MATERIAL AND METHODS

Reagents

LRP1 (C-terminal) antibody against a.a. 4532-4544 of human LRP1 was obtained from Sigma (LRP1-CTD, Cat. No. L2170; St. Louis, MO) and used for Western blotting, immunoprecipitation and immunostaining. Another LRP1 (8G1, Cat. No. ab20384) antibody against a.a. 1-172 was purchased from Abcam (Cambridge, MA) and used for immunostaining. The test for the specificity of the 8G1 and LRP1-CTD antibodies is shown in Figure SII. Antibodies against full length PARP-1 (Cat. No. ab6079) and cleaved PARP-1 (Cat. No. ab72805) were purchased from Abcam and used for Western blotting, and another PARP-1 antibody was purchased from Thermo Scientific (Cat. No. MA3-950; Grand Island, NY) and used for immunostaining. Anti-phospho-CDK2 (Thr160; Cat. No. 2561s), CDK2 (Cat. No. 2546s), phospho-Rb (Ser807/811; Cat. No. 8516s), Rb (Cat. No. 9313s), Lamin B1 (Cat. No. 12586s) and Hsp90 (Cat. No. 4874s) antibodies were purchased from Cell Signaling (Danvers, MA) and used for Western blotting experiments. PARP-1 inhibitor PJ-34 (N-(6-Oxo-5,6-dihydrophenanthridin-2-yl)-(N,N-dimethylamino)acetamide hydrochloride) and γ–secretase inhibitor DAPT N-[N-(3,5-Difluorophenacetyl)-L-alanyl]-S-phenylglycine t-butyl ester were purchased from Sigma (St. Louis, MO).

Mice and oxygen-induced retinopathy (OIR).

LRP1 deletion in endothelium takes place following the mating of $LRP1^{flox/flox}$ ($LRP1^{f/f}$) mice and $Tie2Cre^{+}$ transgenic mice (obtained from Jackson Laboratories, Bar Harbor, ME). All experiments were performed using $LRP1^{f/f}$;Cre^{-} (wild type control) and $LRP1^{f/f}$;Cre^{+} littermates. OIR was induced as previously described[1]. In brief, postnatal day (P) 7 pups with nursing mothers were maintained for 5 days in 75% oxygen, and then placed back in room air (21% oxygen). All experimental procedures on mice were performed according to the National Institutes of Health Guide for the Care and Use of Laboratory Animals and approved by the Institutional Committee for the Use of Animals in Research.

Immunostaining of whole-mounted retinas.

This procedure was performed following the previous protocol[1]. Enucleated eyes were fixed overnight in 4% PFA in PBS with 0.1% Triton X-100 and stored in PBS at 4C. The cornea, sclera, lens and vitreous were removed to isolate retinas and radial incisions were made along the retinal edge. Retinas were then treated with 70% ethanol for 20 minutes, washed for 30 minutes in PBS with 1% Triton X-100. They were blocked for one hour with PBS containing 1% triton X-100 and 5% normal goat serum. Retinas were incubated with TRITC-labeled iso-lectin B4 (Life Technologies), GFAP (Dako Cytomation), NG2 (Chemicon) antibodies. Following washing, Retinas were incubated with Alexa-Fluor secondary antibodies. After several washes, samples were ready for confocal microscopy analysis. 5x confocal images were taken for the analysis of avascularized area. For detailed analysis of angiogenic parameters including vascularized area,

branching points and sprout numbers within the leading edge of the neovascularized area, 20x confocal images were taken and at least 5 random fields of view for vascularized area, and 7 for branching points and sprout numbers per retina were analyzed.

Immunostaining of frozen sections.
This procedure was performed following the previous protocol[1]. Enucleated eyes were fixed overnight in 4% PFA in PBS. Following the incubation with 15% and 30% sucrose gradient, eyes were frozen in OCT mounting media (Andwin Scientific). Cryosections of 6 mm thickness were processed for staining with anti-CD31 (BD Biosciences), LRP1 (8G1, Abcam), Ki67 (Abcam) and DAPI (Life Technologies) antibodies. Confocal images were taken for further quantitative analysis.

Cell culture and transient transfection
HEK 293 cells, $LRP1^{+/+}$ (MEF1; CRL-2214) and $LRP1^{-/-}$ mouse embryonic fibroblast (PEA10 MEF; CRL-2215) cells were obtained from ATCC and cultured in DMEM medium (Life Technologies). Human retinal microvascular endothelial cells (HRECs) were purchased from Cell Systems (Kirkland, WA) and cultured in HREC medium (Cell Systems). Cells from passages 5-8 were used for experiments. ~70% confluent cells in 6-cm dishes were transfected for 3 hours with 2 μg of plasmids using 8 μl of Lipofectamine LTX and 8 μl of Plus reagent (Life Technologies). Two days later, cells were cultured under hypoxia (2% O_2) or room air as a control. For stably expressed LRP1 in 293 cells, 90-100% confluent 293 cells in 10-cm dishes were transfected 24 hours after plating with 2 μg of Flag-tagged LRP1β plasmid using 20 μl of Lipofectamine 2000 (Life Technologies) and then positive cells were selected by using Geneticin (G418; Life Technologies).

siRNA design and transient transfection
The stealth siRNA duplexes were obtained from Life Technologies. The siRNA against human LRP1 is a duplex of 5'- GGGUGGAGAGUAACCUGGAUCAGAU-3'. The control siRNA is the Stealth RNAi negative control duplex (Cat. No. 12935-300) and was purchased from Life Technologies. The siRNAs were transfected into HRECs according to our previous published protocol[2]. Briefly, for each sample, $2x10^5$ HRECs were transfected with 100 pmol siRNA. The experiments with siRNA transfected HRECs were performed two days later.

Immunoprecipitation, GST pull down and immunoblotting analysis
Cells were harvested in lysis buffer (1% Triton X-100, 50 mmol/L Tris (pH 7.4), 150 mmol/L NaCl, 1 mmol/L Na_3VO_4 and 0.1% protease inhibitor mixture; Sigma) and clarified by centrifugation at 15,000 *g*. Equal amounts of proteins were incubated with a specific antibody overnight at 4 °C with gentle rotation. Protein A/G Plus-agarose beads (Santa Cruz Biotechnology) were used to pull down the antibody complexes. GST-tagged recombinant proteins were generated for GST-tagged LRP1 intracellular C-terminal domain (GST-ICD; a.a. 4445-4544 of

human LRP1) and the truncated intracellular C-terminal domain shortened (GST-ICDs; a.a. 4445-4511 of human LRP1) constructs, or GST as a negative control. HREC lysates were mixed with GST-tagged proteins-containing beads overnight at 4°C with gentle rotation. Western blotting analysis was performed with anti-PARP-1 antibody. Immune complexes or GST-pull down complexes were then separated by SDS-PAGE and analyzed by Western blot. The LRP1 (C-terminal) antibody (LRP1-CTD, L2170 from Sigma) is used for all the analysis of immunoprecipitation and immunoblotting.

Subcellular fractionation assay

Subcellular fractions (cytosolic and nuclear) were obtained following previous protocol[3] with a slight modification. HRECs were lysed by hypotonic buffer, and the supernatant (cytosolic) and pellet (nuclear fraction) were separated by centrifugation at 15,000 g for 5 minutes. Purity and consistency of fractions was confirmed using antibodies against markers for different subcellular compartments: anti-HSP90 (to detect cytosolic protein) and anti-Lamin B1 (to detect nuclear protein).

Immunofluorescence and co-localization analysis

Immunofluorescence with cultured HRECs was performed following our previously published protocol[4]. Cells were fixed in 3.7% paraformaldehyde for 10 min at room temperature. After 3 washes with PBS, the cells were sequentially treated with 0.2% Triton X-100 for 5 min (for permeabilization), with 5% boiled serum for 1 hour (for blocking), then with the primary antibody overnight in the blocking solution. After 3 washes, cells were incubated in the dark with secondary antibody conjugated with Alexa Fluor 568 (Life Technologies) in blocking solution for 90 min at 37°C. After 3 washes in PBS, the slides were mounted and the fluorescent signal was visualized by confocal laser scanning microscopy (Zeiss Pascal, Zeiss, Germany). Images for quantitative florescence analysis were acquired with sequential mode to avoid interference between channels and saturation. To quantify the co-localization of LRP1 and PARP-1, each stack optical section was analyzed using the Colocalization_Test, Colocalization_Threshold, and Manders Coefficients plug-ins (developed by Tony Collins at the Wright Cell Imaging Facility in Toronto Canada, http://www.uhnres.utoronto.ca/wcif) for ImageJ software (NIH Image, Bethesda MD). The co-localization of LRP1 with PARP-1 was estimated by the use of Pearson's correlation coefficient. The Pearson coefficient ranges from −1 (total negative correlation) to +1 (total positive correlation). A coefficient of 0 means random correlation. Costes' method was used for automatically setting the threshold based on the scatter plot and minimizing Pearson's coefficient.

In vitro Matrigel angiogenesis assay

Endothelial cell tube formation was analyzed with the Matrigel-based tube formation assay as previously described[3].

Spheroid angiogenesis assay

HRECs were transfected with LRP1 or control siRNAs. Two days later, HREC spheroids were generated as previously described[5-7]. Spheroids were cultured in the polymerized gel of neutralized collagen and carboxymethylcellulose at a ratio of 1:1 and exposed to either 21% or 2% O_2. Images of the sprouts were taken at 4x magnification. The number of sprouts per spheroid was counted. For each experimental condition, at least 10 spheroids were analyzed.

PARP-1 activity assay

PARP-1 activity reactions were carried out following manufacturer's protocol (TREVIGEN, Inc, Gaithersburg, MD). Briefly, HRECs cells were transfected with control or LRP1 siRNA and then incubated at normoxia (21% O_2) or hypoxia (2% O_2) for 2 hours. Cells were harvested and lysed with lysis buffer that is provided by the kit. Protein concentration of the extracts was measured with a BCA protein assay and cell lysates were used immediately for PARP-1 activity assay. Immobilized PAR monoclonal antibody in the wells of a 96-well plate captures cellular PAR and PAR attached to proteins. PAR standards were provided by the kit. Both PAR standards and cell lysates were conjugated with the 96-well plate for 16 hrs in 4°C. The plate was washed 4 times with PBS-Tween (0.1%) buffer and then the plate was incubated with a polyclonal PAR detecting antibody for 2 hours at room temperature, followed by addition of a goat anti-rabbit IgG-HRP secondary antibody for 1 hour. A chemiluminescent HRP substrate yields relative light units (RLU) that directly correlate with the amount of cellular PAR amount. The chemiluminescent readings were taken by a Tecan infinite M200 Pro plate reader.

Flow cytometric analysis of cell cycle with propidium iodide staining of DNA

HRECs cells were harvested and washed in PBS for three times, and then fixed in cold 70% ethanol for 30 minutes at 4°C. Cells were washed with PBS for another three times. Propidium iodide (Life technologies) was added into HRECs with final concentration of 50 ug/ml. DNA contents of the cells were analyzed with flow cytometric analyzer and their cell cycle distribution patterns were determined with ModFit LT software. ~10,000 cells in each sample were analyzed and the percentages of cells at different cell cycle stages were determined.

Statistical analysis

Data are shown as the mean ± SEM for 3 to 4 separate experiments. All datasets have been tested and are appropriate for parametric analysis. Differences were analyzed with Student's *t*-test or ANOVA and followed by a post hoc test with a correction when needed. Values of $p \leq 0.05$ were considered statistically significant.

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