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ORIGINAL RESEARCH ARTICLE

The vasculoprotective effects of resveratrol are mediated via Kruppel-like factor 2 dependent protection of endothelial barrier function

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Abstract

Resveratrol is a naturally occurring polyphenolic compound that is thought to have vasculoprotective properties. Its observed effects are proposed, in part, to be mediated through the induction of endothelial Krüppel-like factor 2 (KLF2) expression. KLF2 is a nuclear transcription factor that is highly expressed within the vascular endothelium. Studies from our laboratory and others have shown that this protein mediates vascular function through its transactivation domain, and its targeted expression promotes vascular health, notably by acting as an important positive regulator of endothelial barrier function. In this study, we demonstrate that resveratrol possesses endothelial barrier protective effects dependent on the presence of KLF2, with several key endothelial tight junction proteins expressed in a KLF2-dependent manner. Collectively, our findings identify KLF2 as essential for resveratrol-mediated endothelial barrier protection, thus further implicating KLF2 as a critical vasculoprotective factor.

Keywords: Resveratrol; Krüppel-like factor 2; Barrier function

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1. Introduction

Resveratrol (3,5,4′ -trihydroxy-trans-stilbene), a polyphenol that is notably present at high concentrations in grapes and red wine, is occasionally used as a dietary supplement, although its beneficial effects remain unproven. It has generated a significant amount of clinical interest for its many beneficial effects on multiple pathologic processes, including hypertension, diabetic cardiomyopathy, atherosclerosis, heart failure, and several types of cancer[1,2]. Studies performed in rodent models have added to a growing body of evidence that suggests the presence of resveratrol which is vasculoprotective. Resveratrol has been shown to improve endothelial dysfunction and decrease vascular inflammation, resulting in reduced ischemic damage in vital organs[2-4]. Recently, resveratrol has been identified as having protective effects on endothelial barrier function in the vasculature of the brain and retina[5,6]. However, the detailed molecular mechanisms by which

resveratrol exerts its barrier protective effects remain largely unknown.

Krüppel-like factors (KLFs) are a family of zinc finger-containing transcription factors that have been shown to have diverse regulatory roles in biological processes, such as cell proliferation, differentiation, and survival, organ development, and metabolism[7]. Several published reports by us and others have demonstrated that KLF2 serves as a nodal regulator of endothelial biology. It promotes a healthy vascular phenotype by affecting key aspects of vascular function and disease[8,9]. Importantly, *in vivo* studies from our laboratory have identified KLF2's ability to induce endothelial tight junction factors (e.g., occludin)[10,11] and protect the integrity of the vascular barrier of the brain, thereby also serving as a stroke protective factor[11]. These studies have firmly established the role of KLF2 as a critical positive regulator of vascular endothelial barrier function.

Interestingly, *in vitro* studies have demonstrated that resveratrol induces KLF2 expression in endothelial cells (ECs), implicating KLF2 as a potential regulator of its beneficial vascular effects[12]. However, the physiologic importance of this regulation *in vivo* and its specific role in mediating vascular endothelial barrier function has not been investigated thus far. In this study, we examine resveratrol's dependence on KLF2 for its protective effects on vascular endothelial barrier function.

2. Materials and methods

2.1. Animals

Postnatal KLF2 knockout mice were generated as previously described[11]. KLF2-floxed mice were crossed with CAG-CreERT^2 strain (Jackson Laboratory) to generate KLF2$^{fl/fl}$-CAG-CreERT^2 mice. Postnatal deletion of KLF2 was induced through intraperitoneal (IP) injection of tamoxifen (0.1 mL at 20 mg/mL, dissolved in sunflower seed oil; T5648, Sigma-Aldrich) to 8 week-old male KLF2$^{fl/fl}$-CAG-CreERT^2 mice. Age-matched male CAG-CreERT^2 mice that were subjected to the same tamoxifen regimen were used as controls. All animals were maintained in a clean animal facility. All mouse studies were approved by an Institutional Animal Care and Use Committee (IACUC) at Case Western Reserve University and were conducted in accordance with the National Institutes of Health (NIH) Guide for the Care and Use of Laboratory Animals.

2.2. Resveratrol pretreatment

Male 17–20-week-old KLF2$^{-/-}$ and control mice were treated with 75 mg/kg of resveratrol (Sigma) in a saline solution of 20% hydroxypropyl-β-cyclodextrin (American Maize-Products Company) through daily gastric gavage for 10 days.

2.3. Stereotactic intracerebral injection of tumor necrosis factor (TNF)-α

Male (15 – 20-week-old) control (tamoxifen-treated CAG-CreERT^2 mice) and postnatal KLF2 knockout mice were stereotactically injected with TNF-α (1 μg/kg in 4 μL 1% bovine serum albumin/phosphate-buffered saline (PBS) over 20 – 30 min) into the striatum using the following coordinates from bregma: anteroposterior, 0.0 mm; lateral, 1.5 mm; and ventral, 2.1 mm.

2.4. *In vitro* cell culture studies and western blotting

Primary human brain microvascular ECs were purchased from cell systems and cultured in endothelial basal medium-2 (EBM-2) that was supplemented with growth factors. Resveratrol was obtained from Sigma. Polyclonal rabbit anti-KLF2 antibody was provided as a gift by Huck-Hui Ng (Singapore). Mouse anti-β-actin antibody was obtained from Santa Cruz Biotechnology. All small interfering RNA (siRNA) oligonucleotides were obtained from Dharmacon. Transfection of siRNA into endothelial cells was carried out as described[10,11]. Protein isolation and western blot analysis using the indicated antibodies were performed as previously described[10].

2.5. Quantitative real-time polymerase chain reaction (RT-PCR)

TRIzol reagent (Invitrogen) was used to extract the total RNA from primary human brain microvascular endothelial cells following the manufacturer's instructions. Two micrograms of total RNA were used for reverse transcription to generate complementary deoxyribonucleic acid (cDNA) using iScript Reverse Transcription kit (Bio-Rad). RT-PCR was performed with Universal SYBR Green PCR Master Mix on Applied Biosystems Step One Real-Time PCR system. Gene expression was normalized to glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) using the ΔΔCt method.

2.6. *In vitro* endothelial permeability assay

In vitro endothelial permeability analysis was performed using a transwell assay as described in our prior publication[11]. To achieve oxygen-glucose deprivation (OGD), cultured endothelial cells were exposed to deoxygenated PBS in a modular incubator chamber flushed with 1% oxygen (O_2), 5% carbon dioxide (CO_2), and 94% nitrogen (N_2), sealed, and placed at 37°C for 30 min, followed by a 2-h exposure to normal culture media under normoxic conditions (21% O_2, 5% CO_2) at 37°C. Immediately on re-exposure to normal media and

normoxia, fluorescein isothiocyanate (FITC)-dextran was added to the transwell insert. Aliquots of medium collected from the bottom chamber and the fluorescence density of samples were analyzed on a microplate fluorometer.

2.7. *In vivo* permeability assay

Following stereotactic injection of TNF-α, tail vein injection of 2% Evans blue dye (EBD; 4 mL/kg) was performed. 2 h later, the mice were euthanized and subjected to saline perfusion, followed by brain tissue removal, weighing, and homogenizing in 50% trichloroacetic acid (TCA). Following EBD extraction, the concentration (ng EBD/mg brain) was determined by fluorescence intensity (excitation 620 nm and emission 680 nm) based on an EBD standard curve.

2.8. Statistical analysis

Data were expressed as mean ± standard error of the mean. One-way analysis of variance (ANOVA) was used to compare the differences across 3 or more levels within 1 variable. When comparing a single variable in multiple groups, one-way ANOVA (followed by Dunnett's multiple comparisons test) was performed. Two-way ANOVA (followed by Dunnett's *post hoc* test) was used for two-factor analysis. Statistical analyses were performed using Prism 9.0 software. $P < 0.05$ was considered statistically significant.

3. Results

3.1. Resveratrol induces KLF2 in microvascular endothelial cells

Previous data have established that resveratrol is able to induce KLF2 expression in human umbilical vein ECs in both time- and dose-dependent manners[12]. We demonstrate similar findings in human primary brain microvascular ECs. The peak induction of *KLF2* mRNA was observed at 8 h after resveratrol treatment (Figure 1A), while all times tested showed significant increase over baseline conditions (0 h). Peak *KLF2* mRNA expression was observed at a concentration of 100 μM, with lower doses also showing upregulation (Figure 1B). Complementary to our mRNA data, resveratrol also induced KLF protein expression in a dose-dependent manner as confirmed by western blot analysis (Figure 1C). Taken together, these data show that resveratrol is a potent activator of KLF2 expression within microvascular ECs.

3.2. Resveratrol's induction of key endothelial tight junction factors is KLF2 dependent

We have recently identified KLF2 to be an important regulator of vascular endothelial barrier function through the upregulation of several key endothelial tight junction factors, including occludin, claudin 12, junctional adhesion molecule 1 (JAM-1), and AF-6/afadin[11]. To determine if resveratrol can induce some or all of these factors and whether the induction is KLF2 dependent, the ability of resveratrol to induce a panel of tight junction factors was assessed in the presence and absence of KLF2. Human primary brain microvascular ECs were transfected with *KLF2* siRNA (siKLF2) or control siRNA (non-specific, NS), followed by resveratrol treatment. Resveratrol specifically induced the expression of occludin (Figure 2B), AF-6 (Figure 2C), and JAM-1 (Figure 2D). No significant changes in the mRNA levels of claudin 3, claudin 5, and zona occludens protein 1 (ZO-1) were observed (data not shown). Following siRNA-mediated KLF2 knockdown, resveratrol's induction of KLF2 expression was significantly inhibited (Figure 2A), and the induction of occludin, AF-6, and JAM-1 was abrogated (Figure 2B–D). These findings show that KLF2 is necessary for resveratrol's induction of several key tight junction factors and suggests that an additional mechanism by which resveratrol exerts its vasculoprotective effects may be through the regulation of endothelial barrier function.

3.3. Resveratrol's protection of endothelial barrier function is KLF2 dependent

To examine the functional impact of resveratrol on endothelial barrier *in vitro*, transwell assays were performed in human primary brain microvascular ECs. The cells were plated onto a transwell, treated with 100 μM of resveratrol for 16 h, and subjected to OGD. The permeability of FITC-dextran was, then, measured to assess barrier integrity. Under these conditions, resveratrol protects against OGD-mediated endothelial barrier disruption with a significant decrease observed in fluorescence intensity when compared to control (Figure 3A). To determine if resveratrol's endothelial barrier protective effects are KLF2 dependent, transwell experiments with OGD were conducted in ECs following siRNA-mediated KLF2 knockdown. Indeed, the protective effects of resveratrol were significantly abrogated in the absence of KLF2 (Figure 3B). When the control siRNA was transfected into microvascular ECs, resveratrol treatment showed decreased FITC-dextran fluorescence under OGD conditions (comparing siControl + vehicle [OGD] versus siControl + resveratrol [OGD]) (Figure 3B). When siRNA specific for KLF was introduced, this effect was eliminated (comparing siKLF2 + vehicle [OGD] versus siKLF2 + resveratrol [OGD]). To determine the physiologic relevance of our findings *in vivo*, EBD incorporation assays were performed on both control and KLF2-deficient mice. Inflammatory cytokine TNFα was stereotactically injected into the striatum of both control and KLF2 knockout mice,

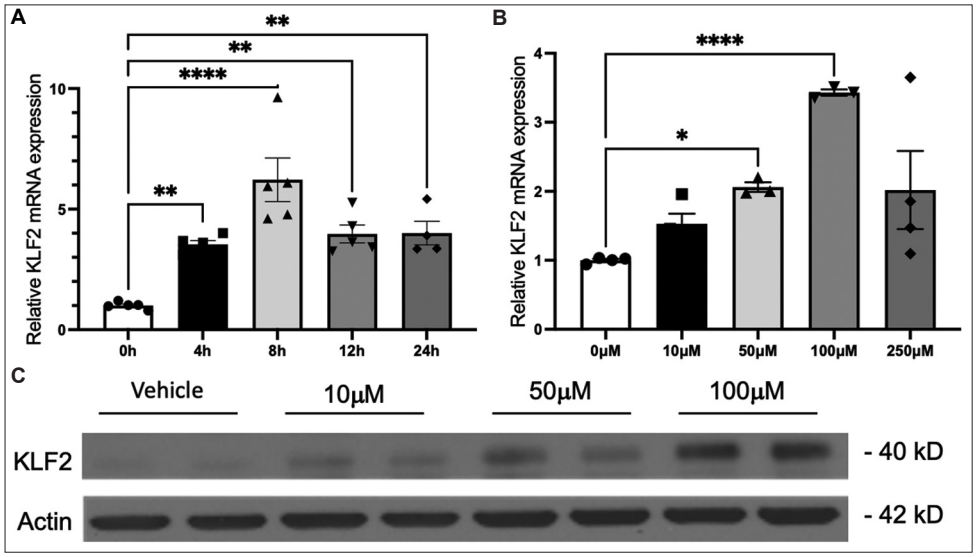

Figure 1. Resveratrol induces Krüppel-like factor 2 (KLF2) expression in human primary brain microvascular endothelial cells (ECs). KLF2 mRNA expression in human primary brain microvascular ECs treated with resveratrol (A) (100 μM dose) at different time intervals (n = 3–4), and (B) (8 h) at different concentrations (n = 3–4), *$P < 0.05$, **$P < 0.005$, ****$P < 0.0001$. (C) Elevation of KLF2 protein in human primary brain microvascular ECs treated with resveratrol (12 h) at different concentrations.

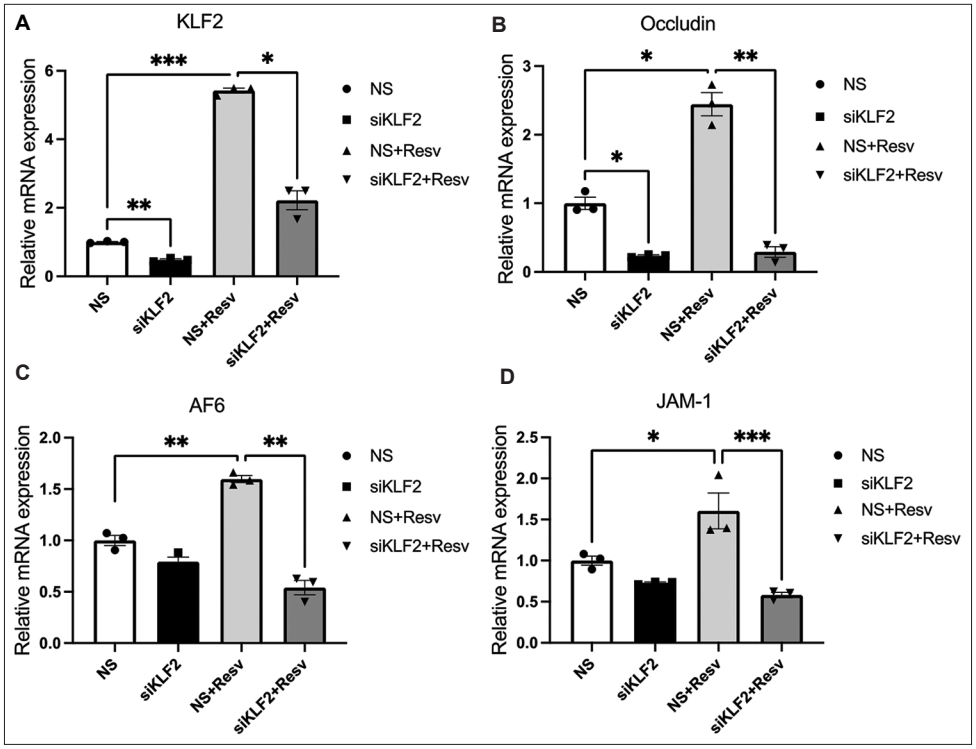

Figure 2. Resveratrol-mediated induction of several key tight junction factors is Krüppel-like factor 2 (KLF2) dependent. Quantitative real-time polymerase chain reaction (RT-PCR) analysis of (A) KLF2, (B) occludin, (C) afadin (AF6), and (D) junctional adhesion molecule 1 (JAM-1) in human primary brain microvascular ECs transfected with small interfering ribonucleic acid (siRNA, siControl, or siKLF2) and treated with resveratrol (100 μM for 8 h). NS: Non-specific siRNA, Resv: Resveratrol. *$P < 0.05$; **$P < 0.005$; ***$P < 0.001$.

followed by tail vein injection of EBD. Brain endothelial barrier function was assessed by the quantification of EBD extracted from the harvested brains. The diminished incorporation of EBD into the brain tissue from control mice verifies the endothelial barrier protective effects of resveratrol *in vivo*. In KLF2-deficient mice, there was a

higher rate of EBD incorporation into the tissue, suggesting an overall increase in barrier dysfunction with resveratrol's protective effect being completely abolished (Figure 4). These findings clearly identify KLF2 as a necessary downstream mediator of resveratrol and an important regulator of its vasculoprotective effects.

4. Discussion

The blood–brain barrier (BBB) is a critical structural and biochemical barrier composed of the endothelium and the surrounding extracellular matrix. The properties of the BBB are primarily due to junctional complexes within the endothelium. These complexes are categorized as either tight junctions, which seal the endothelium and limit paracellular diffusion, or adherens junctions, which regulate EC cell-cell contacts, cytoskeletal association, and intracellular signaling[13]. To maintain cerebral homeostasis and prevent blood-borne molecules from entering the brain, it is essential to maintain a strict separation between the blood and extravascular compartments[14]. Proteins that contribute to the endothelial barrier function (i.e., claudins, occludin, and JAMs) are critical in the maintenance of BBB integrity. Hence, the disruption of these proteins contributes to a broad spectrum of disease

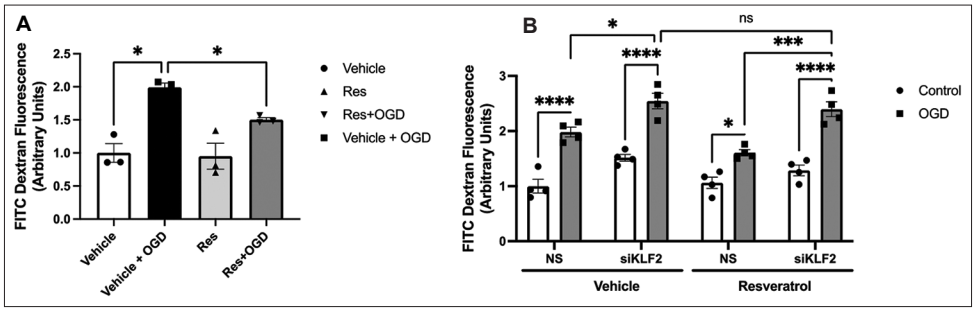

Figure 3. Resveratrol has endothelial barrier protective effects, with this barrier protective function being Krüppel-like factor 2 dependent. (A) *In vitro* permeability analysis quantifying passage of fluorescein isothiocyanate (FITC)-dextran across primary human brain microvascular ECs pre-treated with resveratrol (100 μM for 16 h) or vehicle at baseline (Control) and after 30 min oxygen-glucose deprivation, followed by 2 h of normal media (n = 3/group). (B) Permeability assessment as in (A) using cells transfected with small interfering ribonucleic acid (siRNA, si-NS or siKLF2) (n = 4 per group). NS: Non-specific siRNA. $^{*}P < 0.05$; $^{***}P < 0.001$; $^{****}P < 0.0001$.

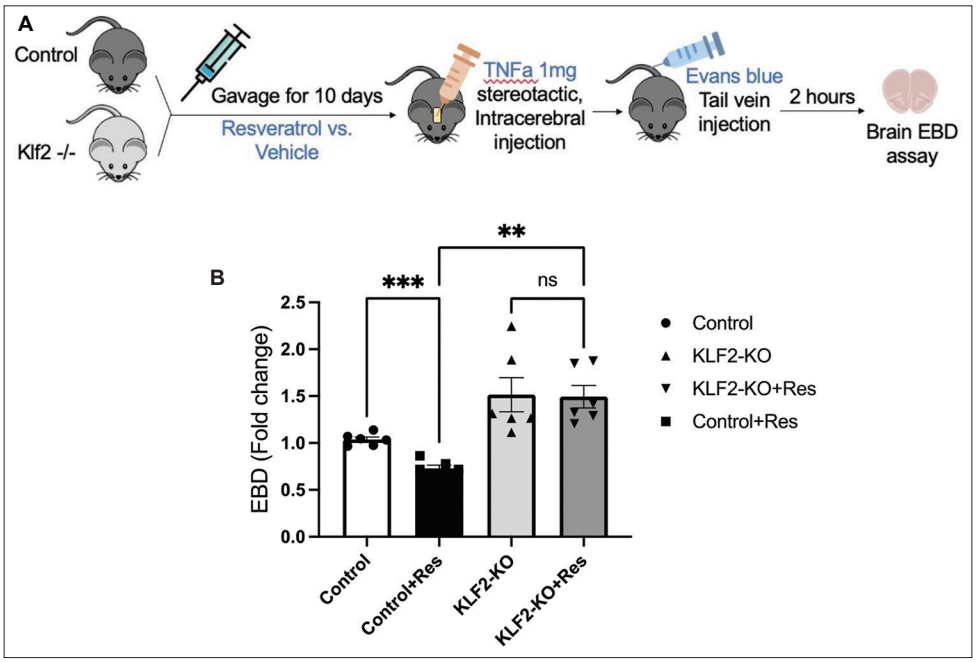

Figure 4. Blood–brain barrier (BBB) protection effect of resveratrol is Krüppel-like factor 2 (KLF2) dependent. (A) Schematic diagram for *in vivo* BBB studies. (B) Quantification of Evans blue dye permeability of brains harvested from control and KLF2 knockout mice after pretreatment with vehicle or resveratrol (75 mg/kg) through gastric gavage for 10 days (n = 6/group). Control: CAG-CreERT^2, KLF2-/-: postnatal KLF2 knockout, Res: Resveratrol. $^{**}P < 0.005$; $^{***}P < 0.001$.

states[15,16]. Therefore, the discovery of compounds that can help maintain or improve the endothelial barrier function is of significant interest and may be helpful in the treatment of diseases such as Alzheimer's or Parkinson's, where BBB impairment can contribute to worsening neurodegenerative states.

Resveratrol is a naturally occurring vasculoprotective compound. Recently, studies have identified additional beneficial aspects and begun to outline the molecular mechanisms of how resveratrol impacts endothelial function[10]. In the present study, we are the first to identify KLF2 as an obligatory factor for resveratrol's induction of key tight junction factors and subsequent endothelial barrier protective effects. KLF2 is a highly expressed transcription factor within ECs. The previous studies have demonstrated that KLF2 critically regulates key aspects of vascular function and disease, including vascular permeability, EC thrombotic function, and angiogenesis. Importantly, in ECs, KLF2 has also been demonstrated to be critical for resveratrol's induction of endothelial nitric oxide synthase (eNOS) and thrombomodulin (TM), both of which are known to play critical roles in inflammation, vasoreactivity, and thrombosis[12]. Taken together, these findings implicate KLF2 as a central regulator of resveratrol's beneficial effects in ECs.

In addition to promoting vascular health through EC signaling, resveratrol's mechanisms of action have been shown to involve other cell types[1]. One limitation of our studies is that they have been performed using global KLF2-deficient mice; therefore, we cannot rule out the contributing factors from non-EC sources. For that reason, since KLF2 is also expressed in cells of the myeloid lineage, future studies in mice with EC-specific KLF2 deficiency are warranted to further pinpoint the importance of resveratrol's regulation of KLF2 in ECs.

Finally, resveratrol has been proposed to be beneficial in vascular diseases, such as atherosclerosis and ischemic stroke[1-3,5]. Similarly, we have demonstrated KLF2 to be a protective factor against atherosclerosis and stroke[11,17]. Studies treating KLF2-deficient mice with resveratrol in these disease models will, further, define its mechanistic pathway of action and how it, or other similar compounds, can be used as an inexpensive and natural way to help treat and/or alleviate symptoms associated with vascular, cerebral, or neurodegenerative disorders.

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None.

Conflict of interest

The authors have declared that no conflicts of interest exist.

Author contributions

Conceptualization: Hong Shi
Formal analysis: Xianming Zhou, Lily Lin, Hong Shi
Investigation: Xianming Zhou, Hong Shi
Writing – original draft: Xianming Zhou, Lily Lin, Hong Shi
Writing – review & editing: Lily Lin, Hong Shi

Ethics approval and consent to participate

All mouse studies were approved by an Institutional Animal Care and Use Committee (IACUC) at Case Western Reserve University (2011-0136) and were conducted in accordance with the National Institutes of Health (NIH) Guide for the Care and Use of Laboratory Animals.

Consent for publication

Not applicable.

Availability of data

Data will be obtained from the corresponding author on reasonable request.

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