent with the in vivo observation (Figure 4B), knockdown of RGC-32 inhibited PI GF expression in trophoblasts (Figure 5G), leading to a blockage of trophoblasts proliferation (Figure 5H). These data indicate that RGC-32 regulates trophoblasts proliferation through modulating PI GF expression.

3.6 RGC-32 regulated VEGFR2 expression via activation of NF-kB

NF-kB and CREB are two important factors that have positive and negative regulatory effects on VEGFR2 expression. To determine the mechanism whereby RGC-32 regulates VEGFR2 expression, we transduced C166 cells with Ad-GFP or Ad-RGC32 and tested if RGC-32 regulates NF-kB and CREB activation. As shown in Figure 6A, RGC-32 overexpression increased NF-kB phosphorylation and nuclear translocation (Supplementary material online, Figure S3). RGC-32 appeared not to affect CREB activation (Figure 6B). To determine whether NF-kB mediates RGC-32-induced VEGFR2 expression, we pre-treated the cells with an NF-kB-specific inhibitor PDTC prior to RGC-32 overexpression. PDTC blocked the effect of RGC-32 on NF-kB phosphorylation (Figure 6C) and nuclear translocation (Supplementary material online, Figure S3), leading to the reduction of RGC-32-induced VEGFR2 mRNA and protein expressions (Figure 6D and E). These data demonstrate that RGC-32 regulates VEGFR2 expression through activating NF-kB signalling in EC.

4. Discussion

Our study has demonstrated for the first time that RGC-32 plays a critical role in mouse placental angiogenesis. Loss of RGC-32 decreases VEGFR2 expression in EC and PI GF expression in trophoblasts, which impairs the angiogenesis of the labyrinth zone and placenta development, leading to foetal growth restriction and even embryonic lethality. Compared with wild-type mice, RGC32−/− mice are significantly smaller in size with a much lighter body weight that are evident since embryonic stage until 4 weeks old due to restricted placenta growth.

The labyrinth zone of the placenta is the place where the nutrients and wastes exchange between foetus and mother. The fully developed placental labyrinth zone composed of highly branched villi is required for the normal and efficient nutrient exchange. Vascular formation of the labyrinth zone is critical for foetal growth and development, in which VEGF family members play important roles. VEGF deficiency is embryonically lethal due to the foetal vascular abnormalities. VEGF promotes angiogenesis through regulating the proliferation, migration and tube formation of EC. VEGF exerts its function mainly through binding to VEGFR2. VEGFR2 is also indispensable for the normal vascular development of the embryos. In contrast to VEGF, PI GF is abundantly expressed in the placenta trophoblasts. It stimulates angiogenesis through VEGFR1. It is reported that PI GF and VEGF may synergistically regulate angiogenesis.

Figure 5 Knockdown of RGC-32 inhibited EC function, PI GF expression and trophoblasts proliferation. (A) RGC-32 knockdown blocked VEGFR2 mRNA expression. C166 cells were transduced with adenovirus expressing GFP (Ctrl) or RGC-32 shRNA (Ad-shRGC32) for 48 h. qPCR was performed to detect mRNA expressions of RGC-32, VEGFR2, and PI GF as indicated. (B) RGC-32 knockdown blocked VEGFR2 protein expression. C166 cells were treated similarly as in (A). VEGFR2 expression was detected by western blot and normalized by α-tubulin. (C) RGC-32 knockdown blocked VEGF-induced EC proliferation. C166 cells were transduced with Ad-GFP (Ctrl) or Ad-shRGC32 followed by treatment with vehicle or VEGF (20 ng/mL) for 48 h. MTT was performed to evaluate EC proliferation. (D) RGC-32 knockdown blocked VEGF-induced EC migration. C166 cells were treated similarly as in (C). The wound healing assay was used to evaluate EC migration. (E) Quantitative analysis of the migration distance. (F) RGC-32 knockdown blocked EC tube formation. Ad-GFP or Ad-shRGC32-transduced C166 cells were seeded on Matrigel for 24 h to observe the capillary-like tube formation. Data shown are representatives of three independent experiments. (G) RGC-32 knockdown inhibited PI GF expression in trophoblasts. HTR-8/SVneo cells were transfected with control (Ctrl) or RGC-32 shRNA plasmid (shRGC32) for 48 h. qPCR was performed to detect mRNA expression of RGC-32 and PI GF. (H) RGC-32 knockdown blocked trophoblasts proliferation. The MTT assay was performed to evaluate the trophoblasts proliferation after the cells were transfected with control (Ctrl) or shRGC32 plasmid for 48 h. *P < 0.05, **P < 0.01 compared with Ctrl group, #P < 0.05 compared with Ctrl + VEGF group (n ≥ 3).
significantly impaired vascular formation probably because RGC32⁻/⁻ repressed the expression of critical components of both VEGF and PlGF angiogenic pathways, i.e. the receptor for the VEGF pathway, VEGFR2 and the ligand for the PlGF pathway. Suppression of both pro-angiogenesis pathways lead to impaired vascularization, foetal growth retardation, and even embryonic death.

A previous report shows that RGC-32 acts as an angiogenic inhibitor dependent on HIF-1α and VEGF. The different effects of RGC-32 between the present and the previous studies are likely due to the different oxygen tensions. Hypoxia dramatically and reversibly induced VEGF.39 In addition, hypoxia induces VEGFR1, but not VEGFR2, expression.40 RGC32⁻/⁻ does not alter HIF-1α and VEGF expression in placenta, suggesting that RGC32⁻/⁻ placenta is in a normal oxygen tension. In addition, RGC-32 appears to regulate the expression of VEGFR2, but not VEGFR1, in EC, which facilitates the canonical VEGF pathway. Interestingly, VEGFR1 can also be expressed as a soluble protein. Soluble VEGFR1 (sVEGFR1) has strong antagonistic activity as it binds to all isoforms of VEGF and PlGF.41 sVEGFR1 can be induced by VEGF and hypoxia that is not altered or present in RGC32⁻/⁻ placenta. Therefore, sVEGFR1 may not be involved in RGC32⁻/⁻-caused defective angiogenesis. Furthermore, RGC-32 regulates PlGF expression and trophoblasts proliferation, further indicating the different mechanisms underlying RGC-32-mediated angiogenesis in placenta during pregnancy and in tissues with hypoxia.

RGC-32 appears to regulate VEGFR2 expression via the NF-kB signalling pathway. Since RGC-32 is not a ligand, it is unlikely to form a complex with VEGFR2 or regulate VEGFR2 stabilization or internalization. RGC-32 has been shown to serve as a co-activator to regulate gene expression.42 RGC-32 can also physically associate with Akt and regulate Akt signalling in EC.15 Our study demonstrates that RGC-32 activates NF-kB, an important regulator for VEGFR2 expression.32 Blockade of NF-kB signalling by its specific inhibitor inhibits RGC-32-induced VEGFR2 expression, demonstrating that RGC-32 regulates VEGFR2 expression via NF-kB activation.

Taken together, our studies have demonstrated that RGC-32 is essential for the normal embryo growth due to its critical role in placental angiogenesis during pregnancy. RGC-32 mediates the placental angiogenesis through regulating VEGFR2 expression in EC and PlGF expression in trophoblasts. RGC-32 may also affect angiogenesis in other vascular systems in addition to placenta.

Supplementary material
Supplementary material is available at Cardiovascular Research online.

Conflict of interest: none declared.

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References
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