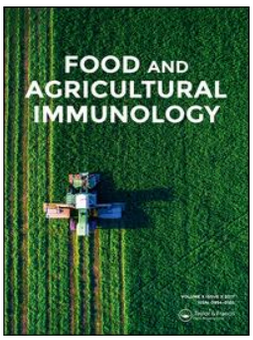

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Glucocerebroside reduces endothelial progenitor cell-induced angiogenesis

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ABSTRACT

The recruitment of bone marrow-derived endothelial progenitor cells (EPCs) facilitates physiological and pathological processes involved in new blood vessel synthesis. Glucocerebroside, an extract of *Cordyceps militaris*, inhibits inflammatory cytokine production and monocyte migration, although its anti-angiogenic properties in human EPCs has remained largely unknown up until now. We describe how glucocerebroside reduces migration as well as tube formation induced by vascular endothelial growth factor (VEGF) stimulation in human EPCs, without affecting cell viability. This inhibitory effect was achieved through the focal adhesion kinase (FAK)/c-Src pathways. We also found that glucocerebroside reduced VEGF-promoted upregulation of the transcription factor Runx2 in the EPCs. The *in vivo* chick embryo chorioallantoic membrane model demonstrated that glucocerebroside reduces new vessel formation. Our investigation is the first to show that glucocerebroside reduces angiogenesis in human EPCs and to describe the underlying mechanisms. Further investigations are needed to examine the effects of glucocerebroside in other angiogenesis-related disorders.

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1. Introduction

Angiogenesis is a progression for synthesis of new capillaries (Folkman, 2006), which is an important step in physiology involving bone remodelling, embryonic development and tissue remodelling. Angiogenesis is also critical for pathological processes, including cancer progression and metastasis, inflammatory diseases and retinopathy (Carmeliet & Jain, 2000; Jain, 2014; Lii et al., 2016), which has encouraged investigations into the reduction of angiogenesis in the treatment of tumours and other angiogenesis-associated diseases (Chung, Lee, & Ferrara, 2010; Su, Huang, & Tang, 2016). So far, around 10 anti-angiogenic agents, including vascular endothelial growth factor (VEGF) and VEGF receptor (VEGFR) antagonists, have received US FDA approval and been introduced into the clinic (Petrovic, 2016; Simone et al., 2017).

It is recognized that endothelial progenitor cells (EPCs) starting in the bone marrow increase postnatal vasculogenesis in physiological and pathological process of new vessel synthesis (Asahara et al., 1999). EPCs are capable of instigating proangiogenic responses, such as cell proliferation, development, migration, invasion and metastasis. Bone marrow EPCs express the cell surface markers CD133, CD34 and VEGFR2, which contribute to new vessel formation (Yoder, 2012). It has been suggested that tumour secretion of VEGF and other growth factors controls EPC mobilization and thus regulates the progression and angiogenesis of certain tumours (Peters et al., 2005). EPCs are also important modulators of the angiogenic switch that regulates tumour metastasis (Jain & Carmeliet, 2012). In clinical studies, elevated circulating EPCs levels have been shown to be associated with various types of malignancies (Nowak et al., 2010; Starzyńska et al., 2013; Yu et al., 2007). Thus, the targeting of EPCs appears promising for the treatment of angiogenesis-associated diseases.

Numerous natural compounds are capable of inhibiting angiogenesis through different mechanisms (Chan, Lien, Lee, & Huang, 2016; Miao, Feng, & Ding, 2012; Su et al., 2013; Wang & Miao, 2013). *Cordyceps militaris*, an entomopathogenic fungus, has long been used to treat inflammatory diseases in humans (Brent et al., 2016). We have previously shown that the *Cordyceps militaris* extract, glucocerebroside, reduces lipopolysaccharide (LPS)-induced production of proinflammatory cytokines, inducible nitric oxide synthase (iNOS) and cyclooxygenase-2 (COX-2) in macrophages (Chiu et al., 2016). Up until now, the roles of glucocerebroside in EPC-associated angiogenesis have been unclear. Here, we describe how glucocerebroside reduced EPC migration and tube formation, without any evidence of cytotoxic activity, via the focal adhesion kinase (FAK)/c-Src pathway. Glucocerebroside also reduced VEGF-induced upregulation of Runx2 (runt-related transcription factor 2) in EPCs. The *in vivo* chick embryo chorioallantoic membrane (CAM) model also demonstrated that glucocerebroside reduces new blood vessel formation. Thus, glucocerebroside shows potential as an angiogenic antagonist that targets EPCs.

2. Materials and methods

2.1. Materials

We purchased FAK, p-FAK, c-Src, p-c-Src, PI3 K, p-PI3 K, Akt, p-Akt, c-Jun, p-c-Jun, Runx2 and β-actin primary antibodies from Santa Cruz Biotechnology (CA, USA). We obtained recombinant human VEGF from PeproTech (Rocky Hill, NJ, USA). We bought

Matrigel from BD Biosciences (Bedford, MA, USA). Transwell® inserts were purchased from Corning (Kennebunk, ME, USA). MV2 basal medium was obtained from PromoCell GmbH (Heidelberg, Germany). FBS was purchased from HyClone, (Logan, UT, USA).

2.2. Cell culture

EPCs were isolated and cultured according to our previous investigations (IRB Reference No. P1000002) (Wang et al., 2015; Wu et al., 2014). Cells were maintained at 37°C in a humidified 5% CO_2 atmosphere.

2.3. MTT assay

Cells were stimulated with glucocerebroside and then treated with MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] for 30 min. The MTT was dissolved in DMSO and absorbance was detected using a microplate reader (BioTek Instruments, Winooski, VT, USA) (Lee et al., 2019; Liu, Chen, Chen, Chang, & Tang, 2016).

2.4. Western blot analysis

EPCs were lysed according to our previous study (Li et al., 2017). Proteins were processed by SDS-polyacrylamide gel electrophoresis and transferred onto polyvinyldifluoride membranes. Blots were blocked, then treated with primary and secondary antibodies. Finally, the blots were visualized using an ImageQuant™ LAS 4000 system (GE Healthcare Life Sciences, Pewaukee, WI, USA) (Huang, Chen, Tsai, Hsu, & Tang, 2012; Liu et al., 2019).

2.5. Measurement of migratory activity in EPC

Cells were applied at a density of 5×10^4/well onto the upper chamber of Transwell inserts and glucocerebroside (1–10 μM) was added to VEGF-containing medium. The migrated cells on the lower side of filters were fixed with 4% formaldehyde, stained with 0.05% crystal violet, then photographed and counted under a microscope (Tsai et al., 2015).

2.6. Measurement of tube formation in EPCs

EPCs (3×10^4 cells) were applied to pre-coated Matrigel plates containing VEGF with glucocerebroside (1–10 μM) for 24 h. Tube formation was photographed and numbers of tube branches were calculated using MacBiophotonics ImageJ software (Hu et al., 2017; Tsai et al., 2015).

2.7. CAM assay

The CAM assay was used to examine angiogenic activity *in vivo*, as according to our previous research (Chen et al., 2017; Wu et al., 2014). The numbers of blood vessel branches were counted by microscopy and photographed using a digital camera. All animal investigations followed approved protocols issued by the China Medical University (Taichung, Taiwan) Institutional Animal Care and Use Committee.

2.8. Statistical analysis

Data are presented as the mean ± the standard error of the mean. Statistical analysis of comparisons between 2 groups was performed using the Student's *t*-test. Statistical comparisons of more than 2 groups were performed using one-way analysis of variance (ANOVA). In all cases, $p < 0.05$ was considered to be statistically significant.

3. Results

3.1. Glucocerebroside does not affect the cell viability of EPCs

Cerebroside derivatives reportedly promote apoptotic signalling in human cancer (Fujiwara et al., 2011; Yazama et al., 2015). The MTT assay was used to examine the viability of human EPCs after glucocerebroside stimulation. Incubation with glucocerebroside (0.3–10 μM) for 24 or 48 h did not affect the viability in human EPCs (Figure 1). These data indicate that glucocerebroside is not cytotoxic in human EPCs.

3.2. Glucocerebroside reduces VEGF-enhanced migration and tube formation of EPCs

Migration of EPCs via the capillary basement membrane is a critical step in new blood vessel formation (Ammendola et al., 2015). We therefore used the Transwell assay to analyze the role of glucocerebroside in EPC migration. Treatment of EPCs with VEGF enhanced their migration activity; this was dose-dependently inhibited by glucocerebroside (Figure 2). The tube formation assay is a widely used *in vitro* mimic of vessel

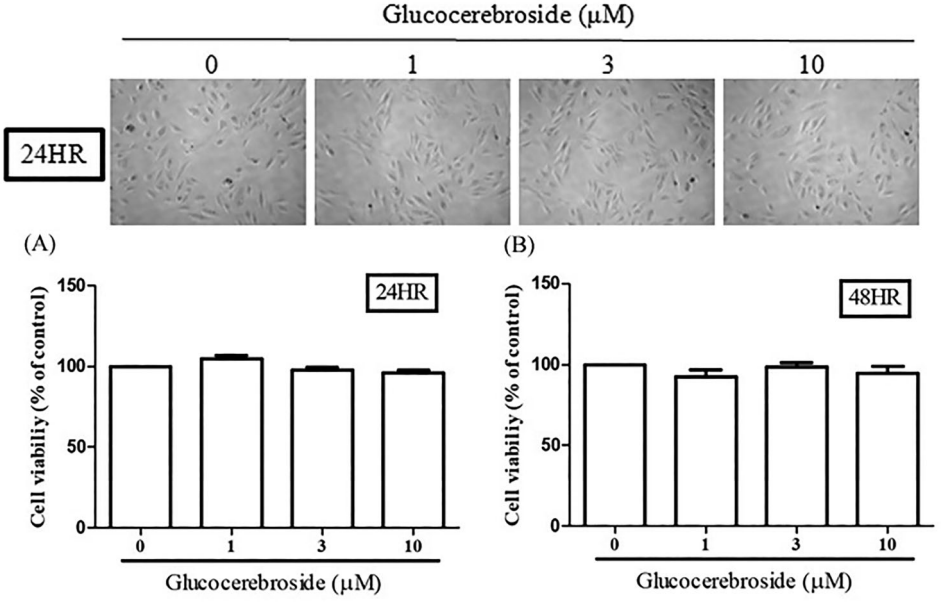

Figure 1. Glucocerebroside does not affect the viability of human EPCs. EPCs were incubated with the indicated concentrations of glucocerebroside for 24 or 48 h and cell viability was determined using the MTT assay. Data represent the mean ± S.E.M.

formation. This assay showed that incubation of EPCs with VEGF enhanced reorganization and formation of capillary-like structures. In addition, glucocerebroside significantly reduced VEGF-promoted EPC tube formation (Figure 3), suggesting that glucocerebroside has anti-angiogenic effects in human EPCs.

3.3. Glucocerebroside reduces VEGF-induced FAK and c-Src activation

FAK activation reportedly controls EPC angiogenesis (Li et al., 2017; Tsai et al., 2017). We therefore examined whether glucocerebroside reduces EPC angiogenesis through the FAK pathway. Stimulation of EPCs with VEGF facilitated phosphorylation of FAK, while glucocerebroside reduced VEGF-augmented FAK phosphorylation (Figure

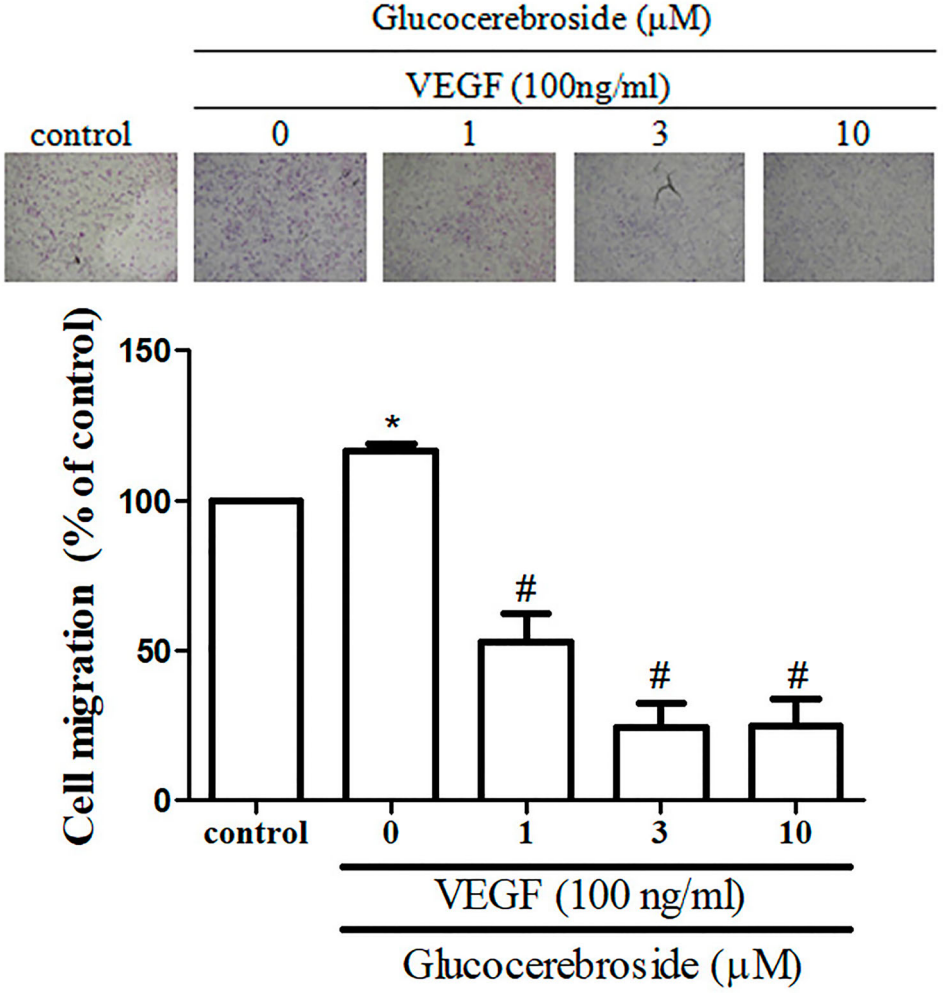

Figure 2. Glucocerebroside reduces VEGF-induced migration of human EPCs. EPCs were stimulated with or without VEGF (100 ng/mL) with or without the indicated concentrations of glucocerebroside for 24 h. Cell migration was examined by the Transwell migration assay. Data represent the mean ± S.E.M. *, $p < 0.05$ compared with the control group; #, $p < 0.05$ compared with the VEGF-treated group.

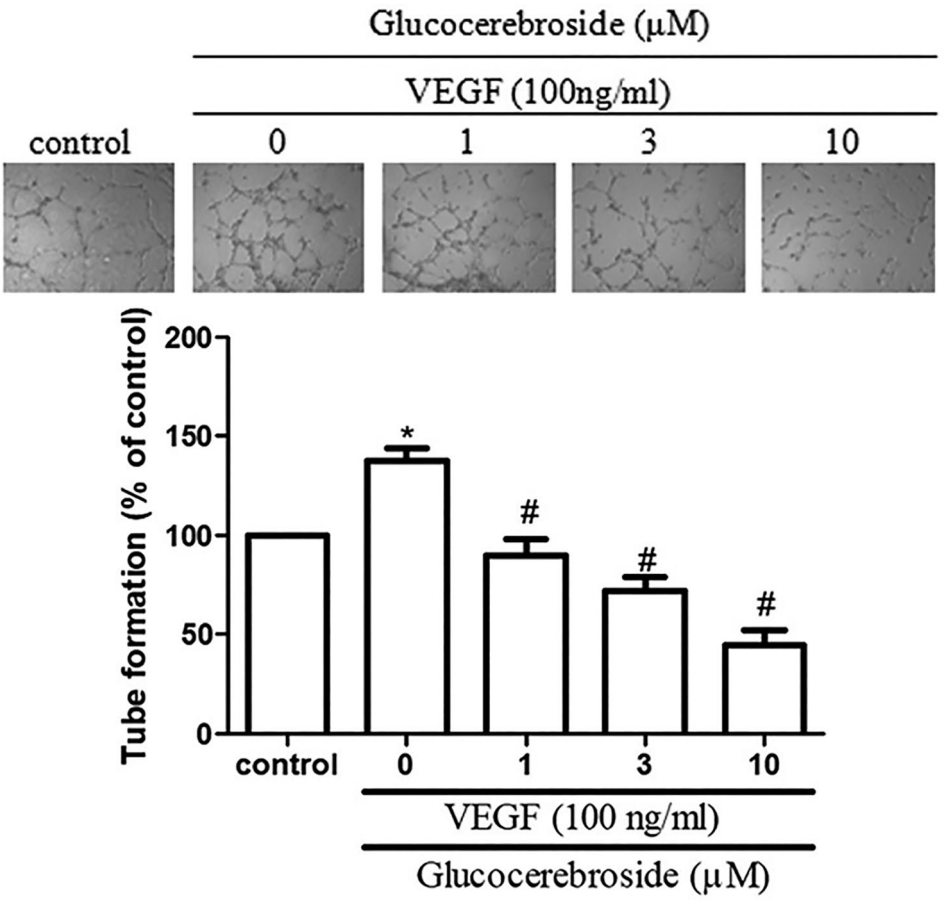

Figure 3. Glucocerebroside reduces VEGF-induced tube formation of human EPCs. EPCs were stimulated with or without VEGF (100 ng/mL) with or without the indicated concentrations of glucocerebroside for 24 h. Capillary-like structure formation was examined using the tube formation assay. Data represent the mean ± S.E.M. *, $p < 0.05$ compared with the control group; #, $p < 0.05$ compared with the VEGF-treated group.

4A). Moreover, c-Src has been implicated in the downstream signalling of FAK activation (Lin et al., 2013; Wang et al., 2017). In this study, VEGF increased c-Src phosphorylation, which was antagonized by glucocerebroside (Figure 4B). It is well established that the PI3K and Akt signalling cascade controls several biological effects of endothelial cells in the angiogenesis process (Chen et al., 2019; Lee et al., 2015), although we found that glucocerebroside failed to affect the phosphorylation of the PI3K and Akt pathway (Figure 4C and D). According to our findings, FAK and c-Src activation are required by glucocerebroside for successful reduction of VEGF-induced EPC angiogenesis.

3.4. Glucocerebroside reduces VEGF-promoted Runx2 activation in EPCs

Recent reports indicate that the cancer-related transcription factor Runx2 regulates EPC differentiation and angiogenesis (Li et al., 2019). We therefore examined whether Runx2

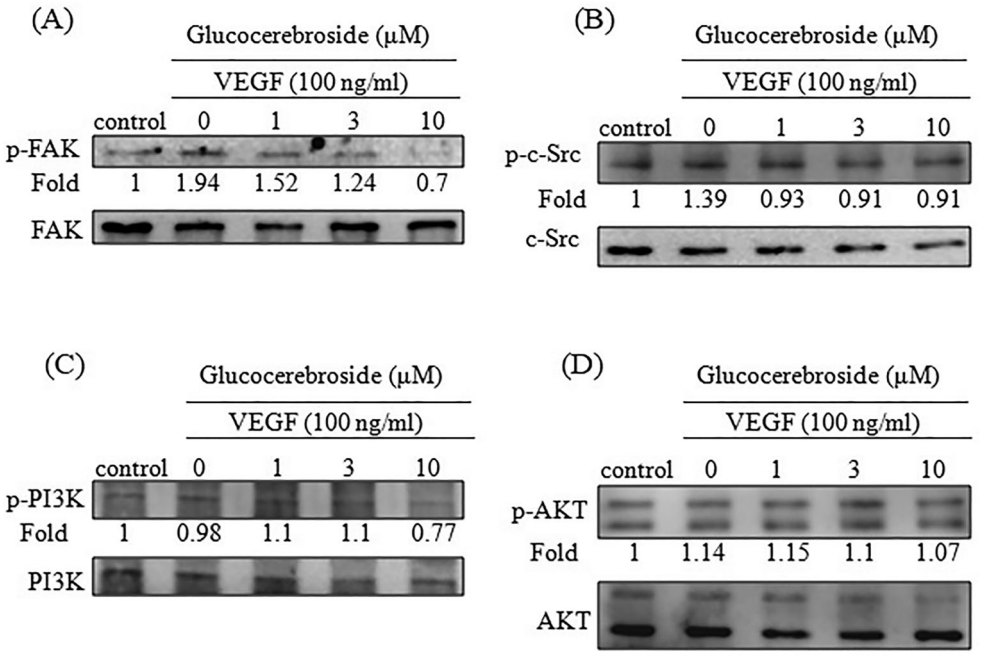

Figure 4. Glucocerebroside suppress the FAK/c-Src pathway in human EPCs. EPCs were incubated with VEGF-A (100 ng/mL) and the indicated concentrations of glucocerebroside for 24 h. FAK, c-Src, PI3 K and Akt phosphorylation was examined by Western blot analysis. Data represent the mean ± S.E.M. *, $p < 0.05$ compared with the control group; #, $p < 0.05$ compared with the VEGF-treated group.

is involved in glucocerebroside-promoted inhibition of angiogenesis driven by EPCs. We found that VEGF-promoted Runx2 upregulation was reduced by glucocerebroside treatment (Figure 5A). Moreover, the activator protein-1 (AP-1) transcription factor and a related increase in c-Jun expression is implicated in the angiogenic activity of endothelial cells (Huang et al., 2017). However, we failed to find any glucocerebroside-induced effects upon VEGF-mediated c-Jun phosphorylation (Figure 5B). We conclude that glucocerebroside reduces VEGF-induced EPC angiogenesis via Runx2 activation, not via AP-1 transcriptional activity.

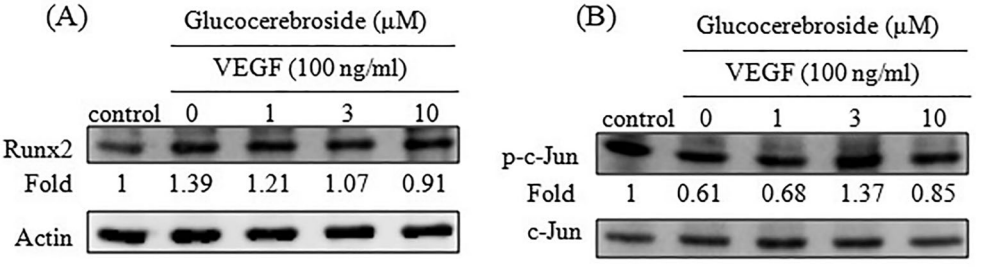

Figure 5. Glucocerebroside reduces Runx2 activation in human EPCs. EPCs were incubated with VEGF-A (100 ng/mL) and the indicated concentrations of glucocerebroside for 24 h. Runx2 and c-Jun expression was examined by Western blot analysis. Data represent the mean ± S.E.M. *, $p < 0.05$ compared with the control group; #, $p < 0.05$ compared with the VEGF-treated group.

3.5. *Glucocerebroside reduces angiogenesis* in vivo

Next, we used the CAM model to investigate *in vivo* anti-angiogenic effects. The results show that VEGF promotes vessel formation in CAM and that glucocerebroside reduces this activity (Figure 6). Thus, glucocerebroside appears to reduce *in vivo* angiogenesis.

4. Discussion

Therapeutic angiogenesis for ischemic disorders induces the progress of new blood vessels from pre-existing vessels, enabling the flow of blood to ischemic tissue. The angiogenic activities of EPCs constitute an important contribution to neovascularization (Asahara et al., 1999). They demonstrate a high potential to differentiate into mature endothelial cells *in vitro* and exhibit *in vivo* angiogenic ability in ischemic tissues (Asahara et al., 1997). Several chemoattractants, including VEGF, recruit circulating EPCs from the

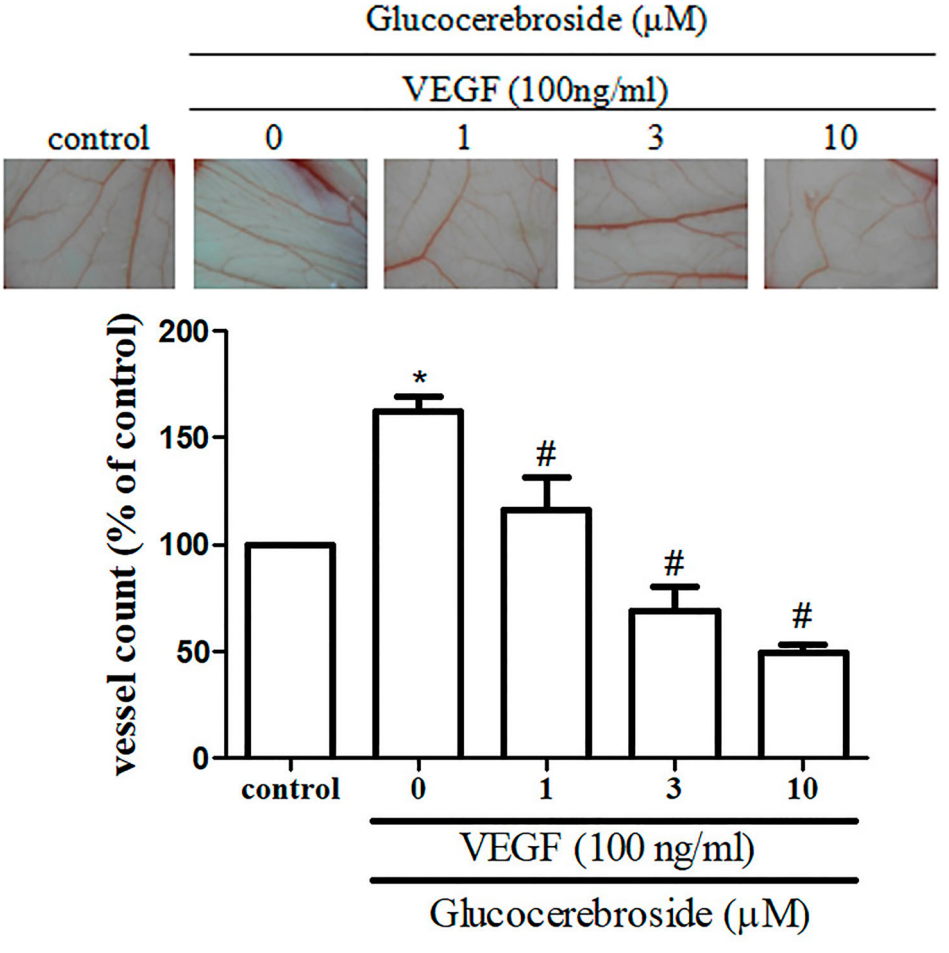

Figure 6. Glucocerebroside reduces vessel formation *in vivo*. Five-day-old fertilized chick embryos were treated with VEGF (100 ng/mL) with the indicated concentrations of glucocerebroside. After 3 days, the CAMs were examined by microscopy and photographed. Data represent the mean ± S.E.M. *, $p < 0.05$ compared with the control group; #, $p < 0.05$ compared with the VEGF-treated group.

bone marrow into the bloodstream, subsequently controlling integrity and promoting tissue repair (Carmeliet, 2005). Chemotherapy agents induce the mobilization of circulating EPCs and their subsequent "homing" into the cancer (Shaked et al., 2006). Thus, EPC-targeted treatment may help to antagonize angiogenesis-promoted tumour development and metastasis. In this study, we report that glucocerebroside reduced VEGF-facilitated migration and tube formation of human EPCs, without exhibiting any cytotoxic activity. Our evidence reveals the involvement of the FAK, c-Src and Runx2 signalling cascade in the anti-angiogenic effects of glucocerebroside. We also found that glucocerebroside reduces new blood vessel formation *in vivo*, suggesting that this agent is a potential therapeutic candidate for angiogenesis-related diseases.

FAK activation regulates VEGF-induced control of angiogenesis (Hosseini et al., 2019) and c-Src phosphorylation is implicated in the angiogenic functions of EPCs (Di et al., 2013). In this study, we found that glucocerebroside reduced FAK and c-Src phosphorylation, suggesting that the FAK and c-Src pathways mediate the inhibitory effects of glucocerebroside. The PI3 K/Akt signalling mechanism has also been implicated in the regulation of VEGF-dependent angiogenesis (Liu et al., 2014). However, we failed to find any evidence in support of glucocerebroside affecting the phosphorylation of PI3 K and Akt, suggesting that the FAK/c-Src signalling cascade, not the PI3 K/Akt pathway, mediates glucocerebroside-induced reduction of EPC angiogenesis.

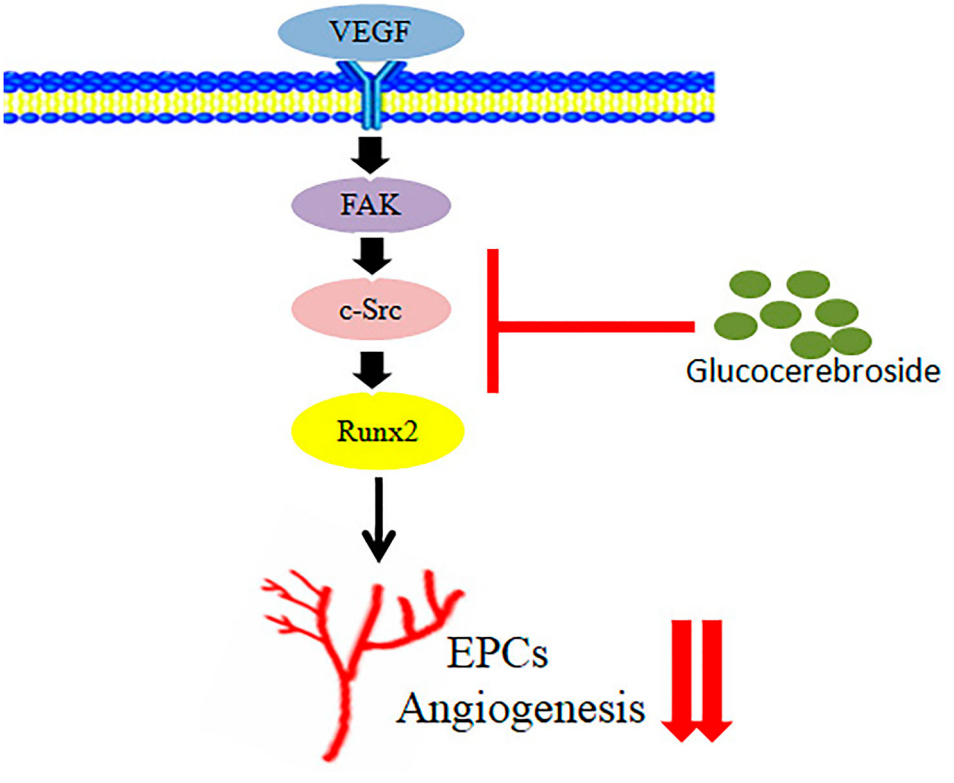

Figure 7. Schema depicting how glucocerebroside reduces angiogenesis in human EPCs. Glucocerebroside inhibits EPCs angiogenesis via the downstream signalling of FAK, c-Src and Runx2.

The transcription factor Runx2 mediates angiogenesis activated by the FAK/c-Src signalling cascade (Kim, Kim, Kim, Seong, & Kim, 2018; Li et al., 2019; Tsai, Huang, Yang, & Tang, 2012). Here, we found that treatment of EPCs with VEGF increased Runx2 activation, which was reduced by glucocerebroside. Interestingly, glucocerebroside had no effect upon another transcription factor, AP-1, which mediates c-Jun phosphorylation in human EPCs. Thus, the transcription factor Runx2, but not AP-1, plays a critical role in glucocerebroside-regulated reduction of angiogenesis.

Glucocerebroside, an extract of *Cordyceps militaris*, exhibits anti-inflammatory activity in human synovial fibroblasts (Liu et al., 2017). However, the role of glucocerebroside in EPC angiogenesis has been unclear up until now. Our report is the first to show that glucocerebroside reduces VEGF-induced EPC migration and tube formation and that the downstream signalling of FAK, c-Src and Runx2 is inhibited via the glucocerebroside-mediated reduction of *in vitro* and *in vivo* angiogenesis activities (Figure 7). Notably, glucocerebroside did not affect cell viability of human EPCs. Glucocerebroside deserves to be examined further for the treatment of angiogenesis-associated diseases.

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Disclosure statement

No potential conflict of interest was reported by the authors.

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