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KLF11 mediates PPARγ cerebrovascular protection in ischaemic stroke

Ke-Jie Yin,[1],* Yanbo Fan,[1],* Milton Hamblin,[1] Jifeng Zhang,[1] Tainqing Zhu,[1] Siming Li,[2] John R. Hawse,[3] Malayannan Subramaniam,[3] Chao-Zhong Song,[4] Raul Urrutia,[5] Jiandie D. Lin[2] and Y. Eugene Chen[1]

1 Cardiovascular Centre, Department of Internal Medicine, University of Michigan Medical Centre, Ann Arbor, MI 48109, USA
2 Life Sciences Institute and Department of Cell and Developmental Biology, University of Michigan Medical Centre, Ann Arbor, MI 48109, USA
3 Department of Biochemistry and Molecular Biology, Mayo Clinic, Rochester, MN 55905, USA
4 Department of Medicine/Haematology, Institute for Stem Cell and Regenerative Medicine, University of Washington, Seattle, WA 98195, USA
5 Division of Gastroenterology and Hepatology, Mayo Clinic College of Medicine, Rochester, MN 55901, USA

*These authors contributed equally to this work.

Correspondence to: Y. Eugene Chen, MD, PhD,
Cardiovascular Centre,
Department of Internal Medicine,
University of Michigan Medical Centre,
NCRC Bld 26, Rm 361S,
2800 Plymouth Rd, Ann Arbor,
MI 48109,
USA
E-mail: echenum@umich.edu

Correspondence may also be addressed to: Ke-Jie Yin, MD, PhD. E-mail: kejie@umich.edu

Peroxisome proliferator-activated receptor gamma (PPARγ) is emerging as a major regulator in neurological diseases. However, the role of (PPARγ) and its co-regulators in cerebrovascular endothelial dysfunction after stroke is unclear. Here, we have demonstrated that (PPARγ) activation by pioglitazone significantly inhibited both oxygen–glucose deprivation-induced cerebral vascular endothelial cell death and middle cerebral artery occlusion-triggered cerebrovascular damage. Consistent with this finding, selective (PPARγ) genetic deletion in vascular endothelial cells resulted in increased cerebrovascular permeability and brain infarction in mice after focal ischaemia. Moreover, we screened for (PPARγ) co-regulators using a genome-wide and high-throughput co-activation system and revealed KLF11 as a novel (PPARγ) co-regulator, which interacted with (PPARγ) and regulated its function in mouse cerebral vascular endothelial cell cultures. Interestingly, KLF11 was also found as a direct transcriptional target of (PPARγ). Furthermore, KLF11 genetic deficiency effectively abolished pioglitazone cytoprotection in mouse cerebral vascular endothelial cell cultures after oxygen–glucose deprivation, as well as pioglitazone-mediated cerebro-vascular protection in a mouse middle cerebral artery occlusion model. Mechanistically, we demonstrated that KLF11 enhanced (PPARγ) transcriptional suppression of the pro-apoptotic microRNA-15a (*miR-15a*) gene, resulting in endothelial protection in cerebral vascular endothelial cell cultures and cerebral microvasculature after ischaemic stimuli. Taken together, our data demonstrate that recruitment of KLF11 as a novel (PPARγ) co-regulator plays a critical role in the cerebrovascular protection after ischaemic insults. It is anticipated that elucidating the coordinated actions of KLF11 and (PPARγ) will provide new insights into understanding the molecular mechanisms underlying (PPARγ) function in the cerebral vasculature and help to develop a novel therapeutic strategy for the treatment of stroke.

Introduction

Stroke is one of the leading causes of death and disability in the USA. However, the development of effective therapies for stroke has been limited (Stapf and Mohr, 2002; Moskowitz et al., 2010). PPARγ is a ligand-activated nuclear transcription factor that functions to improve metabolic syndrome and neural activity through regulation of lipid and glucose metabolism and facilitation of anti-inflammatory signalling. To date, thiazolidinediones (thiazolidinedione, rosiglitazone and pioglitazone), a class of synthetic PPARγ ligands, show efficacy in treating type 2 diabetes clinically (Yki-Jarvinen, 2004), and they appear to reduce brain injury following stroke in rodent models (Sundararajan et al., 2005; Luo et al., 2006; Tureyen et al., 2007; Wilcox et al., 2007; Zhao et al., 2009). However, the therapeutic efficacy and potential of thiazolidinediones have been compromised because of increased risk of peripheral oedema, weight gain and hepatotoxicity (Yki-Jarvinen, 2004), as well as increased cardiovascular events by rosiglitazone treatment (Nissen and Wolski, 2007). Hence, uncovering the molecular mechanisms underlying PPARγ function in depth is essential and will lead to the development of novel and better PPARγ drugs with greater efficacy and reduced side effects.

Generally, PPARγ exerts its effects on gene expression by recruiting co-activators or co-repressors to the transcriptional complex of a gene, leading to transcriptional activation or repression. Thus, PPARγ-triggered effects are largely dependent on the specificity and activity of recruited co-regulators (Powell et al., 2007; Ricote and Glass, 2007; Yu and Reddy, 2007). To date, several co-regulators have been found to mediate the effects of PPARγ in energy metabolism and cardiovascular function (Qi et al., 2003; Koppen et al., 2009; Leidi et al., 2009).

The Kruppel-like factor family, Sp1-like zinc finger transcription factors, consists of 17 members to date (Black et al., 2001; Kaczynski et al., 2003). Recent studies have implicated several Kruppel-like factor family members (KLF2, KLF4, KLF5 and KLF6) in developmental and pathophysiological vascular processes (Suzuki et al., 2005). KLF11 is a member of the Kruppel-like factor family with high expression in various human tissues, including brain vasculature. Accumulating evidence has shown that KLF11 is involved in the regulation of cholesterol metabolism, cell growth and differentiation, and cell death and may have potent effects in diabetes (Neve et al., 2005; Suzuki et al., 2005; Gohla et al., 2008; Fernandez-Zapico et al., 2009). Of importance, emerging data suggest that KLF11 gene single-nucleotide polymorphisms are associated with type 2 diabetes (Neve et al., 2005; Fernandez-Zapico et al., 2009). Indeed, maturity onset diabetes of the young 7, an early-onset type 2 diabetes mellitus, is caused by mutations in the KLF11 gene (Fernandez-Zapico et al., 2009). Up to now, the role of KLF11 in cerebral vascular biology is undetermined.

In this study we define a synergistic role of KLF11 as a novel PPARγ co-regulator in ischaemic cerebral vascular injury. We expect that these results will set the basis for future investigations regarding the critical role of PPARγ co-regulators in the pathogenesis of stroke and eventually reveal KLF11 and other PPARγ co-regulators as novel pharmacological targets for modulating PPARγ signalling pathways in the treatment of stroke.

Materials and methods

Experimental mice

In this study, vascular endothelial cell-selective PPARγ conditional knockout (EC-Pγ cKO) mice were generated by breeding vascular endothelial-cadherin (cdh5)-Cre transgenic mice (Jackson laboratory, cdh5$^{Cre/+}$, Cat#: 006137) with PPARγ-floxed homozygous mice (PPARγ$^{flox/flox}$) (Chang et al., 2009). Two groups of mice were selected for this study: (i) EC-Pγ cKO mice with a genotype of cdh5-Cre-transgenic and PPARγ-floxed homozygous (cdh5$^{Cre/+}$/PPARγ$^{flox/flox}$, Fig. 2); and (ii) littermate control mice with a genotype of cdh5 wild-type and PPARγ-floxed homozygous (cdh5$^{+/+}$/PPARγ$^{flox/flox}$, PPARγ-floxed control, Fig. 2). KLF11 knockout mice (Song et al., 2005) were kindly provided by Dr Song at the University of Washington. Generally, EC-Pγ cKO and KLF11 knockout mice are viable and fertile with normal appearance, behaviour, growth and litter size. The animal study protocol was approved by the University of Michigan Animal Care and Use Committee.

Mouse model of transient focal cerebral ischaemia

Focal cerebral ischaemia was induced in mice by intraluminal middle cerebral artery occlusion as described previously (Yin et al., 2010a, b, 2011). Briefly, mice were anaesthetized with ketamine and xylazine. A 2-cm length of a 6-0 rounded tip nylon suture was gently advanced from the external carotid artery up to the internal carotid artery until regional cerebral blood flow was reduced to <16% of baseline. After 30 min of middle cerebral occlusion, blood flow was restored by removing the suture, and the mice were allowed to recover for 24 h. Changes in cerebral blood flow, arterial blood gases, mean arterial pressure and heart rate were monitored in animals 30 min before, during and 30 min after middle cerebral artery occlusion. The rectal temperature was controlled at $37.0 \pm 0.5°C$ during surgery.

Measurement of infarct volume and neurological deficit

Infarct area was measured from the brain slices using 2% 2,3,5-triphenyltetrazolium chloride staining. The infarct volume was calculated using a derived formula (Swanson et al., 1990). Following cerebral ischaemia, mice were also tested for neurological deficits and scored on a 5-point scale (Yin et al., 2010b, 2011).

Quantitation of Evans blue extravasation

Mice were injected with 100 μl of 4% Evans blue via tail vein 23 h after middle cerebral artery occlusion (Hamer *et al.*, 2002; Manaenko *et al.*, 2011). One hour later, animals were perfused with PBS, and the brains were removed. Each hemisphere was then homogenized in *N*, *N*-dimethylformamide and centrifuged at 25 000 *g* for 45 min. The supernatants were collected, and quantitation of Evans blue extravasation in each hemisphere was determined from the formula: $\{A_{620}$ nm $- [(A_{500}$ nm $+ A_{740}$ nm$)/2]\}$/mg wet weight. Background Evans blue levels in the non-ischaemic hemisphere were subtracted from the ischaemic hemisphere ipsilateral to the middle cerebral artery occlusion (Yin *et al.*, 2010*a*, 2011).

Isolation of cerebral vessels

The mouse brains were removed, and cerebral microvessels were isolated using a previously described method (Yin *et al.*, 2006). The final vessel pellet was stored at $-80°C$ until various biochemical assays were performed.

Cell cultures

Cerebral cortex from KLF11 knockout or wild-type mice was homogenized, filtered and sequentially digested with collagenase B, then collagenase/dispase, followed by centrifugation in 40% Percoll solution. The second band containing microvessels was collected and plated onto collagen-coated dishes. Mouse cerebral vascular endothelial cells (4–15 passages) were grown to 85–95% confluency before use (Yin *et al.*, 2002*a*).

Oxygen–glucose deprivation

To mimic ischaemia-like conditions *in vitro*, mouse cerebral vascular endothelial cell cultures were exposed to oxygen–glucose deprivation for various times (Yin *et al.*, 2002*a*). In some experiments, cerebral vascular endothelial cells were treated with pioglitazone (0–10 μM) overnight in the presence or absence of infection with an adenovirus carrying PPARγ, small-hairpin PPAR, the KLF11 gene or green fluorescent protein (GFP) before oxygen–glucose deprivation exposure.

Assessment of cell death, DNA fragmentation and caspase 3 activity

The extent of mouse cerebral vascular endothelial cell death was assessed by MTT (3-(4,5-dimethylthiazol-2-yl)-2, 5-diphenyl-tetrazolium bromide) and lactate dehydrogenase assays (Yin *et al.*, 2002*a*). Genomic DNA was extracted from isolated cerebral microvessels, and DNA fragmentation was measured by using a commercial apoptotic DNA-ladder kit (Roche). Caspase 3 activity in isolated cerebral microvessels was detected by a commercial Caspase-3 Colorimetric Activity Assay Kit (Millipore).

Real-time polymerase chain reaction

A quantitative real-time reverse transcriptase–PCR was carried out with a Bio-Rad thermocycler and a SYBR® green kit (Bio-Rad). Specific primers used for the reaction are as follows: KLF11 forward, 5′-gggga-gaagcctttacctg-3′, KLF11 reverse, 5′-agcctgggatcttcttggtt-3′; CD36 (Cluster of Differentiation 36) forward, 5′-ggaaactgctatcatgactggg-3′, CD36 reverse, 5′-ttgctgtgtagccaagaactcca-3′. The relative messenger RNA expression was normalized by 18S RNA levels (Yin *et al.*, 2011).

TaqMan® microRNA assay for identification of microRNA-15a

Total RNA was isolated from treated mouse cerebral vascular endothelial cell cultures or isolated cerebral vessels by using a miRNeasy Mini Kit (Qiagen). Reverse transcription was performed using the specific TaqMan® MicroRNA Reverse Transcription Kit (Applied Biosystems). PCR reactions were then conducted using the TaqMan® MicroRNA Assay Kit (Applied Biosystems). The relative microRNA levels were normalized to endogenous *SnoRNA 202* expression for each sample (Yin *et al.*, 2010*a*).

Western blot

Samples from the cerebral cortex, cerebral vessels or cerebral vascular endothelial cell cultures were homogenized in lysis buffers, and total protein was isolated as described previously (Wu *et al.*, 2009). Equal amounts of protein were electrophoresed onto a 10–15% SDS–PAGE and transferred to polyvinyldifluoride membranes. The membranes were blocked in 5% non-fat milk in Tris-buffered saline, then incubated with various primary antibodies, including PPARγ (1:200; Santa Cruz) and actin (1:500; Santa Cruz), for 1–2 h at room temperature. After incubation with secondary antibodies conjugated with alkaline phosphatase (Promega) at room temperature for 1 h, the colour reaction was developed by the ProtoBlot® AP System (Promega) according the manufacturer's instructions.

Co-immunoprecipitation

Cerebral vascular endothelial cell cultures were lysed in lysis buffer (Roche Applied Science) and centrifuged at 12 000*g* for 15 min at 4°C. The supernatants were pre-cleared with protein G plus agarose for 1 h at 4°C, and then incubated with an anti-KLF11 (Novus) or an anti-PPARγ (Santa Cruz) polyclonal antibody overnight at 4°C. Normal IgG was used for a negative control. The immunocomplexes were pulled down by incubation with protein G plus agarose for 1 h at 4°C and washed four times with wash buffer. The samples were separated by SDS–PAGE and analysed by immunoblotting using an anti-PPARγ antibody (1:200; Santa Cruz) or an anti-KLF11 antibody (1:500; Novus) (Yin *et al.*, 2002*b*).

Adenovirus-mediated gain or loss of PPARγ or KLF11 function in cerebral vascular endothelial cells

We used a previously described method that facilitates adenoviral vector construction (He *et al.*, 1998). Briefly, the expressed sequence of the mouse *Pparg* or *Klf11* gene was amplified and then cloned into a pCMVTrack vector. For the generation of an adenovirus carrying the small hairpin PPARγ gene, the suppressing sequence targeting the mouse *Pparg* gene or a non-effective 29mer small hairpin GFP cassette was cloned into the same vector. These plasmids were then co-transformed with the AdEasy™ vector into *Escherichia coli*. The clones from *in vivo* recombination were isolated, digested and used for transfection in HEK293 cells. Transfected cells were collected 7–10 days later, and viruses were purified by a CsCl gradient. The generated adenovirus was used to infect cerebral vascular endothelial cells for

48–72 h, and comparisons were made using an adenovirus carrying the GFP gene (Yin *et al.*, 2010*a*).

Luciferase reporter assays

Cerebral vascular endothelial cell cultures were transiently co-transfected using Lipofectamine® 2000 with a plasmid containing the luciferase gene under the control of three tandem PPAR response elements (PPRE) (PPRE $\times$ 3 TK-luciferase) as previously described (Schopfer *et al.*, 2005) or pGL 4.10 luciferase vectors carrying a 1.9-kb microRNA-15a (*miR-15a*) promoter fragment containing either wild-type or mutant PPRE binding sites at a location of $-1593/-1573$ bp (Yin *et al.*, 2010*a*). The pRL-TK vector was used as an internal control for transfection. After transfection, cells were cultured for 4 h in Opti-MEM® I and then treated with an adenovirus carrying PPARγ, KLF11 or GFP for an additional 48 h. A dual luciferase assay kit from Promega was used to measure the luciferase activity of cells with a luminometer (Victor II; Perkin-Elmer).

Genome-wide screening for PPARγ co-regulators

To screen for novel PPARγ co-regulators, a genome-wide co-activation system, which covered ~48% of all predicted transcriptional factors and co-regulators in the human genome, was used in the current study as described previously (Li *et al.*, 2008). These 1146 transcription factor open reading frames were fused to the Gal4-DBD (DNA-binding domain) vector using the Gateway cloning system to generate Gal4–transcription factor plasmids for co-activational studies (Li *et al.*, 2008). For co-activation assays, AD293 cells seeded in 96-well plates were transiently transfected with a UAS–luciferase reporter (10 ng) and individual Gal4–transcription factor plasmids (10–20 ng) in the presence or absence of pcDNA3.1 Flag-PPARγ (100 ng) using Lipofectamine® 2000 Reagent. Luciferase activity was measured 48 h after transfection.

Statistical analysis

Quantitative data are expressed as mean $\pm$ standard deviation (SD) or standard error of the mean (SEM) based on at least three independent experiments of triplicate samples. Differences among three or more groups were statistically analysed by one-way ANOVA followed by Bonferroni's *post hoc* test. Comparisons between two experimental groups were based on a two-tailed *t*-test. A *P*-value < 0.05 was considered significant.

Results

## PPARγ activation reduces ischaemia-induced cerebral vascular injury *in vitro* and *in vivo*

Although thiazolidinediones have been reported to protect the neuron from ischaemic insults (Tureyen *et al.*, 2007; Wilcox *et al.*, 2007), the role of thiazolidinediones in the cerebral vasculature after ischaemia is largely unexplored. To identify this, we first explored the effects of the PPARγ agonist, pioglitazone, on oxygen–glucose deprivation-induced cerebral vascular endothelial cell death and investigated their potential link to the PPARγ receptor by manipulating PPARγ gene expression through adenoviral gene transfer. As shown in Fig. 1, activation of PPARγ by pioglitazone significantly reduced oxygen–glucose deprivation-induced cerebral vascular endothelial cell death in a dose-dependent manner, as evidenced by MTT assays (Fig. 1A) and lactate dehydrogenase quantitation (Fig. 1B). Gain of PPARγ function by infection of an adenovirus carrying PPARγ in cerebral vascular endothelial cells potentiated pioglitazone-mediated cytoprotection after 16 h of oxygen–glucose deprivation exposure, whereas loss of PPARγ function by adenovirus-mediated PPARγ RNA interference reversed this effect (Fig. 1A and B). Of note, adenoviral GFP gene transfer into cerebral vascular endothelial cells had no effect on oxygen–glucose deprivation-induced cell death, whereas adenoviral gain or loss of PPARγ function itself statistically reduces or increases oxygen–glucose deprivation-induced cerebral vascular endothelial cell death, respectively (Fig. 1A and B). As expected, the success of the adenovirus-mediated gain or loss of PPARγ function approach is documented by the significantly increased or reduced PPARγ protein levels (Supplementary Fig. 1A) and expression of CD36, a PPARγ target gene (Supplementary Fig. 1B). These findings clearly demonstrate that pioglitazone-mediated cytoprotection after oxygen–glucose deprivation is PPARγ-dependent. Moreover, the essential role of pioglitazone in vasoprotection against cerebral ischaemia was further evaluated in mice by administering pioglitazone immediately after 1 h middle cerebral artery occlusion and quantitatively measuring Evans blue extravasation 24 h later. As shown in Fig. 1C, pioglitazone significantly attenuated ischaemia-triggered increases in cerebrovascular permeability. Taken together, our data demonstrate that PPARγ activation by pioglitazone plays a vascular protective role in ischaemic stroke.

Endothelial cell-selective PPARγ deletion potentiates blood–brain barrier disruption and brain injury in mice after focal cerebral ischaemia

To investigate the role of PPARγ in the endothelium, we generated EC-Pγ cKO mice (Fig. 2) by crossing PPAR$\gamma^{\text{flox/flox}}$ mice with cdh5-Cre transgenic mice (Alva *et al.*, 2006). The western blot, as shown in Fig. 2C, confirms that PPARγ protein expression was completely absent in the cerebral microvessels of EC-Pγ cKO mice. It is noteworthy that no differences in systolic blood pressure (Fig. 2D) and heart rate (Fig. 2E) were observed between mice with endothelial cell-selective PPARγ gene deficiency and their littermate wild-type control mice.

To determine the role of endothelial cell-specific PPARγ function in ischaemic brain injury, EC-Pγ cKO and littermate control mice were subjected to transient middle cerebral artery occlusion for 30 min followed by 24 h reperfusion ($n = 8$–10). Cerebral infarction, neurological outcomes and cerebral vascular permeability were determined by 2% 2,3,5-triphenyltetrazolium chloride staining and Evans blue extravasation, respectively. In comparison with the littermate control mice, EC-Pγ cKO mice showed a larger cerebral infarct volume (Fig. 3A and B) and a significantly more severe neurological deficit (Fig. 3C) in response to ischaemic insults. Of significance, endothelial cell-selective PPARγ deletion
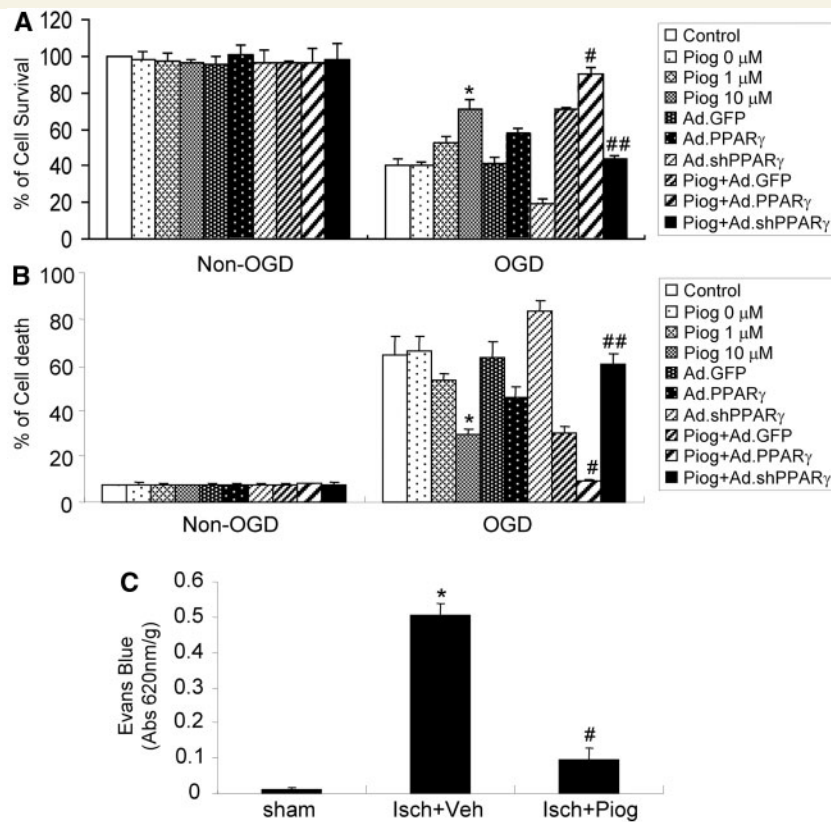
Figure 1 Pioglitazone provides vasoprotection *in vitro* and *in vivo*. (**A** and **B**) Mouse cerebral vascular endothelial cells were treated with pioglitazone (Piog) at different doses with or without infection of an adenovirus carrying PPARγ, small-hairpin PPARγ (shPPARγ) or GFP for 48 h, and then subjected to 16 h oxygen–glucose deprivation (OGD) exposure. Cell viability was determined by MTT assays (**A**) and the lactate dehydrogenase method (**B**). Pioglitazone significantly reduced oxygen–glucose deprivation-induced cerebral vascular endothelial cell death in a dose-dependent manner. Gain of PPARγ function by infection of an adenovirus (Ad) carrying PPARγ in cerebral vascular endothelial cells potentiated pioglitazone-mediated cytoprotection after 16 h of oxygen–glucose deprivation exposure, whereas loss of PPARγ function by adenovirus-mediated PPARγ RNA interference reversed this effect. (**C**) Mice were treated with pioglitazone (intraperitoneally 2 mg/kg) at the start of 24 h of middle cerebral artery reperfusion after 1 h of middle cerebral artery occlusion ($n = 6$). Cerebrovascular permeability was determined by Evans blue extravasation. Pioglitazone significantly attenuated ischaemia-triggered increases in cerebrovascular permeability. Data are expressed as mean ± SEM. *$P < 0.05$ versus oxygen–glucose deprivation or Sham group. #$P < 0.05$ versus oxygen–glucose deprivation + pioglitazone + Ad.GFP; ##$P < 0.05$ versus ischemia + vehicle (Isch + Veh) group.

also aggravated ischaemia-induced blood–brain barrier disruption by increasing cerebrovascular permeability (Fig. 3D). However, the cerebral blood flow in EC-Pγ cKO mice had no significant changes at 30 min before, during and after middle cerebral artery occlusion compared with their control mice (Supplementary Fig. 2). Taken together, these results suggest that endothelial PPARγ plays a critical role in the maintenance of vascular structure and function. Loss of PPARγ function in the cerebral vasculature exacerbates ischaemic cerebrovascular and brain damage.

Genome-wide screening for PPARγ co-regulators

Co-regulators are required for nuclear receptor function (Glass and Rosenfeld, 2000; McKenna and O'Malley, 2002). In the current study, we used a genome-wide co-activation system (Fig. 4A) to define the key PPARγ co-regulators contributing to PPARγ-mediated vasoprotection in the cerebral vasculature during

ischaemic stroke (Li *et al.*, 2008). During the initial screening, we identified 12 co-regulators, including MED31, p300, SERTAD1, NFE2L2, RXRalpha, SURB7, IRF6, DR1, FLJ31951, PCBD1, PGC-1 and KLF11. Interestingly, 3 of these 12 cofactors (e.g. PGC-1, RXRalpha and p300) are well-documented PPARγ cofactors (Powell *et al.*, 2007; Ricote and Glass, 2007; Yu and Reddy, 2007), thus validating our approach and suggesting that other cofactors identified in this screening are likely novel cofactors for PPARγ. Among them, KLF11 was selected as a focus in this study, and its transcriptional activity could be augmented by PPARγ >2-fold in these co-activation reporter assays (Fig. 4B).

KLF11 physically binds to PPARγ and enhances its activity in cerebral vascular endothelial cells

To confirm that KLF11 is a PPARγ co-regulator, we performed co-immunoprecipitation studies to identify the physical interaction of
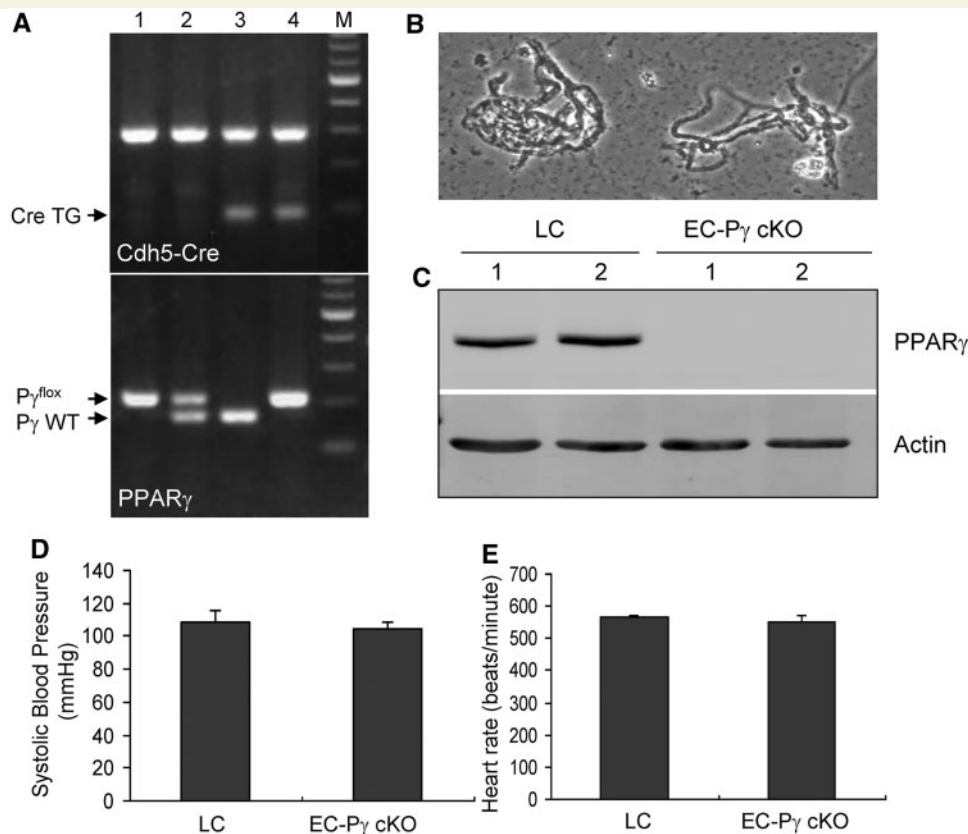
Figure 2 Generation and identification of endothelial cell-selective PPARγ conditional knockout mice. (**A**) Genomic PCR genotyping showed that endothelial cell-selective PPARγ conditional knockout (EC-Pγ cKO, lane 4) mice and littermate control mice (LC, lane 1) were generated by crossing cdh5-Cre transgenic mice (Cre TG) with PPARγ^flox/flox mice. (**B**) Microscopic view of isolated cerebral microvessels. (**C**) Western blot analysis showing PPARγ expression in lysates from cerebral vessels of EC-Pγ cKO and littermate control mice. Cre-mediated recombination in endothelial cells resulted in a lack of PPARγ expression in the cerebral vessels of EC-Pγ cKO mice. There is no statistical significance in blood pressure (**D**) and heart rate (**E**) between EC-Pγ cKO mice and littermate control mice. Results shown are representative of three separate experiments with similar results.

KLF11 with PPARγ. As shown in Fig. 4C, KLF11 strongly binds to PPARγ in mouse cerebral vascular endothelial cells, suggesting a potential regulation of KLF11 on PPARγ function. Moreover, we used a PPRE-luciferase activity assay to determine whether KLF11 functionally enhances PPARγ transcriptional activity. As shown in Fig. 4D, KLF11 does not activate PPRE activity at basal levels, but significantly enhances PPRE activity induced by adenovirus-mediated overexpression of PPARγ in mouse cerebral vascular endothelial cells. Furthermore, activation of PPARγ by its agonist, pioglitazone, significantly elevated KLF11 messenger RNA expression levels (Fig. 4E). These findings document that KLF11 is a functional PPARγ co-regulator and modulates PPARγ activity in the cerebral endothelium.

To further study the mechanisms of pioglitazone activation of KLF11 transcription in mouse cerebral vascular endothelial cell cultures, we performed a functional analysis of potential PPRE sites in the mouse KLF11 promoter. As demonstrated in Supplementary Fig. 3A, we identified two PPRE sites in the promoter of the KLF11 gene at locations of −768/−745 bp (PPRE2) and −271/−248 bp (PPRE1). To determine the functionality of these two PPREs in the KLF11 promoter, we cloned a 914-bp segment from the promoter

region of the mouse KLF11 gene into a luciferase reporter vector, and the transcriptional response of the KLF11 promoter to PPARγ was performed using a luciferase transcriptional assay. Cerebral vascular endothelial cell cultures were transfected with a 914-bp promoter (KLF11 PPRE wild-type) or the corresponding site-directed mutated promoter on the identified PPREs (KLF11 PPRE1 and two mutations). Cells were co-infected with adenoviral PPARγ or adenoviral GFP in the presence or absence of pioglitazone pre-treatment. As shown in Supplementary Fig. 3B, pioglitazone and/or adenoviral overexpression of PPARγ significantly increase KLF11 promoter-driven luciferase reporter activity. Moreover, luciferase activities from the KLF11 promoter driven by the mutated PPRE1, not the mutated PPRE2, did not respond to pioglitazone treatment or PPARγ overexpression, indicating that the predicted PPRE1 site in the promoter region of KLF11 is responsible for PPARγ transactivation of KLF11 expression *in vitro*. These results suggested that activation of PPARγ transactivates KLF11 messenger RNA expression. Consistent with this finding, quantitative PCR data further showed that endothelial cell-selective gene deficiency of PPARγ resulted in a significant reduction of KLF11 messenger RNA levels in the cerebral vasculature at both pre-ischaemic and
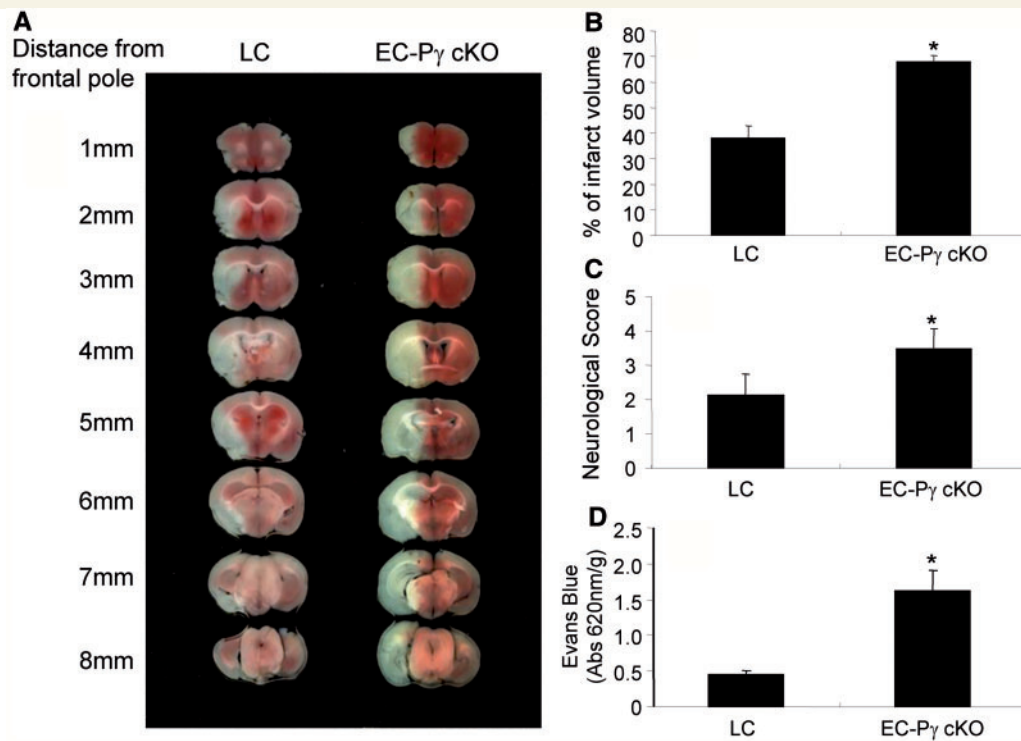
Figure 3 The effect of endothelial cell PPARγ on ischaemia-induced brain infarction and cerebrovascular permeability. Endothelial cell-selective PPARγ conditional knockout (EC-Pγ cKO) and littermate control (LC) mice were subjected to 30 min middle cerebral artery occlusion and 24 h reperfusion. Two per cent 2,3,5-triphenyltetrazolium chloride-stained coronal sections are shown at different brain levels posterior to the frontal pole (**A**). Quantitative analysis was performed on infarct volume (**B**) and neurological deficits (**C**) in mice after stroke. Quantitative analysis of cerebrovascular permeability was determined 1 h after Evans blue injection (**D**). Compared with littermate control mice, EC-Pγ cKO mice show potentiated ischaemia-induced brain infarction ($n = 8$), worsened neurological outcomes ($n = 8$) and decreased cerebrovascular permeability ($n = 7$). Data are expressed as mean $\pm$ SD. $*P < 0.05$ versus the littermate control group.

ischaemic conditions in comparison with control mice (Supplementary Fig. 3C).

KLF11 genetic deficiency abolishes pioglitazone-mediated cerebrovascular protection against ischaemic insults *in vitro* and *in vivo*

To define the potential role of KLF11 in pioglitazone-mediated endothelial protection *in vitro*, primary cerebral vascular endothelial cell cultures were isolated from KLF11 knockout and wild-type mice and then subjected to oxygen–glucose deprivation exposure in the presence or absence of pioglitazone treatment. As demonstrated in Fig. 5, PPARγ activation by pioglitazone significantly increased cell survival after oxygen–glucose deprivation in cerebral vascular endothelial cells from wild-type mice. However, pioglitazone cytoprotection was completely abolished in cerebral vascular endothelial cells from KLF11 knockout mice (Fig. 5A and B). Interestingly, KLF11 genetic deficiency itself also statistically increased cerebral vascular endothelial cell death after oxygen–glucose deprivation exposure (Fig. 5A and B).

To further determine KLF11 as a key PPARγ co-regulator in pioglitazone-mediated vasoprotection *in vivo*, KLF11 knockout

and wild-type mice were treated with pioglitazone or vehicle control, and then followed by middle cerebral artery occlusion. As shown in Fig. 5C–E, pioglitazone effectively attenuated cerebral ischaemia-activated caspase 3 activity (Fig. 5C), DNA fragmentation in cerebral microvessels (Fig. 5D) and improved cerebrovascular permeability in wild-type mice (Fig. 5E). However, these vasoprotective roles of PPARγ activation by pioglitazone were abolished in mice with genetic deletion of KLF11 (Fig. 5). These data suggest that endothelial KLF11 is an essential co-regulator of PPARγ for pioglitazone-induced vascular protection after ischaemic cerebral insults.

PPARγ activation in endothelial cells reduces proapoptotic miR-15a levels in the mouse cerebral vasculature and cerebral vascular endothelial cell cultures after ischaemic insults

It is well-established that apoptotic vascular cell death contributes to the development of ischaemic cerebrovascular damage (Barone and Feuerstein, 1999; Yin *et al.*, 2010*a, b*). To determine whether activation of PPARγ affects post-ischaemic vascular degeneration,
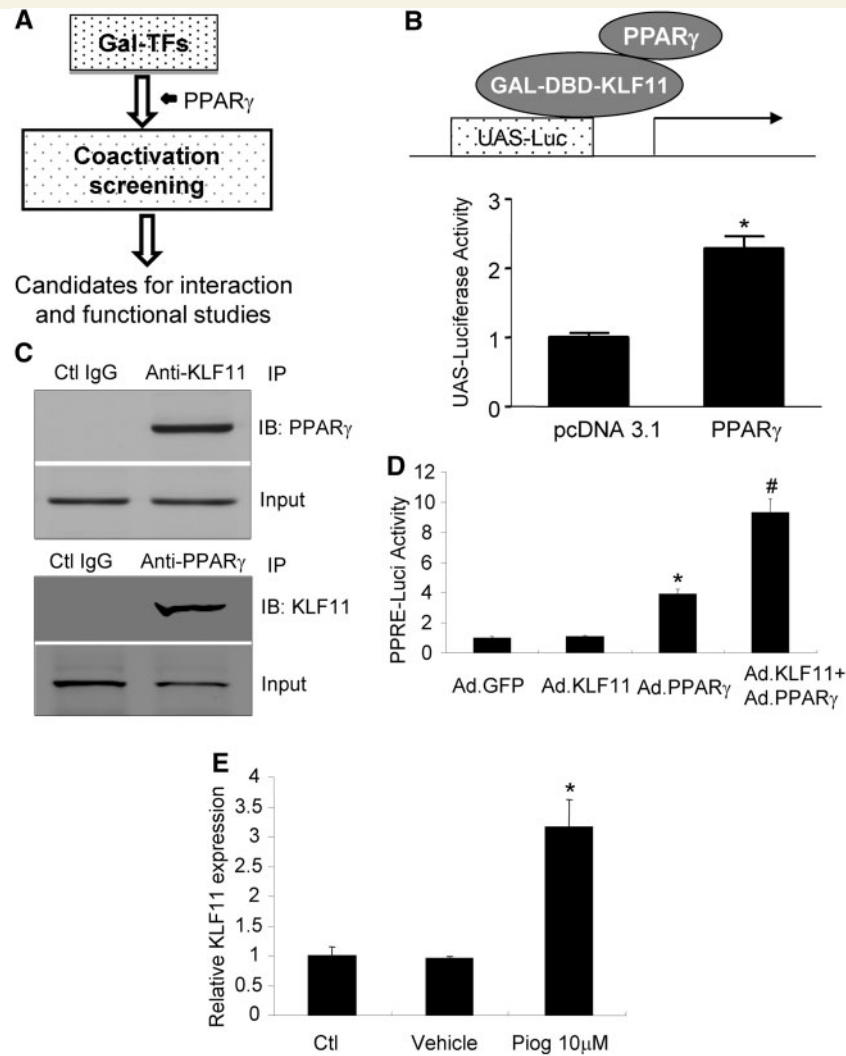
Figure 4 Identification of KLF11 as a novel PPARγ co-regulator. (**A**) Schema of genome-wide co-activation analysis. (**B**) UAS–luc, KLF11–Gal4–DBD and PPARγ expression vectors were co-transfected into HEK293 cells, and then luciferase activity was determined. (**C**) Total protein was isolated from mouse cerebral vascular endothelial cells, and then a co-immunoprecipitation assay was performed for KLF11–PPARγ interaction. KLF11 strongly binds to PPARγ in mouse cerebral vascular endothelial cells. (**D**) The 3 × PPRE–luc and adenoviral KLF11 vectors were co-transfected into mouse cerebral vascular endothelial cells in the presence of adenoviral GFP or PPARγ, and PPRE transcriptional activity was determined. KLF11 does not affect PPRE activity at basal levels, but it increases PPARγ adenoviral-mediated PPRE transcriptional activity in mouse cerebral vascular endothelial cells. (**E**) Mouse cerebral vascular endothelial cell cultures were treated with vehicle or pioglitazone at indicated doses, and total RNA was isolated for real-time PCR. Activation of PPARγ by its agonist, pioglitazone, significantly elevated KLF11 messenger RNA expression levels. Data are expressed as mean ± SEM. *$P < 0.05$ versus pcDNA 3.1 or Ad.GFP or vehicle control group. #$P < 0.05$ versus Ad.PPARγ group. IB = immunoblotting; IP = immunoprecipitation.

we examined the effects of pioglitazone on the expression of a newly identified cerebrovascular pro-apoptotic microRNA (Yin et al., 2010) in mice after 24 h of middle cerebral artery occlusion. As indicated in Fig. 6A, middle cerebral artery occlusion caused a significant increase in miR-15a expression, and this effect was almost completely abolished by treatment with pioglitazone. Similarly, cerebral vascular endothelial cell activation of PPARγ by pioglitazone also significantly decreased oxygen–glucose deprivation-induced elevations of the miR-15a level in vitro (Fig. 6B). Of note, pioglitazone inhibition of the miR-15a gene was significantly reversed by adenoviral loss of PPARγ function in cerebral vascular endothelial cell cultures (Fig. 6B). These data clearly

demonstrate that miR-15a is one of the potential downstream targets of pioglitazone suppression, which may contribute to pioglitazone-induced vascular protection after ischaemic insults. These findings also indicate that pioglitazone protects the cerebral vasculature through a microRNA-related mechanism.

KLF11 enhances PPARγ transcriptional repression of miR-15a expression

In our previous publication (Yin et al., 2010a), we identified a putative PPRE binding site in the promoter of mouse miR-15a at
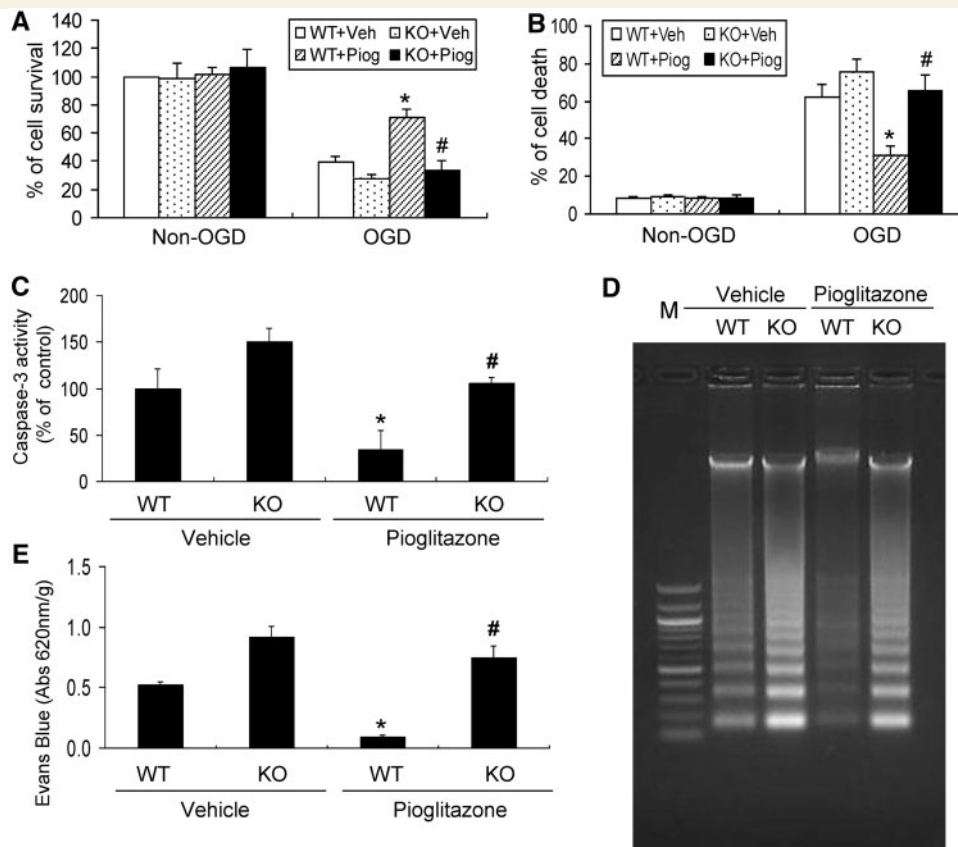
Figure 5 The effect of KLF11 genetic deficiency on pioglitazone-mediated endothelial protection in cerebral vascular endothelial cell cultures and the cerebral vasculature after ischaemic insults. Primary mouse cerebral vascular endothelial cells were cultured from KLF11 knockout or wild-type mice and then treated with 10 μM pioglitazone overnight before 16 h of oxygen–glucose deprivation exposure. Cell viability was determined by MTT assays (**A**) and the lactate dehydrogenase method (**B**). Kruppel-like factor knockout or wild-type mice (n = 6) were treated with pioglitazone (intraperitoneal 2 mg/kg) immediately after 30 min middle cerebral artery occlusion. After 24 h middle cerebral artery reperfusion, cerebral microvessels were isolated from the brain and then subjected to measurement of caspase 3 activity (**C**) and DNA fragmentation (**D**) as described in the 'Materials and methods' section. Cerebrovascular permeability was also determined 1 h after injection from Evans blue extravasation (n = 6) in mice after stroke (**E**). Pioglitazone treatment significantly attenuates caspase 3 activation and DNA fragmentation in the cerebral microvasculature and improves cerebrovascular permeability in wild-type mice after middle cerebral artery occlusion. However, pioglitazone-mediated vasoprotection was abolished in KLF11 knockout mice. Data are expressed as mean ± SD. *$P < 0.05$ versus wild-type (WT) + vehicle group. #$P < 0.05$ versus WT + pioglitazone group.

the location of −1593 to −1573 bp, and documented that PPARδ, another PPAR subtype, binds directly to the PPRE site in the miR-15a promoter region and represses its expression. Using the same strategies, we further investigate the possibility that PPARγ may also suppress miR-15a expression in a transcriptional manner, and KLF11 may synergistically modulate this effect. As shown in Fig. 7A, mouse cerebral vascular endothelial cells were transfected with a luciferase reporter vector carrying a 1.9-kb miR-15a promoter with wild-type PPRE (miR-15a PPRE Wt) or mutated PPRE as indicated (miR-15a PPRE mut). Cells were co-infected with adenoviral KLF11, PPARγ, KLF11 and PPARγ, or adenoviral GFP. As shown in Fig. 7B, PPARγ overexpression significantly reduced transcriptional activity of the miR-15a promoter, whereas KLF11 overexpression had no effect on miR-15a transcriptional activity by itself, but it significantly increased PPARγ transcriptional suppression of miR-15a. Of significance, luciferase activities from the miR-15a promoter driven by the mutated PPRE did not

respond to KLF11 and/or PPARγ overexpression, indicating that the predicted PPRE site in the promoter region of miR-15a is specifically responsible for KLF11/PPARγ coordinated transcriptional repression of miR-15a expression *in vitro*. Consistent with our transcriptional assay data, quantitative PCR data further showed that PPARγ overexpression by adenovirus significantly reduced miR-15a expression in mouse cerebral vascular endothelial cells, whereas additional KLF11 obviously enhanced this effect (Fig. 7C). Taken together, these data suggest that KLF11 functions as a novel PPARγ co-regulator and synergistically enhances PPARγ transcriptional repression of miR-15a.

Discussion

In this study, we found that PPARγ activation by pioglitazone plays a protective role in the mouse cerebral endothelium after
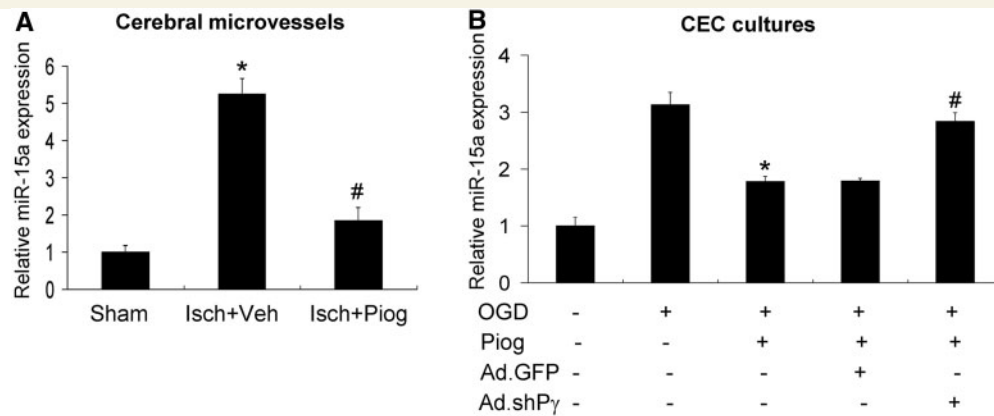
Figure 6 Potential targets of PPARγ activation in the cerebral vasculature after ischaemic insults. (**A**) Mice were treated with pioglitazone (intraperitoneal 2 mg/kg) after 30 min middle cerebral artery occlusion. After 24 h middle cerebral artery reperfusion, cerebral microvessels were isolated from the brain, and total RNA was extracted for real-time PCR. Cerebral ischaemia resulted in a significant increase in miR-15a expression, and this effect was almost completely abolished by treatment with pioglitazone. (**B**) Mouse cerebral vascular endothelial cell (CEC) cultures were treated with 10 μM pioglitazone for 24 h with or without infection of an adenovirus (Ad) carrying small-hairpin PPARγ or GFP for 48 h. Total RNA was then isolated, and miR-15a levels were examined by real-time PCR. Data are expressed as mean ± SEM. *$P < 0.05$ versus oxygen–glucose deprivation (OGD) or sham group. #$P < 0.05$ versus oxygen–glucose deprivation + pioglitazone + Ad.GFP or ischaemia + vehicle group.

in vitro and *in vivo* cerebral ischaemic insult. This novel finding is strongly confirmed by our experimental evidence, demonstrating that endothelial cell-selective deletion of PPARγ led to severer blood–brain barrier breakdown, neuronal loss and neurological deficits in mice after focal cerebral ischaemia. Moreover, we further defined that KLF11 can bind to PPARγ and function as its co-activator to enhance PPARγ activity in the cerebral vasculature. Genetic deficiency of the KLF11 gene appears to abolish pioglitazone-mediated cytoprotection in cultured cerebral vascular endothelial cells after oxygen–glucose deprivation, as well as cerebrovascular protection in mice following focal cerebral ischaemia. Furthermore, we revealed that miR-15a is a direct downstream target of KLF11/PPARγ synergistic vasoprotection. To our knowledge, our experiments have provided the first evidence that KLF11 synergistically co-operates with PPARγ to reduce ischaemic cerebral vascular damage.

Recently, PPARγ neuroprotection in stroke has been shown in rodent models (Sundararajan *et al.*, 2005; Luo *et al.*, 2006; Tureyen *et al.*, 2007; Zhao *et al.*, 2009) and humans (Wilcox *et al.*, 2007), thus making PPARγ activators potentially ideal treatment for ischaemic brain injury. During cerebral ischaemia, it is well-recognized that the cerebral microvasculature is among the major targets of ischaemic brain insults. However, the specific roles of PPARγ in the pathogenesis of stroke-induced cerebrovascular injury have not yet been systematically investigated *in vitro* and *in vivo*; although PPARγ is highly expressed in the vascular wall and has recently emerged as an important determinant of vascular structure and function (Chen *et al.*, 2003; Hamblin *et al.*, 2009). Gamboa *et al.* (2010) observed that in addition to decreasing brain infarction volume, pioglitazone and rosiglitazone also reduced intercellular adhesion molecule-immunoreactive vessels in brain sections of middle cerebral artery occlusion-induced rats, implying that thiazolidinediones may have some vascular effects

after ischaemic stroke. In the current study, we show that pioglitazone significantly reduced oxygen–glucose deprivation-induced cerebral vascular endothelial cell death, and gain or loss of PPARγ function by adenovirus either potentiated or attenuated pioglitazone-mediated cytoprotection in cerebral vascular endothelial cell cultures. Pioglitazone-mediated cytoprotection is further verified in mice because pioglitazone significantly reduced cerebral ischaemia-triggered increases in cerebrovascular permeability. Thus, our data clearly demonstrate that PPARγ activation by pioglitazone plays a vasculoprotective role in ischaemic stroke.

PPARγ is widely expressed in brain parenchymal cells (neurons, microglia, astrocytes, oligodendrocytes), as well as cerebral vascular cells (endothelial cells and smooth muscle cells) (Chen *et al.*, 2003; Moreno *et al.*, 2004; Hamblin *et al.*, 2009), implying that there are multiple cell targets for PPARγ agonists in the brain. Recently, PPARγ activation by thiazolidinedione compounds has been shown to be effective in reducing brain damage and improving neurological outcomes in rodent focal cerebral ischaemia (Sundararajan *et al.*, 2005; Luo *et al.*, 2006; Tureyen *et al.*, 2007; Zhao *et al.*, 2009). Mechanistically, PPARγ-triggered brain protection after ischaemic stroke may include neuronal and vascular mechanisms (Bordet *et al.*, 2006; Culman *et al.*, 2007). Up to now, the cell type responsible for PPARγ-mediated brain protection has not been clearly established. Our work here focuses on endothelial cell PPARγ-mediated cerebral vascular protection, which turns out to contribute to brain protection after focal cerebral ischaemia. However, we cannot exclude the possibilities that PPARγ in other neural elements may also play an important role in brain protection after ischaemic stimuli. Indeed, others have recently addressed neuronal PPARγ-mediated brain protection in the same stroke model by using neuron-selective PPARγ conditional knockout mice (Zhao *et al.*, 2009). Thus, it is our viewpoint that PPARγ-mediated brain protection after ischaemic stroke is a
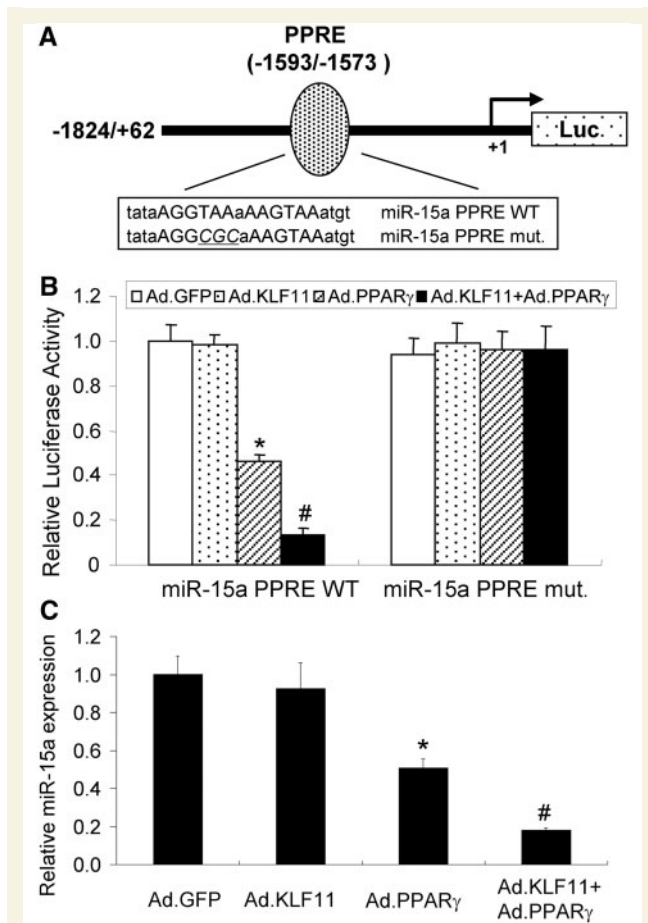
Figure 7 KLF11 enhances PPARγ transcriptional repression of miR-15a in mouse cerebral vascular endothelial cells. (**A**) Schematic representation of a pGL 4.10 luciferase reporter plasmid containing ~1.9 kb of the 5′ upstream promoter sequence of the mouse miR-15a gene. A putative PPRE binding site was demonstrated in the promoter of miR-15a at locations of −1593/−1573 bp (PPRE). The transcription start site is numbered as +1. (**B**) Luciferase reporter assays were performed by transfecting mouse cerebral vascular endothelial cells with a pGL 4.10 luciferase plasmid carrying either a miR-15a wild-type promoter (miR-15a PPRE Wt) or a miR-15a promoter with a mutated PPRE (miR-15a PPRE mut.). Transcriptional activity is reduced in the miR-15a wild-type promoter, but not in the PPRE mutant promoter, on adenoviral gain of PPARγ function. Of note, adenoviral gain of KLF11 function itself has no effect on miR-15a expression, but it significantly enhances PPARγ transcriptional suppression of miR-15a. (**C**) Mouse cerebral vascular endothelial cells were infected or co-infected with an adenovirus carrying PPARγ, KLF11 or GFP for 48 h. Total RNA was then isolated, and miR-15a levels were examined by real-time PCR. Data are expressed as mean ± SEM. *$P < 0.05$ versus Ad.GFP group. #$P < 0.05$ versus Ad.PPARγ group. Results shown are representative of three separate experiments with similar results.

cumulative effect of PPARγ neuroprotection and cerebrovascular protection.

The biological effects of PPARγ signalling depend on the levels of PPARγ expression, the presence of ligands and also the

availability of co-regulators. The recruitment of co-regulators to the PPARγ receptor, including co-activators or co-repressors, appears to be the key step in regulating PPARγ activity (Burgermeister *et al.*, 2006; Powell *et al.*, 2007; Ricote and Glass, 2007; Yu and Reddy, 2007; Hamblin *et al.*, 2009). These co-regulators are critical mediators for regulating PPARγ-driven transcriptional activation or repression of PPARγ target genes. Although PPARγ agonists and the PPARγ receptor have been found to be protective in ischaemic brain damage, the role of PPARγ co-regulators in stroke is less explored. Interestingly, it has been recently suggested that the involvement of PPARγ in the pathogenesis of focal cerebral ischaemia may be dependent on the coordination of co-regulators. For instance, LMO4 has been identified as an essential hypoxia-inducible co-regulator required for PPARγ signalling in neurons (Schock *et al.*, 2008). Hence, PPARγ co-regulators may become potential pharmacological targets to optimize the effects of PPARγ in the clinical setting. Indeed, the concept of selective PPARγ modulators for better treatment of diabetes and diabetic cardiovascular or cerebrovascular complications has been recently raised in the field (Hamblin *et al.*, 2009, Villacorta *et al.*, 2009).

Multiple functions of KLF11 have been previously identified in both physiological and pathophysiological conditions, including cell proliferation, apoptosis and diabetes (Neve *et al.*, 2005; Suzuki *et al.*, 2005; Gohla *et al.*, 2008; Fernandez-Zapico *et al.*, 2009). However, its role in cerebral vascular biology and pathologies remains unknown. In the current study, we have uncovered the contributory and synergistic roles of PPARγ and KLF11 in ischaemia-induced injury in both cultured cerebral vascular endothelial cells and the cerebral microvasculature. We are the first to identify KLF11 as a novel PPARγ co-regulator that enhances PPARγ transcriptional activity in mouse cerebral vascular endothelial cells. Of note, Kruppel-like factor activity is also subject to PPARγ transactivation, thus establishing a biological KLF11–PPARγ positive feedback. Moreover, we found that genetic deficiency of KLF11 abolishes PPARγ activation-triggered reduction of endothelial cell death in cerebral vascular endothelial cell cultures after oxygen–glucose deprivation; also, we found that genetic deletion of KLF11 significantly attenuates PPARγ inhibition of cerebrovascular permeability in mouse brains following focal cerebral ischaemia. These results provide novel insights into a better understanding of PPARγ and KLF11 function in the cerebral vasculature, as well as characterization of novel molecular mechanisms of PPARγ-mediated vasoprotection following cerebral ischaemia.

In addition to KLF11 enhancement of PPARγ function as a PPARγ co-activator in the cerebral endothelium, KLF11 has been shown to recruit the Sin3 histone deacetylase chromatin remodelling complex and trans-repress cytosolic phospholipase $A_2\alpha$, a prostaglandin E2 (PGE$_2$) biosynthetic enzyme in cultured adenocarcinoma cell lines (Buttar *et al.*, 2010). In pancreatic cancer cells, hyperactive extracellular signal-regulated kinase counteracts transforming growth factor (TFG)-β-induced cell growth inhibition through inhibition KLF11–Smad3 binding or disruption of KLF11–Smad3 complex formation (Buck *et al.*, 2006). In neuronal cells, KLF11 differentially couples to histone acetyltransferase and histone methyltransferase chromatin remodelling pathways to transcriptionally regulate dopamine D2 receptor gene expression (Seo

et al., 2012). Moreover, KLF11 also has direct transcriptional regulation of other genes, such as oxidative stress genes *SOD2* and catalase 1 (Fernandez-Zapico *et al.*, 2003), Smad7 loop (Ellenrieder *et al.*, 2004), Bcl-xL (Wang *et al.*, 2007), uncoupling protein 1 (Yamamoto *et al.*, 2010), monoamine oxidase A (Grunewald *et al.*, 2012), to mediate neoplastic transformation, cell proliferation and apoptosis, brown adipocyte differentiation and neuronal stress, respectively. These previous studies combined with our findings here suggest the diversity and complexity of KLF11 function in a tissue-specific manner in response to various pathophysiological stimuli.

KLF11/PPARγ-coordinated vasculoprotection may involve multiple mechanisms, including the regulation of vascular cell apoptosis (Hamblin *et al.*, 2009). We recently documented that miR-15a-mediated apoptosis contributes to cerebral endothelial injury after cerebral ischaemia (Yin *et al.*, 2010*a*). Here, we hypothesized that pioglitazone-mediated endothelial cytoprotection may also be associated with an anti-apoptotic mechanism. Indeed, under *in vitro* or *in vivo* stroke conditions, oxygen–glucose deprivation or middle cerebral artery occlusion resulted in ~3 - or 5-fold increase in miR-15a expression in cerebral vascular endothelial cell cultures or cerebral microvessels, respectively. However, pioglitazone treatment significantly reduced ischaemic insult-induced miR-15a expression. This effect is PPARγ-dependent, since gain of PPARγ function mediated pioglitazone reduction of miR-15a levels, whereas loss of PPARγ function reversed this effect. Furthermore, using a functional analysis of the PPRE binding site in the miR-15a promoter region, we are the first to reveal that PPARγ directly represses miR-15a in a transcriptional manner. Of significance, KLF11 can synergistically enhance PPARγ transcriptional suppression of miR-15a expression in the cerebral endothelium. These findings suggest that KLF11 may mediate PPARγ function through suppression of miR-15a-related mechanisms, and thereby protect the vascular endothelium against ischaemic insults.

The recently identified cerebral protective role of thiazolidinediones (rosiglitazone and pioglitazone) in experimental stroke models may render these agents as potentially ideal treatment for the ischaemic brain. This notion is further supported by the PROspective pioglitAzone Clinical Trial showing that pioglitazone provides a beneficial effect for reducing the risk of stroke recurrence in patients with type 2 diabetes mellitus (Wilcox *et al.*, 2007). However, rosiglitazone has been reported to be associated with increased cardiovascular morbidity and mortality, potentially limiting its future clinical application for the treatment of diabetes and diabetes-associated cerebrovascular complications (Nissen and Wolski, 2007; Kaul *et al.*, 2010). Therefore, it is our view that a more rational drug design based on the discovery and characterization of novel molecular targets that modulate PPARγ will likely lead to the development of more efficacious and safer PPAR drugs. PPARγ co-regulators are critical and promising molecular targets for modulating PPARγ function, and targeting these co-regulators may be critical for ultimately attenuating any adverse side effects of thiazolidinediones. Our study here has defined an important synergistic role of KLF11 as a novel PPARγ co-regulator in brain vasculature and ischaemic cerebral vascular injury. We anticipate that our current findings will eventually help us to define KLF11 and other PPARγ co-regulators as novel pharmacological targets for modulating PPARγ signalling cascades in stroke therapy. Expectedly, development of better PPARγ drugs that display more favourable pharmacological outcomes with reduced side effects may provide a novel therapy for stroke-induced cerebrovascular and neuronal damage.

Funding

Supplementary material

Supplementary material is available at *Brain* online.

References

Alva JA, Zovein AC, Monvoisin A, Murphy T, Salazar A, Harvey NL, et al. VE-Cadherin-Cre-recombinase transgenic mouse: a tool for lineage analysis and gene deletion in endothelial cells. Dev Dyn 2006; 235: 759–67.

Barone FC, Feuerstein GZ. Inflammatory mediators and stroke: new opportunities for novel therapeutics. J Cereb Blood Flow Metab 1999; 19: 819–34.

Black AR, Black JD, Azizkhan-Clifford J. Sp1 and kruppel-like factor family of transcription factors in cell growth regulation and cancer. J Cell Physiol 2001; 188: 143–60.

Bordet R, Ouk T, Petrault O, Gele P, Gautier S, Laprais M, et al. PPAR: a new pharmacological target for neuroprotection in stroke and neurodegenerative diseases. Biochem Soc Trans 2006; 34 (Pt 6): 1341–6.

Buck A, Buchholz M, Wagner M, Adler G, Gress T, Ellenrieder V. The tumor suppressor KLF11 mediates a novel mechanism in transforming growth factor beta-induced growth inhibition that is inactivated in pancreatic cancer. Mol Cancer Res 2006; 4: 861–72.

Burgermeister E, Schnoebelen A, Flament A, Benz J, Stihle M, Gsell B, et al. A novel partial agonist of peroxisome proliferator-activated receptor-gamma (PPARgamma) recruits PPARgamma-coactivator-1alpha, prevents triglyceride accumulation, and potentiates insulin signaling in vitro. Mol Endocrinol 2006; 20: 809–30.

Buttar NS, DeMars CJ, Lomberk G, Rizvi S, Bonilla-Velez J, Achra S, et al. Distinct role of Kruppel-like factor 11 in the regulation of prostaglandin E2 biosynthesis. J Biol Chem 2010; 285: 11433–44.

Chang L, Villacorta L, Zhang J, Garcia-Barrio MT, Yang K, Hamblin M, et al. Vascular smooth muscle cell-selective peroxisome proliferator-activated receptor-gamma deletion leads to hypotension. Circulation 2009; 119: 2161–9.

Chen YE, Fu M, Zhang J, Zhu X, Lin Y, Akinbami MA, et al. Peroxisome proliferator-activated receptors and the cardiovascular system. Vitam Horm 2003; 66: 157–88.

Culman J, Zhao Y, Gohlke P, Herdegen T. PPAR-gamma: therapeutic target for ischemic stroke. Trends Pharmacol Sci 2007; 28: 244–9.

Ellenrieder V, Buck A, Harth A, Jungert K, Buchholz M, Adler G, et al. KLF11 mediates a critical mechanism in TGF-beta signaling that is inactivated by Erk-MAPK in pancreatic cancer cells. Gastroenterology 2004; 127: 607–20.

Fernandez-Zapico ME, Mladek A, Ellenrieder V, Folch-Puy E, Miller L, Urrutia R. An mSin3A interaction domain links the transcriptional activity of KLF11 with its role in growth regulation. EMBO J 2003; 22: 4748–58.

Fernandez-Zapico ME, van Velkinburgh JC, Gutierrez-Aguilar R, Neve B, Froguel P, Urrutia R, et al. MODY7 gene, KLF11, is a novel p300-dependent regulator of Pdx-1 (MODY4) transcription in pancreatic islet beta cells. J Biol Chem 2009; 284: 36482–90.

Gamboa J, Blankenship DA, Niemi JP, Landreth GE, Karl M, Hilow E, et al. Extension of the neuroprotective time window for thiazolidinediones in ischemic stroke is dependent on time of reperfusion. Neuroscience 2010; 170: 846–57.

Glass CK, Rosenfeld MG. The coregulator exchange in transcriptional functions of nuclear receptors. Genes Dev 2000; 14: 121–41.

Gohla G, Krieglstein K, Spittau B. Tieg3/Klf11 induces apoptosis in OLI-neu cells and enhances the TGF-beta signaling pathway by transcriptional repression of Smad7. J Cell Biochem 2008; 104: 850–61.

Grunewald M, Johnson S, Lu D, Wang Z, Lomberk G, Albert PR, et al. Mechanistic role for a novel glucocorticoid-KLF11 (TIEG2) protein pathway in stress-induced monoamine oxidase A expression. J Biol Chem 2012; 287: 24195–206.

Hamblin M, Chang L, Fan Y, Zhang J, Chen YE. PPARs and the cardiovascular system. Antioxid Redox Signal 2009; 11: 1415–52.

Hamer PW, McGeachie JM, Davies MJ, Grounds MD. Evans Blue Dye as an in vivo marker of myofibre damage: optimising parameters for detecting initial myofibre membrane permeability. J Anat 2002; 200 (Pt 1): 69–79.

He TC, Zhou S, da Costa LT, Yu J, Kinzler KW, Vogelstein B. A simplified system for generating recombinant adenoviruses. Proc Natl Acad Sci USA 1998; 95: 2509–14.

Kaczynski J, Cook T, Urrutia R. Sp1- and Kruppel-like transcription factors. Genome Biol 2003; 4: 206.

Kaul S, Bolger AF, Herrington D, Giugliano RP, Eckel RH. Thiazolidinedione drugs and cardiovascular risks: a science advisory from the American Heart Association and American College of Cardiology Foundation. Circulation 2010; 121: 1868–77.

Koppen A, Houtman R, Pijnenburg D, Jeninga EH, Ruijtenbeek R, Kalkhoven E. Nuclear receptor-coregulator interaction profiling identifies TRIP3 as a novel peroxisome proliferator-activated receptor gamma cofactor. Mol Cell Proteomics 2009; 8: 2212–26.

Leidi M, Mariotti M, Maier JA. Transcriptional coactivator EDF-1 is required for PPARgamma-stimulated adipogenesis. Cell Mol Life Sci 2009; 66: 2733–42.

Li S, Liu C, Li N, Hao T, Han T, Hill DE, et al. Genome-wide coactivation analysis of PGC-1alpha identifies BAF60a as a regulator of hepatic lipid metabolism. Cell Metab 2008; 8: 105–17.

Luo Y, Yin W, Signore AP, Zhang F, Hong Z, Wang S, et al. Neuroprotection against focal ischemic brain injury by the peroxisome proliferator-activated receptor-gamma agonist rosiglitazone. J Neurochem 2006; 97: 435–48.

Manaenko A, Chen H, Kammer J, Zhang JH, Tang J. Comparison Evans Blue injection routes: intravenous versus intraperitoneal, for measurement of blood-brain barrier in a mice hemorrhage model. J Neurosci Methods 2011; 195: 206–10.

McKenna NJ, O'Malley BW. Combinatorial control of gene expression by nuclear receptors and coregulators. Cell 2002; 108: 465–74.

Moreno S, Farioli-Vecchioli S, Ceru MP. Immunolocalization of peroxisome proliferator-activated receptors and retinoid X receptors in the adult rat CNS. Neuroscience 2004; 123: 131–45.

Moskowitz MA, Lo EH, Iadecola C. The science of stroke: mechanisms in search of treatments. Neuron 2010; 67: 181–98.

Neve B, Fernandez-Zapico ME, Ashkenazi-Katalan V, Dina C, Hamid YH, Joly E, et al. Role of transcription factor KLF11 and its diabetes-associated gene variants in pancreatic beta cell function. Proc Natl Acad Sci USA 2005; 102: 4807–12.

Nissen SE, Wolski K. Effect of rosiglitazone on the risk of myocardial infarction and death from cardiovascular causes. N Engl J Med 2007; 356: 2457–71.

Powell E, Kuhn P, Xu W. Nuclear receptor cofactors in PPARgamma-mediated adipogenesis and adipocyte energy metabolism. PPAR Res 2007; 2007: 53843.

Qi C, Surapureddi S, Zhu YJ, Yu S, Kashireddy P, Rao MS, et al. Transcriptional coactivator PRIP, the peroxisome proliferator-activated receptor gamma (PPARgamma)-interacting protein, is required for PPARgamma-mediated adipogenesis. J Biol Chem 2003; 278: 25281–4.

Ricote M, Glass CK. PPARs and molecular mechanisms of transrepression. Biochim Biophys Acta 2007; 1771: 926–35.

Schock SC, Xu J, Duquette PM, Qin Z, Lewandowski AJ, Rai PS, et al. Rescue of neurons from ischemic injury by peroxisome proliferator-activated receptor-gamma requires a novel essential cofactor LMO4. J Neurosci 2008; 28: 12433–44.

Schopfer FJ, Lin Y, Baker PR, Cui T, Garcia-Barrio M, Zhang J, et al. Nitrolinoleic acid: an endogenous peroxisome proliferator-activated receptor gamma ligand. Proc Natl Acad Sci USA 2005; 102: 2340–5.

Seo S, Lomberk G, Mathison A, Buttar N, Podratz J, Calvo E, et al. Kruppel-like factor 11 differentially couples to histone acetyltransferase and histone methyltransferase chromatin remodeling pathways to transcriptionally regulate dopamine D2 receptor in neuronal cells. J Biol Chem 2012; 287: 12723–35.

Song CZ, Gavriilidis G, Asano H, Stamatoyannopoulos G. Functional study of transcription factor KLF11 by targeted gene inactivation. Blood Cells Mol Dis 2005; 34: 53–9.

Stapf C, Mohr JP. Ischemic stroke therapy. Ann Rev Med 2002; 53: 453–75.

Sundararajan S, Gamboa JL, Victor NA, Wanderi EW, Lust WD, Landreth GE. Peroxisome proliferator-activated receptor-gamma ligands reduce inflammation and infarction size in transient focal ischemia. Neuroscience 2005; 130: 685–96.

Suzuki T, Aizawa K, Matsumura T, Nagai R. Vascular implications of the Kruppel-like family of transcription factors. Arterioscler Thromb Vasc Biol 2005; 25: 1135–41.

Swanson RA, Morton MT, Tsao-Wu G, Savalos RA, Davidson C, Sharp FR. A semiautomated method for measuring brain infarct volume. J Cereb Blood Flow Metab 1990; 10: 290–3.

Tureyen K, Kapadia R, Bowen KK, Satriotomo I, Liang J, Feinstein DL, et al. Peroxisome proliferator-activated receptor-gamma agonists induce neuroprotection following transient focal ischemia in normotensive, normoglycemic as well as hypertensive and type-2 diabetic rodents. J Neurochem 2007; 101: 41–56.

Villacorta L, Schopfer FJ, Zhang J, Freeman BA, Chen YE. PPARgamma and its ligands: therapeutic implications in cardiovascular disease. Clin Sci (Lond) 2009; 116: 205–18.

Wang Z, Spittau B, Behrendt M, Peters B, Krieglstein K. Human TIEG2/KLF11 induces oligodendroglial cell death by down-regulation of Bcl-XL expression. J Neural Transm 2007; 114: 867–75.

Wilcox R, Bousser MG, Betteridge DJ, Schernthaner G, Pirags V, Kupfer S, et al. Effects of pioglitazone in patients with type 2 diabetes with or without previous stroke: results from PROactive (PROspective pioglitAzone Clinical Trial In macroVascular Events 04). Stroke 2007; 38: 865–73.

Wu JS, Lin TN, Wu KK. Rosiglitazone and PPAR-gamma overexpression protect mitochondrial membrane potential and prevent apoptosis by upregulating anti-apoptotic Bcl-2 family proteins. J Cell Physiol 2009; 220: 58–71.

Yamamoto K, Sakaguchi M, Medina RJ, Niida A, Sakaguchi Y, Miyazaki M, et al. Transcriptional regulation of a brown adipocyte-specific gene, UCP1, by KLF11 and KLF15. Biochem Biophys Res Commun 2010; 400: 175–80.

Yin KJ, Chen SD, Lee JM, Xu J, Hsu CY. ATM gene regulates oxygen-glucose deprivation-induced nuclear factor-kappaB DNA-binding activity and downstream apoptotic cascade in mouse cerebrovascular endothelial cells. Stroke 2002a; 33: 2471–7.

Yin KJ, Deng Z, Hamblin M, Xiang Y, Huang H, Zhang J, et al. Peroxisome proliferator-activated receptor delta regulation of miR-15a in ischemia-induced cerebral vascular endothelial injury. J Neurosci 2010a; 30: 6398–408.

Yin KJ, Deng Z, Hamblin M, Zhang J, Chen YE. Vascular PPAR{delta} Protects against stroke-induced brain injury. Arterioscler Thromb Vasc Biol 2011; 31: 574–81.

Yin KJ, Deng Z, Huang H, Hamblin M, Xie C, Zhang J, et al. miR-497 regulates neuronal death in mouse brain after transient focal cerebral ischemia. Neurobiol Dis 2010b; 38: 17–26.

Yin KJ, Hsu CY, Hu XY, Chen H, Chen SW, Xu J, et al. Protein phosphatase 2A regulates bim expression via the Akt/FKHRL1 signaling pathway in amyloid-beta peptide-induced cerebrovascular endothelial cell death. J Neurosci 2006; 26: 2290–9.

Yin KJ, Lee JM, Chen SD, Xu J, Hsu CY. Amyloid-beta induces Smac release via AP-1/Bim activation in cerebral endothelial cells. J Neurosci 2002b; 22: 9764–70.

Yki-Jarvinen H. Thiazolidinediones. N Engl J Med 2004; 351: 1106–18.

Yu S, Reddy JK. Transcription coactivators for peroxisome proliferator-activated receptors. Biochim Biophys Acta 2007; 1771: 936–51.

Zhao X, Strong R, Zhang J, Sun G, Tsien JZ, Cui Z, et al. Neuronal PPARgamma deficiency increases susceptibility to brain damage after cerebral ischemia. J Neurosci 2009; 29: 6186–95.