Supplemental Material

Title: Genetic Deletion of endothelial MicroRNA-15a/16-1 Promotes Cerebral Angiogenesis and Neurological Recovery in Ischemic Stroke through Src Signaling Pathway

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Supplemental Method

Methods to prevent bias and exclusion criteria

All experiments were reported in compliance with the updated ARRIVE guidelines 2.0 (ARRIVE, Animal Research: Reporting in Vivo Experiments)[1]. Animals were randomly assigned with a lottery-drawing box to different experimental groups, including the middle cerebral artery occlusion (MCAO) procedure, neurobehavioral evaluation, histological assessments, etc. Surgical operations and stroke outcome assessments were performed by investigators blinded to mouse genotype and experimental group assignments. Animals that showed one or more the following signs were excluded: (1) received a neurological score less than 1 (0, no visible neurological deficits; 1, forelimb flexion), (2) did not show a >75% cerebral blood flow (CBF) reduction or a <60% CBF reperfusion over baseline levels during MCAO surgery, (3) lack of a brain infarct based on immuno-histological evaluation, (4) did not ambulate/swim during water-maze test, (5) had greater than 20% weight loss, or were moribund and unable to attend normal physiological needs such as eating, drinking, and grooming, (6) died prior to behavioral test or sample collection (see detailed information of excluded mice in **Fig. S2**).

Determination of Sample Size

Mouse numbers required for the *in vivo* studies were determined by a power analysis based on our preliminary results and previous experiences with the murine MCAO/reperfusion model with long-term outcome assessments in the Pittsburgh Institute of Brain Disorders and Recovery. For example, to detect a 30% decrease in infarct volume or neurological deficits with 80% power at an α value of 0.05 (two-tailed T-test), ~6–8 mice were needed per group; for immunohistochemistry, qPCR, and western blotting, ~4–5 samples were required to detect a 30% change after MCAO with 80% power ($\beta = 0.8$, $\alpha = 0.05$).[2, 3] However, occasionally more mice were needed to ensure adequate experimental numbers after consideration of sample loss (animal mortality, exclusion of animals) during these complex *in vivo* experiments to achieve statistical significance among the treatment groups.

Drug treatment

The Src inhibitor AZD0530 was prepared and administered to experimental mice as previously described[4] with some modifications. Briefly, AZD0530 was first dissolved in DMSO at a concentration of 50 mg/ml to make a stock solution, and then the stock solution was mixed with corn oil at a ratio of 5% DMSO: 95% corn oil. For AZD0530 treatment, mice were administered daily with AZD0530 (20 mg/kg) at 3-21 d after MCAO by oral gavage. For vehicle treatment, mice were administered daily with the same amount of DMSO and corn oil (5%: 95%) mixture at 3-21 d after MCAO by oral gavage (**Fig. S1**).

Morris water maze test

The Morris water maze test was performed at 22-27 d after MCAO to evaluate the long-term cognitive functions as previously described.[3, 5, 6] Briefly, a circular platform (11 cm ⌀) was submerged in one quadrant of the circular pool (109 cm ⌀) of opaque water. To examine spatial learning ability, each mouse was placed into the pool from one of the three different start points (3 trials) and allowed to swim for a maximum of 60 s to locate the hidden platform. The time when the mouse found the hidden platform (escape latency) was recorded for each trial. When each trial ended, the mouse was allowed to stay on the platform for 20 s to help it to remember the external spatial cues displayed around the room. Mice were trained for three consecutive days before and tested 22-26 d after MCAO (three trials per day). To evaluate spatial memory, a single 60 s probe trial was performed at 27 d after MCAO when the platform was removed. An investigator recorded the time that each mouse spent in the target quadrant where the platform had previously been placed. Data are expressed as the percentage of the total testing time of 60 s (Duration in goal quadrant).

Measurements of brain atrophy

At 28 d after tMCAO, mice were deeply anesthetized and transcardially perfused with 0.9% NaCl followed by 4% paraformaldehyde in PBS. Brains were collected and cryoprotected in 30% sucrose in PBS for two days, and serial coronal brain sections (25 μm thickness) were prepared on a microtome (HM450, Thermo Scientific). Brain sections were transferred to cryoprotectant and stored at -20°C. After a series washes, six equally spaced (1 mm) brain sections were blocked by 5% donkey serum in PBS for 1 h, followed by overnight incubation with rabbit polyclonal anti-microtubule-associated protein 2 (MAP2, 1:500, EMD Millipore) antibody at 4°C. Then the brain sections were incubated with donkey anti-rabbit secondary antibody (1:1000, Jackson ImmunoResearch Laboratories). Images were then captured with an inverted Nikon Diaphot-300 fluorescence microscope equipped with a SPOT RT slider camera and Meta Series Software 5.0 (Molecular Devices, Sunnyvale, CA). Brain atrophy was measured on MAP2-stained sections encompassing the MCA territory using the NIH ImageJ software. These areas were summed and multiplied by the distance between sections (1 mm) to yield a final atrophy volume in mm^3. Tissue atrophy was calculated as the volume of the contralateral hemisphere minus the MAP2-positive ipsilateral hemisphere. Tissue atrophy percentage was calculated as the atrophy volume in the ispisilateral hemisphere to the total volume of contralateral hemisphere.

Quantitative real-time PCR

As previously described, total RNA was isolated from the 0.9% NaCl perfused mouse cerebral cortex[5, 7, 8]. Quantitative real-time reverse transcriptase PCR was performed with a Bio-Rad CFX connect thermocycler, iScript cDNA synthesis kit (ThermoFisher Scientific), and iTaq Universal SYBR Green Supermix (Bio-Rad). Specific primers used for the PCR reaction are presented in detail in Table S1. The relative mRNA expression was calculated by the $2^{-\Delta\Delta Ct}$ method and

normalized to mouse cyclophilin RNA levels. PCR experiments were repeated at least 3 times, each using separate brain cortex samples.

Western Blotting

At 28 d after tMCAO, mice were euthanized in a CO_2 chamber and then transcardially perfused with 0.9% NaCl. Brains were collected, and the cortices were isolated on ice for future tests or subjected to western blots immediately as described.[5, 9-11] Briefly, brain samples were homogenized in 1x RIPA buffer (Thermo Scientific) containing Mini Protease Inhibitor Cocktail (cOmplete™, Sigma) and Phosphatase Inhibitor Cocktail II (Abcam). Protein concentrations were determined by the Bio-Rad Protein Assay (Bradford, Bio-Rad). Equal amounts of protein (60 μg/lane) were loaded into 4-15% precast gels (Bio-Rad) and transferred to polyvinylidene difluoride (PVDF, Bio-Rad) membranes. Membranes were blocked for 1 h with 0.1% TBST (0.1% Tween-20) buffer plus 5% (w/v) bovine serum albumin (BSA) and incubated overnight at 4°C with primary antibodies diluted in 5% BSA (dissolved in 0.1% TBST). Membranes were then incubated with secondary antibodies diluted in 5% BSA (dissolved in 0.1% TBST) for 1 h and developed using a Pierce® ECL Western blotting detection kit (Thermo Scientific) and Amersham High-Performance Chemiluminescence Films (GE Healthcare). The primary antibodies used are detailed in **Table S2**; secondary HRP (horseradish peroxidase)-conjugated antibodies used were anti-rabbit IgG (1: 2000) (Cell Signaling; Beverly, MA, USA), and anti-mouse IgG (1: 2000) (Cell Signaling). The ImageJ software was used to quantify the western blot signals. Whole WB images were shown in **Fig. S9**, **Fig. S10**, **Fig. S11** and **Fig. S12**.

Statistical analyses

All data are presented as mean ± SD. The Gaussian distribution of each variable was checked using the Shapiro-Wilk test if $N<30$ and Kolmogorov-Smirnov test if $N \geq 30$. Non-normally distributed data were analyzed by nonparametric tests: Mann-Whitney test for two groups, and Kruskal-Wallis test followed by Dunn's post hoc analysis for three or more groups. Normally distributed data were analyzed using parametric tests: two-tailed Student's t-test between two groups, and one-way ANOVA followed by Bonferroni's multiple comparison tests for three or more groups. Experiments consisting of two categorically independent variables and one dependent variable were analyzed by two-way ANOVA followed by Bonferroni's multiple comparison tests. Statistical analyses for survival rate were performed by Log-rank (Mantel-Cox) test and Gehan-Breslow-Wilcoxon test. A $p<0.05$ was considered to be statistically significant. All these statistical analyses and graphic representations were obtained by using the GraphPad Prism 7.0 software.

Supplemental Figures and Legends:

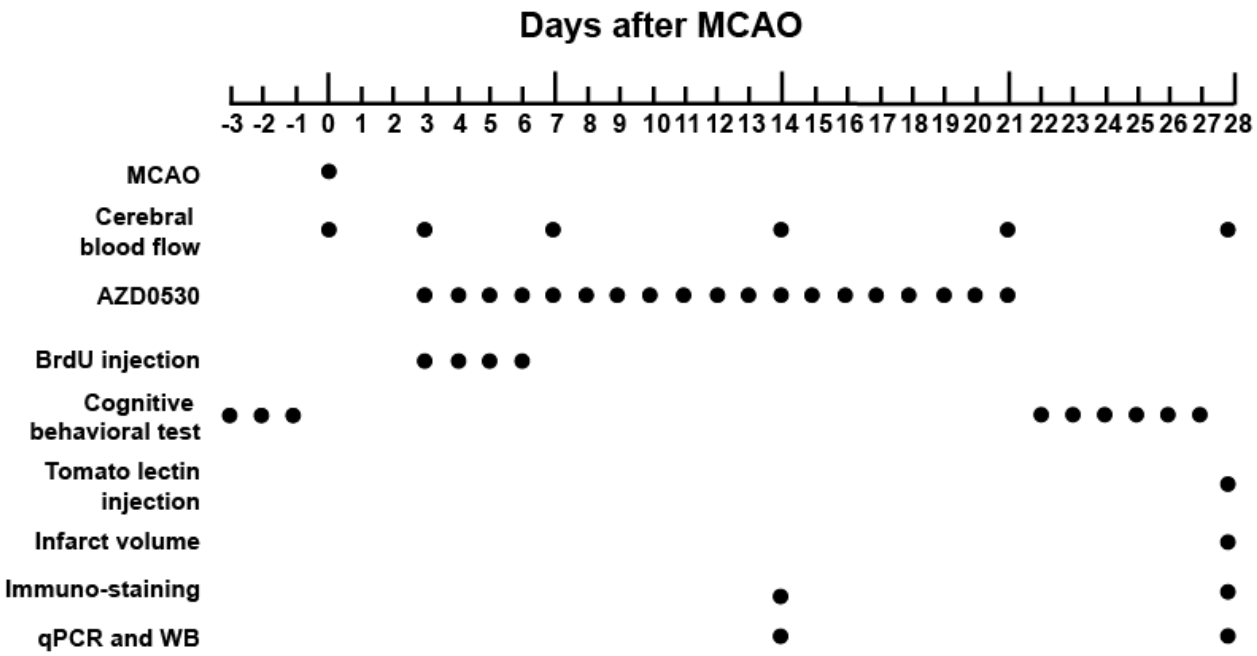


Fig. S1. Schematic diagram of experimental design. Mice were subjected to 1 h transient middle cerebral artery occlusion (MCAO). Cognitive behavioral test was performed up to 27 d after tMCAO. The Src inhibitor AZD0530 (20 mg/kg) was administered daily at 3-21 d after MCAO by oral gavage. To detect newly proliferated cells, BrdU was injected intraperitoneally twice per day with an interval of 6 h at 3-6 d after MCAO. To examine functional blood vessels, tomato lectin was injected transcardially 5 min before euthanasia at 28 d after MCAO. Cerebral blood flow was monitored by laser speckle imaging at 15 min before MCAO, 15 min after the onset of MCAO, and 15 min, 3 d, 7 d, 14 d, 21 d, and 28 d after the onset of reperfusion. Immunohistological and biochemical analysis utilized brain sections or samples were harvested at 28 d after MCAO.

Experimental gropus	Excluded animal number	Time points	Reason
Vehicle animals	1	Day 0	CBF did not show a >75% reduction after MCAO
Vehicle animals	1	Day 0	CBF <60% reperfusion over baseline levels after MCAO
AZD0530 animals	1	Day 0	CBF <60% reperfusion over baseline levels after MCAO
Vehicle animals	4	Day 0	Death during surgery
AZD0530 animals	5	Day 0	Death during surgery
Vehicle animals	2	Day 1	Death
AZD0530 animals	3	Day 1	Death
Vehicle animals	2	Day 2	No visible neurological deficits
AZD0530 animals	2	Day 2	No visible neurological deficits
AZD0530 animals	1	Day 3	Death
Vehicle animals	2	Day 4	Death
Vehicle animals	1	Day 5	Moribund and unable to eat and drink, euthanized
Vehicle animals	2	Day 5	Death
AZD0530 animals	1	Day 5	Death
AZD0530 animals	1	Day 6	Death
AZD0530 animals	1	Day 7	Death
Vehicle animals	1	Day 8	Death
AZD0530 animals	1	Day 9	Death
AZD0530 animals	2	Day 10	Death
AZD0530 animals	1	Day 25	Reluctant to swim in water-maze test

Fig. S2. Animal exclusion for WT or endothelium-targeted miR-15a/16-1 deletion mice treated with vehicle or AZD0530. All animals that fulfilled the exclusion criteria were excluded from the data analyses.

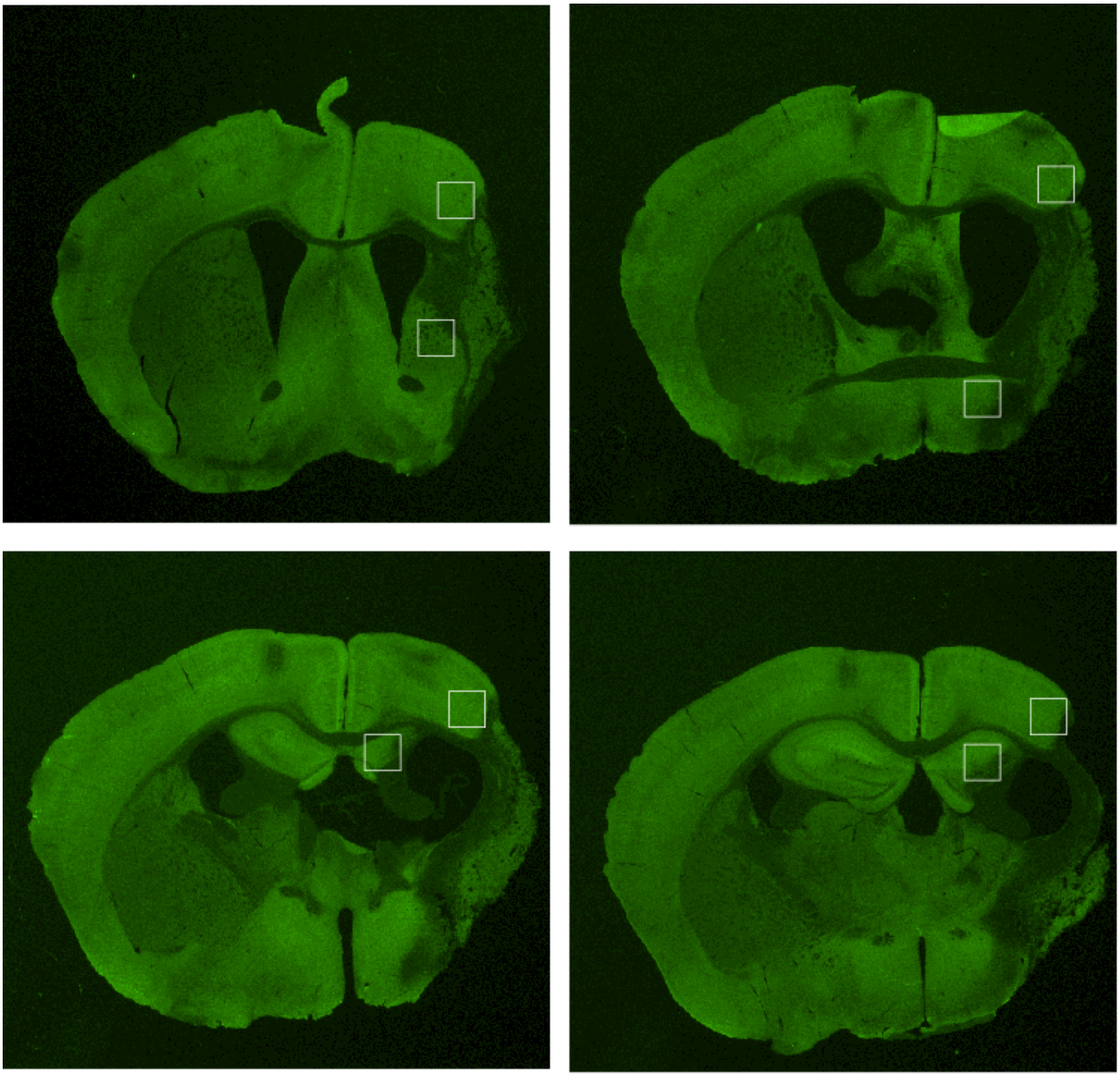
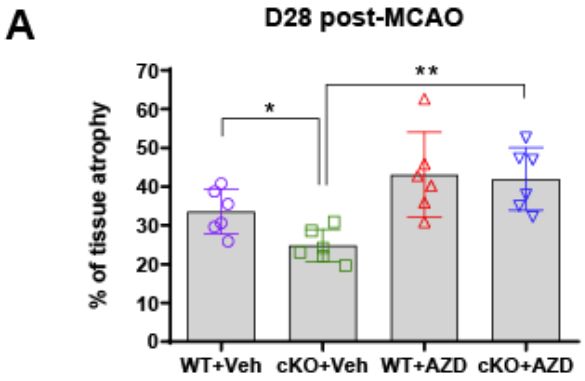
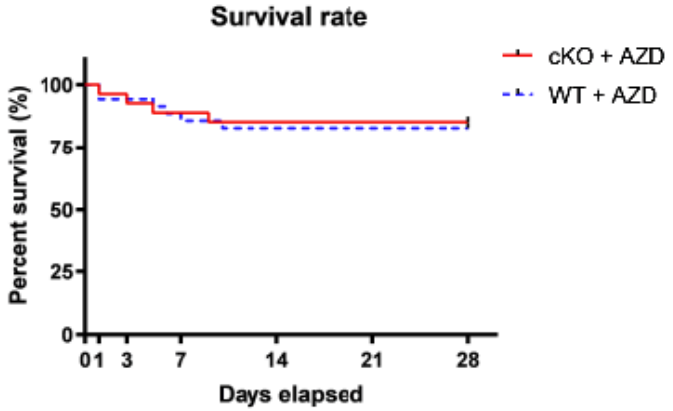


Fig. S3. Regions of interest in the peri-infarct areas that were selected for confocal microscopy. Mouse brains were harvested at 14 or 28 d after MCAO. Four equally spaced (1 mm) brain sections were selected for each animal, and MAP2 immunofluorescent staining was used to determine healthy and infarct brain tissue after stroke. The white-squared areas are the regions of interest in the peri-infarct areas that were selected for confocal microscopy.



B



Days elapsed	cKO+AZD	WT+AZD
0	100.00	100.00
1	96.30	94.29
3	92.59	
5	88.89	91.43
6		88.57
7		85.71
9	85.19	
10		82.86
28	85.19	82.86

Fig. S4. Brain atrophy percentage and sequential tracing of survival rate in WT or endothelium-targeted miR-15a/16-1 deletion mice treated with AZD0530 following cerebral ischemia. EC-miR-15a/16-1 cKO mice and WT littermate controls were subjected to 1h MCAO followed by 28d of reperfusion. Vehicle (Veh) or AZD0530 (AZD, 20 mg/kg) was administered daily to both genotypes 3-21d after MCAO by oral gavage. ***A***, Brain atrophy percentage of the ipsilateral hemisphere to contralateral hemisphere was calculated for microtubule-associated protein 2 (MAP2) immunostaining in **Fig 2*E,F***. Data are expressed as the mean ± SD. n = 6 for each group. $^{*}p < 0.05$ and $^{**}p < 0.05$ as indicated. Statistical analysis was performed by one-way ANOVA and followed by Bonferroni's multiple comparison tests. ***B***, The survival curve of EC-miR-15a/16-1 WT and cKO mice after MCAO was traced and each death event was recorded immediately. No survival rate difference was found between two genotypes of mice following MCAO and treated with AZD0530. n = 35 for WT+AZD mice and n = 27 for cKO+AZD mice. Statistical analyses were performed by Log-rank (Mantel-Cox) test (p = 0.8209) and Gehan-Breslow-Wilcoxon test (p = 0.8332).

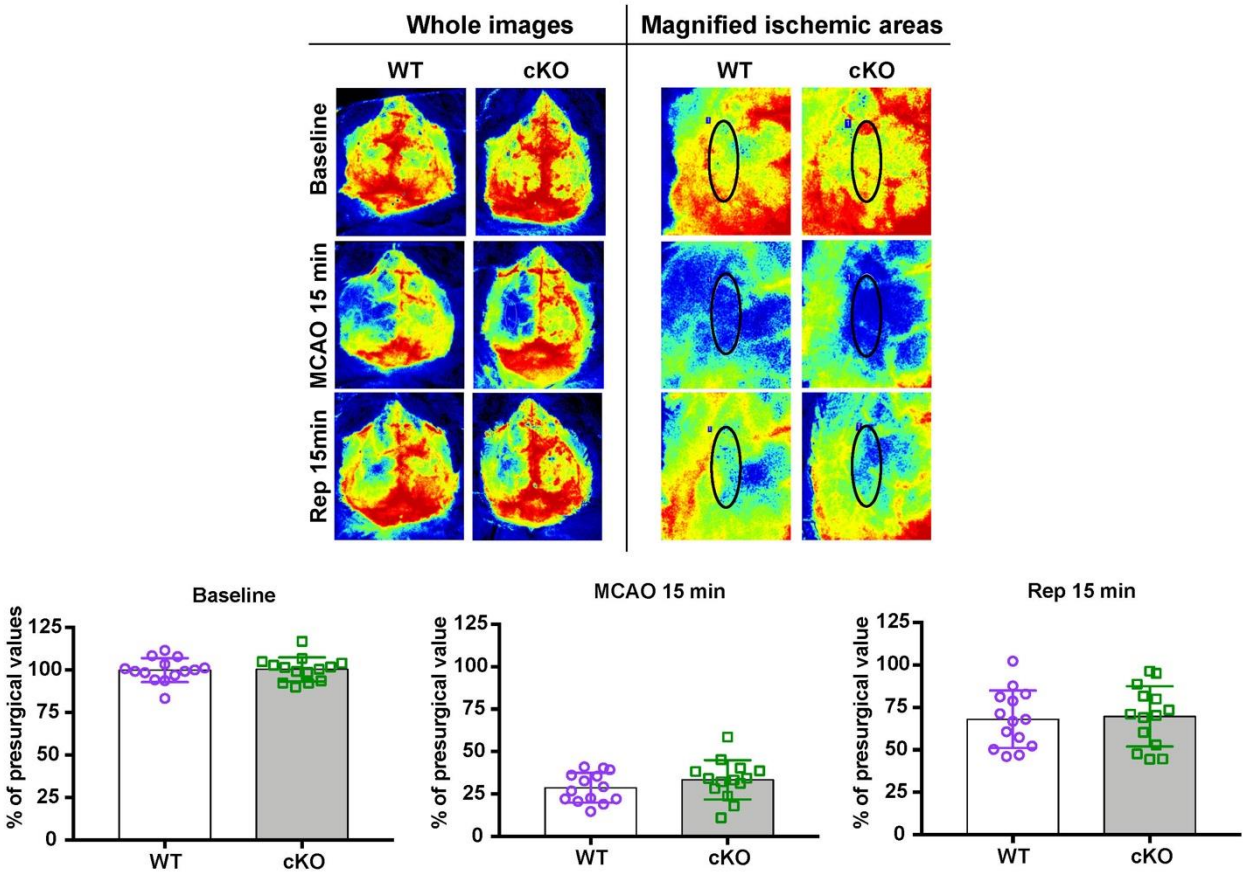


Fig. S5. The regional cerebral blood flow during MCAO surgery in EC-miR-15a/16-1 WT and cKO mice before AZD0530 treatment. Laser speckle imager was used to measure the regional CBF in EC-miR-15a/16-1 WT and cKO mice during MCAO surgery. Representative CBF images were shown at 15 min before cerebral ischemia (Baseline), 15 min after ischemia (MCAO 15 min) and 15 min after reperfusion (Rep 15 min). Two identical elliptical ROIs were selected as indicated on the ipsilateral and contralateral hemispheres. The relative CBF was first determined as the ratio of ischemic to non-ischemic values, and then normalized to the pre-MCAO baseline for each animal. Quantitative data analyses showed similar changes in regional CBF between EC-miR-15a/16-1 WT and cKO mice. Data are expressed as mean ± SD; n = 14/ group; statistical analyses were performed by two-tailed Student's t-test.

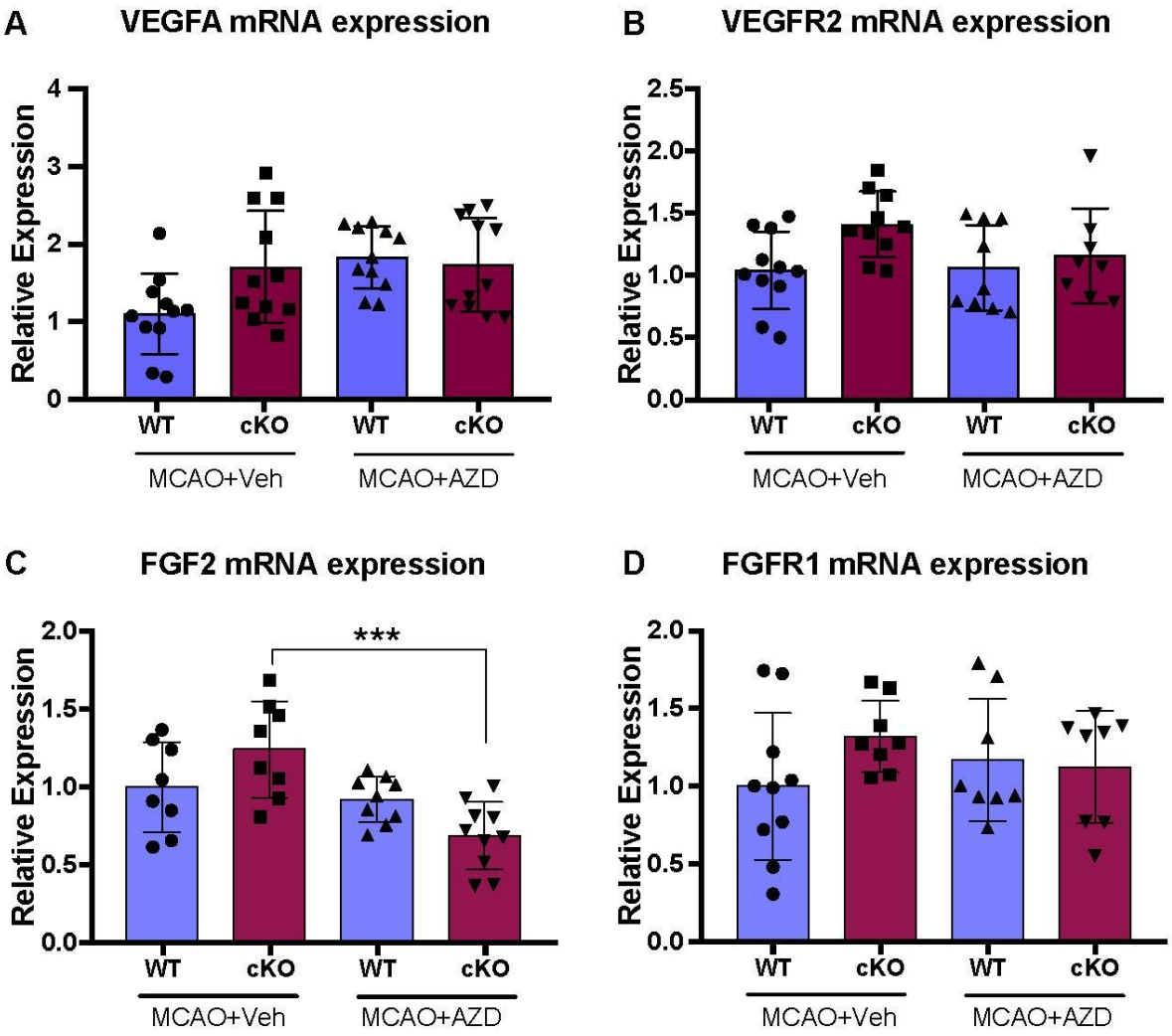


Fig. S6. Effects of AZD0530 treatment on the mRNA expression of VEGFA, FGF2 and their receptors VEGFR2 and FGFR1 in the ischemic cortical brains of EC-miR-15a/16-1 WT and cKO mice. Under Vehicle (Veh) treatment, qPCR data showed statistically significant up-regulated mRNA expression for VEGFA (***A***) and VEGFR2 (***B***), and enhanced trends for FGF2 (***C***) and FGFR1 (***D***), in the cerebral cortex of EC-miR-15a/16-1 cKO mice compared to WT controls. AZD0530 treatment statistically significantly down-regulated mRNA expression for FGF2 (***C***) in EC-miR-15a/16-1 cKO mice, without statistically significantly altered the mRNA expression of other angiogenic factors in the ischemic cerebral cortex in EC-miR-15a/16-1 WT or cKO mice. Data are expressed as mean ± SD; n = 8-10/group; $^{***}p < 0.0001$ as indicated; statistical analyses were performed by one-way ANOVA followed by Bonferroni's multiple comparison tests.

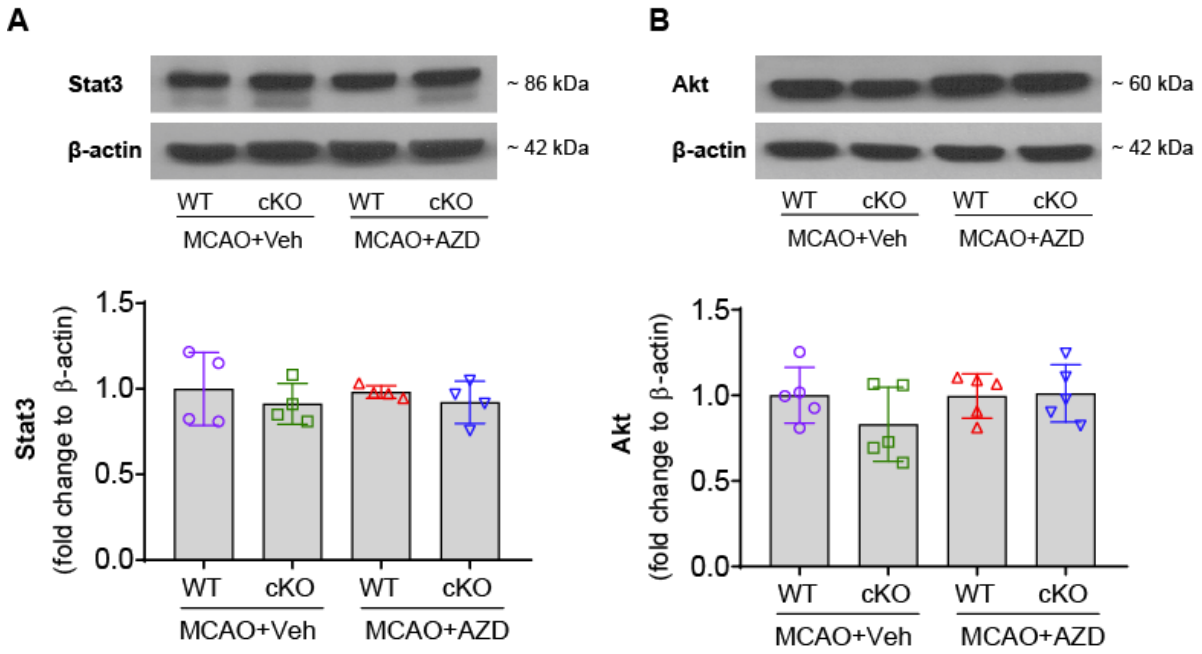


Fig. S7. AZD0530 treatment does not affect the total protein levels of Stat3 and Akt in the ischemic brains of WT and EC-targeted miR-15a/16-1 cKO mice. EC-miR-15a/16-1 cKO mice and WT littermate controls were subjected to 1h MCAO followed by 14d reperfusion. Vehicle (Veh) or AZD0530 (AZD, 20 mg/kg) was administered daily to both genotypes at 3-14d after MCAO by oral gavage. Total proteins were isolated from the ipsilateral cortex of ischemic brains, and western blotting was carried out to detect the total protein expression of Stat3 and Akt. ***A-B***, representative western blotting images and quantitative analysis show that genetic deletion of miR-15a/16-1 in endothelium did not affect the total protein levels of Stat3 (***A***) and Akt (***B***), and the administration of AZD0530 did not affect the total protein levels of Stat3 (***A***) and Akt (***B***) for WT and cKO groups, either. n = 4/group for ***A*** and n = 5/group for ***B***. Statistical analysis was performed by one-way ANOVA followed by Bonferroni's multiple comparison tests.

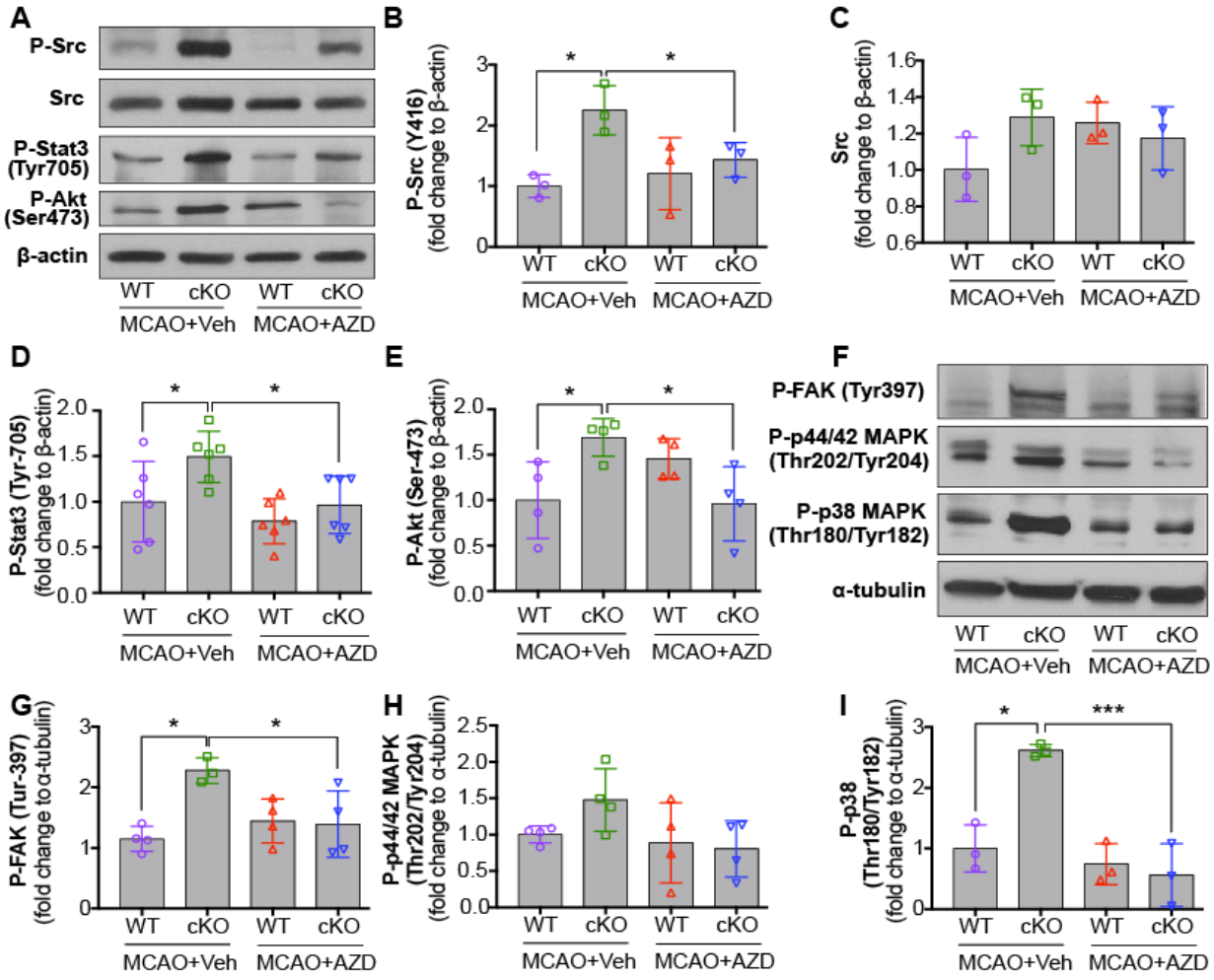


Fig. S8. AZD0530 treatment blocks the Src signaling pathway in the ischemic brains of EC-targeted miR-15a/16-1 cKO mice. EC-miR-15a/16-1 cKO mice and WT littermate controls were subjected to 1h MCAO and 28d reperfusion. Vehicle (Veh) or AZD0530 (AZD, 20 mg/kg) was administered daily to both genotypes at 3-21d after MCAO by oral gavage. Total proteins were isolated from the ipsilateral cortex of ischemic brains, and western blotting was carried out to detect the expression of proteins in the Src signaling pathway. ***A-I***, representative western blotting images (***A,F***) and quantitative analysis (***B,C,D,E,G,I***) indicated endothelial-selective deletion of the miR-15a/16-1 cluster upregulated the Src signaling pathway by enhancing the expressing of phosphorylated-Src (Y416) (***B***), phosphorylated-Stat3 (Tyr-705) (***D***), phosphorylated-Akt (Ser-473) (***E***), phosphorylated-FAK (Tur-397) (**G**), and phosphorylated-p38 (Thr180/Tyr182) (***I***), whereas AZD0530 effectively downregulated the Src signaling pathway by downregulating phosphorylation of Src (***B***) and its downstream mediators (***D,E,G,I***) in the ischemic brains of EC-miR-15a/16-1 cKO mice. Data are expressed as the mean ± SD. n = 3-4/group for ***B,C,E,G,H,I***. n = 6/group for ***D***. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ as indicated. Statistical analysis was performed by one-way ANOVA followed by Bonferroni's multiple comparison tests.

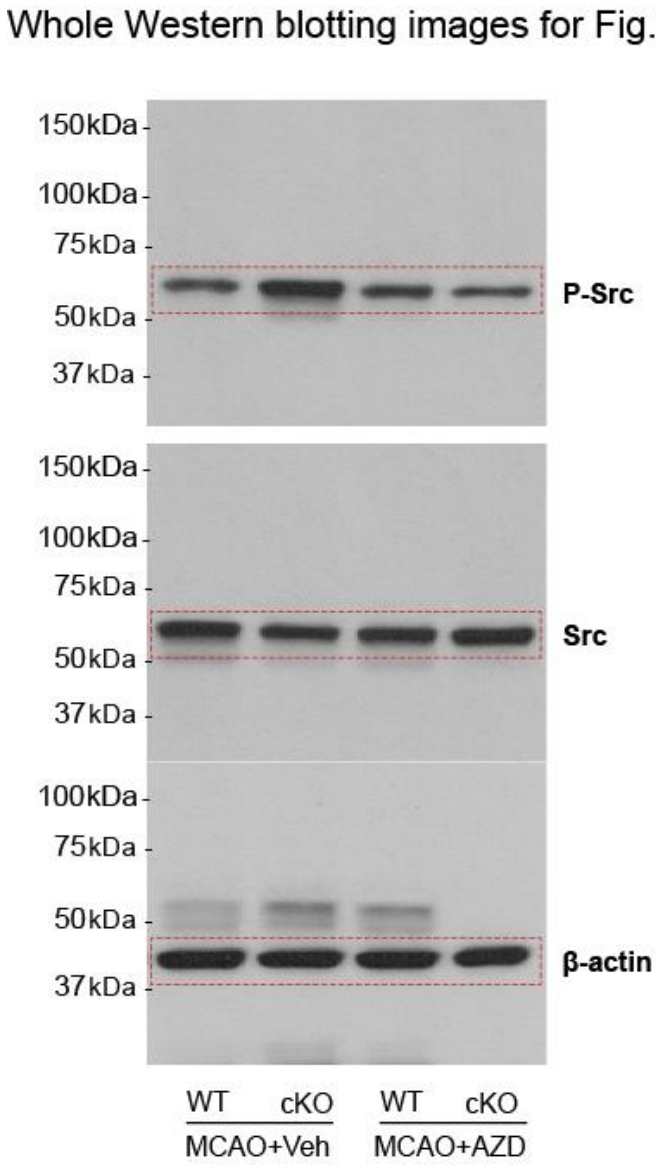


Fig. S9. Images of full-length blots presented in Fig. 1.

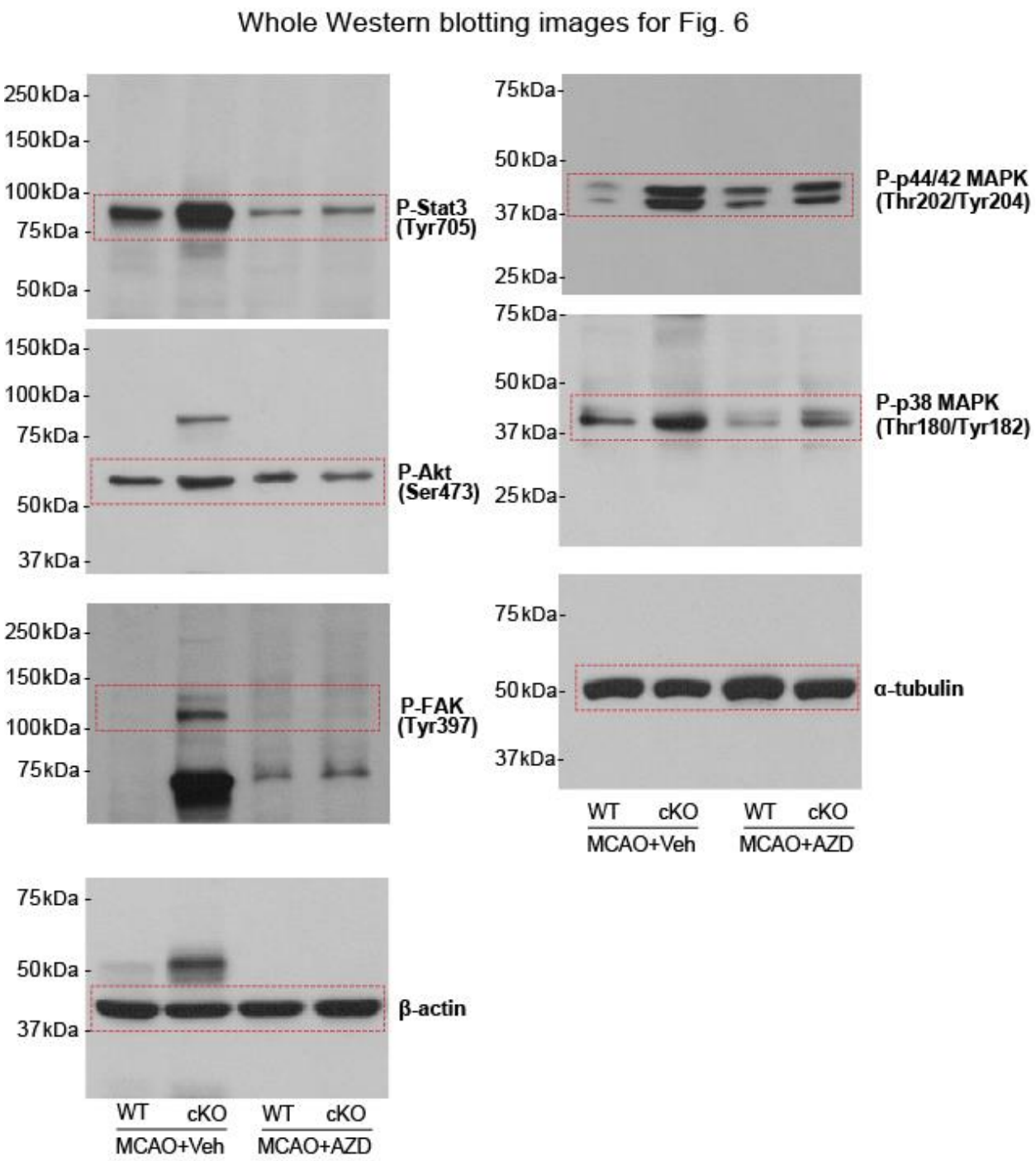


Fig. S10. Images of full-length blots presented in Fig. 6.

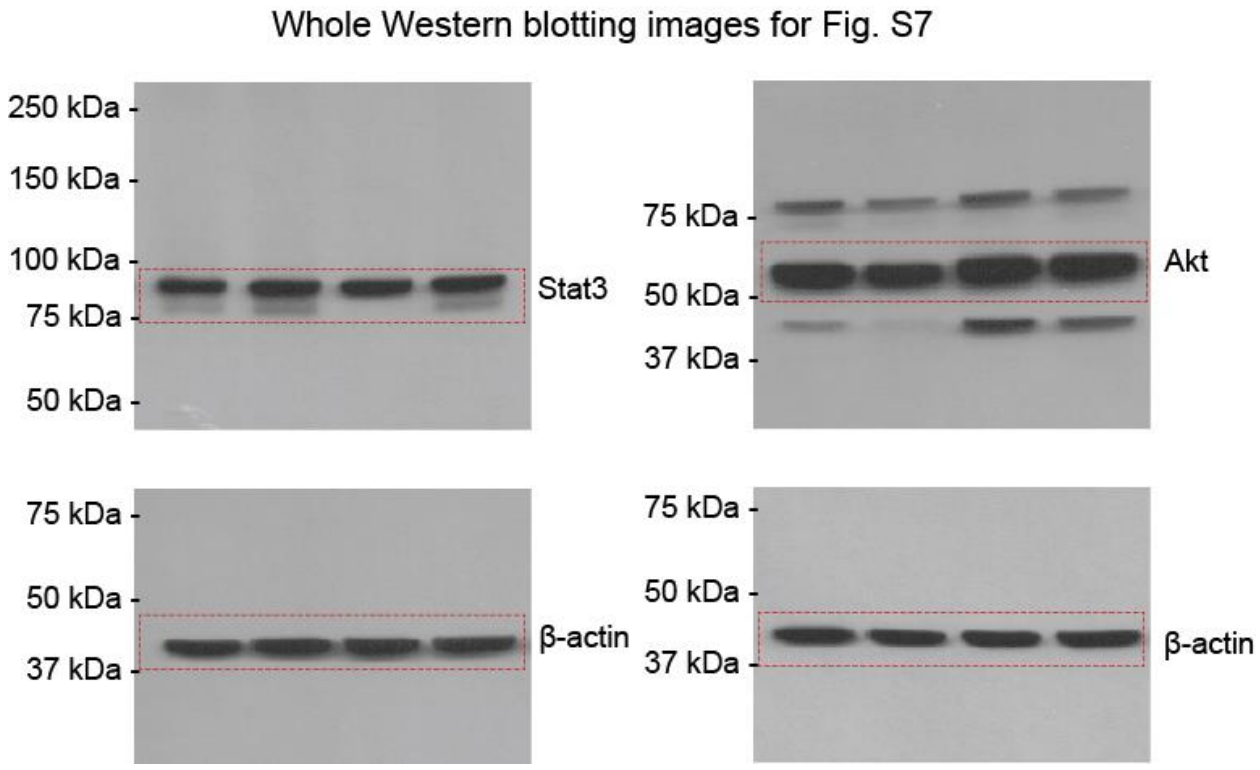


Fig. S11. Images of full-length blots presented in Fig. S7.

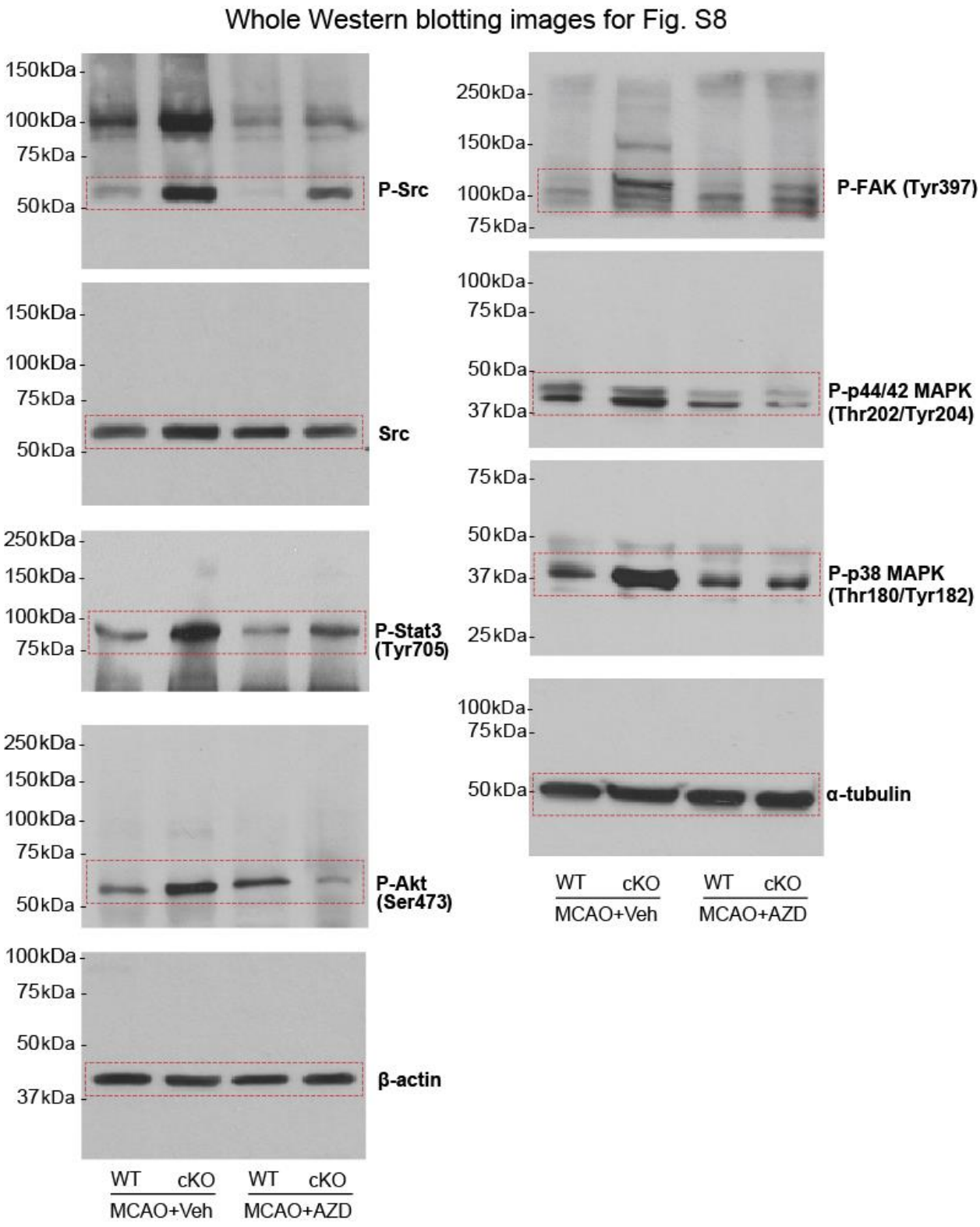


Fig. S12. Images of full-length blots presented in Fig. S8.

Table S1. List of primer sequences

Gene	Primer sequence
mVEGF-A164	forward, 5'-AACAAAGCCAGAAAATCACTGTGA-3'
	reverse, 5'-CGGATCTTGGACAAACAAATGC-3'
mVEGFR2 (Flk-1)	forward, 5'-TTTGGCAAATACAACCCTTCAGA-3'
	reverse, 5'-GCAGAAGATACTGTCACCACC-3'
mFGF	forward, 5'-GAGTTGTGTCTATCAAGGGAGTG-3'
	reverse, 5'-CCGTCCATCTTCCTTCATAGC-3'
mFGFR1	forward, 5'- ACTCTGCGCTGGTTGAAAAAT-3'
	reverse, 5'- GTAGCCTCCAATTCGGTGGTC-3'
cyclophilin	forward, 5'-ACTCCTCATTTAGATGGGCATCA-3'
	reverse, 5'-GAGTATCCGTACCTCCGCAAA-3'

Table S2. List of primary antibodies

Antibodies	Host species	Dilution used	Companies	Ref #
mouse CD31	Rat	1:200	BD Pharmingen (San Diego, CA, USA)	553370
mouse microtubule-associated protein 2 (MAP2)	Rabbit	1:500	EMD Millipore (Burlington, MA, USA)	AB5622
5-bromo-2'-deoxyuridine (BrdU)	mouse	1:200	BD Pharmingen	555627
Src (36D10)	Rabbit	1:2000	Cell Signaling (Beverly, MA, USA)	2109
Phospho-Src Family (Tyr416)	Rabbit	1:1000	Cell Signaling	2101
Phospho-Stat3 (Tyr705)	Rabbit	1:1500	Cell Signaling	9145
Phospho-Akt (Ser473)	Rabbit	1:1000	Cell Signaling	4060
Phospho-p38 MAPK (Thr180/Tyr182)	Rabbit	1:1000	Cell Signaling	4511
Phospho-p44/42 MAPK (Erk1/2) (Thr202/Tyr204)	Rabbit	1:1000	Cell Signaling	4377
Phospho-FAK (Tyr397)	Rabbit	1:500	Cell Signaling	3283
Stat3	Rabbit	1:2000	Cell Signaling	4904
Akt	Rabbit	1:2000	Cell Signaling	9272
α-Tubulin	Mouse	1:2000	Cell Signaling	3873
β-actin	Mouse	1:2000	Sigma-Aldrich	A5441

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