**Supplementary material**

**Identification of two phosphorylation sites essential for Annexin A1 in blood-brain barrier protection after experimental intracerebral hemorrhage in rats**

**Supplementary Materials and Methods**

**Antibodies**

Antibodies against ANXA1 (ab115770, ab137745), albumin (ab106582), GFP (ab6556), and rhANXA1 (ab92966) were from Abcam. Antibody against cleaved caspase3 (9661) was from Cell Signaling Technology. Antibodies against p-Ser (sc-81514), p-Thr (sc-5267), p-Tyr (sc-7020), FPR2 (sc-66901), β-Tubulin (sc-9140) and β-actin (sc-47778) were from Santa Cruz Biotechnology. FOX3-NeuN antibody-Neuronal cell marker (ab104224) and vWF antibody-endothelial cell marker (ab6994) were from Abcam. Secondary antibodies for western blot analysis, including goat anti-rabbit IgG-HRP (sc-2004) and goat anti-mouse IgG-HRP (sc-2005) from Santa Cruz Biotechnology, rabbit anti-goat IgG-HRP (GP016029) from Genetech, and goat Anti-chicken IgY-HRP (A00165) from Genscript. Normal rabbit IgG (sc-2027), normal mouse IgG (sc-2025) and normal goat IgG (sc-2028) were from Santa Cruz Biotechnology. Secondary antibodies for immunofluorescence, including Alexa Fluor-555 donkey anti-rabbit IgG antibody (A31572), Alexa Fluor-488 donkey anti-goat IgG antibody (A21206) and Alexa Fluor-555 donkey anti-mouse IgG antibody (A31570), were from Invitrogen.

**ANXA1 siRNA sequences**

(I) Sense: 5‘ GGAUGAAACACUUAAGAAA dTdT 3‘

Antisense: 3‘ dTdT CCUACUUUGUGAAUUCUUU 5‘

(II) Sense: 5‘ GACGUAAACGUGUUCAAUA dTdT 3‘

Antisense: 3‘ dTdT CUGCAUUUGCACAAGUUAU 5‘

(III) Sense: 5‘ GUUCUGAAAUUGACAUGAA dTdT 3‘

Antisense: 3‘ dTdT CAAGACUUUAACUGUACUU 5‘

**TUNEL and FJB staining**

Briefly, TUNEL staining was performed on paraffin embedded brain sections (4 μm thick) by In Situ Cell Death Detection Kit (Roche, 11684795910) according to the manufacturer’s instructions. Brain sections were observed under a fluorescence microscope, and Image J software was used to analyze TUNEL staining. For FJB staining, after being dewaxed, the sections were incubated with 0.06% KMnO4 solution in dark at room temperature for 15 min. The sections were then washed with PBS (5 min/time) for 3 times, and were incubated with FJB working solution (containing 0.1% acetic acid) for 60 min. Subsequently, the sections were washed 3 times and air-dried at room temperature in dark. Finally, brain sections were observed under a fluorescence microscope, and Image J software was used to analyze the positive rate of FJB. The quantitative analysis was performed by an observer who was blind to the experimental group.

**Cell viability**

Briefly, hCMEC/D3 cells were plated in 96-well plates at a density $1 \times 10^6$/ml and grown for approximately 24 h. When cell density reached 70–80% confluence, cells were exposed to indicated treatments. And then, cells were fixed with 50% trichloroacetic acid and stained with a Sulforhodamine B (SRB) Solution, as described previously [1]. Finally, SRB was measured by absorbance at 565 nm wavelength using a BioRad Micro plate reader. Assay was performed in triplicate and repeated at least three independent times.

**Immunoprecipitation analysis**

Firstly, the brain samples were lysed in ice-cold RIPA lysis buffer (Beyotime, China). For immunoprecipitation, the lysate was incubated with specific antibodies or rabbit IgG (negative control) overnight at 4℃ with agitation. Protein A+G Sepharose beads were then added to each immune complex and the lysate-bead mixture was incubated for 4 h at 4℃ with rotary agitation. The beads

were washed three times with RIPA lysis buffer and then eluted with 4× SDS loading buffer. Western blot assay were then performed for further protein separation and detection.

**Ponceau S staining**

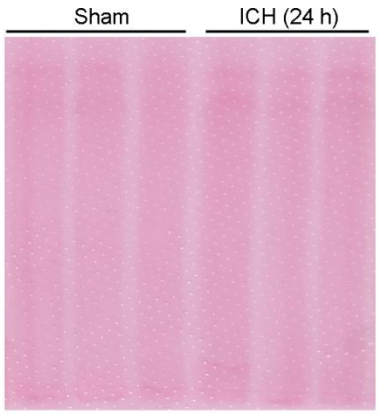
Briefly, the total protein samples form isolated microvessels (50 μg/lane) were loaded on a SDS-polyacrylamide gel, separated, and electrophoretically transferred to a polyvinylidene difluoride membrane. Then, the membrane was covered with ponceau S solution and agitated for about 30 s. Subsequently, the membrane was washed with $ddH_2O$ until bands become visible. Finally, the membrane was captured, and the level of total protein was analyzed by use of Image J program. The quantitative analysis was performed by an observer who was blind to the experimental group.

**Supplementary References**

1. Paximadis P, Najy AJ, Snyder M and Kim HR. The interaction between androgen receptor and PDGF-D in the radiation response of prostate carcinoma. *The Prostate*. 2016.

**Supplementary Figures and Supplementary Figure legends**

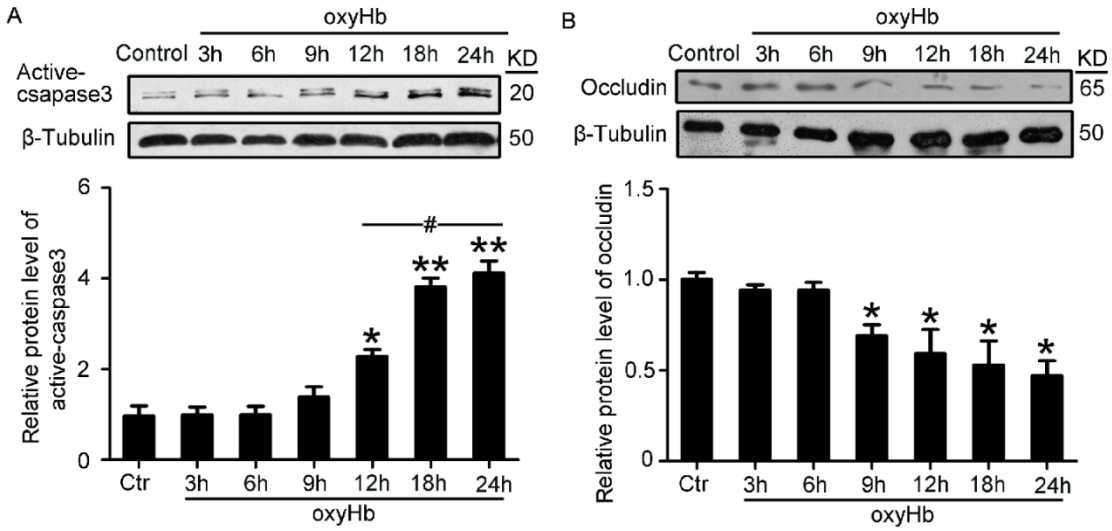
**Supplementary Figure.1**




**Supplementary Figure.1** Ponceau S staining for total protein determination. The total protein samples form isolated microvessels (50 μg/lane) were loaded on a SDS-polyacrylamide gel, separated, and

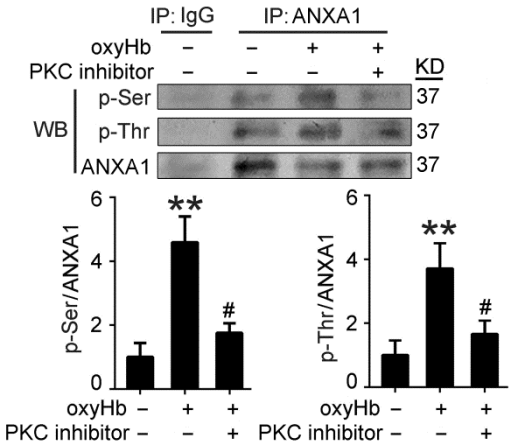
electrophoretically transferred to a polyvinylidene difluoride membrane. Then, the membrane was stained with ponceau S and captured. Three representative lands of sham group and ICH (24 h) group were shown.

**Supplementary Figure.2**



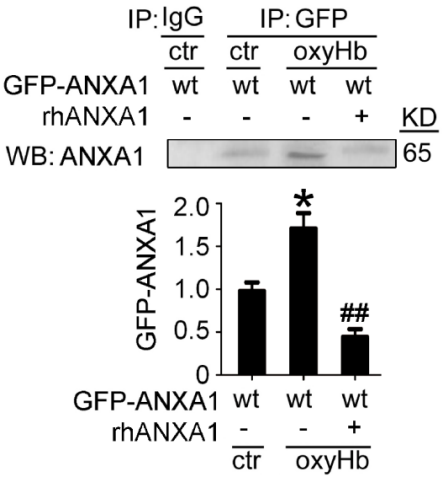

**Supplementary Figure.2** Time course of the protein levels of active-caspase3 and occludin in oxyHb-treatment hCMEC/D3 cells. Western blot analysis and quantification of the protein levels of active-caspase3 (***A***) and occludin (***B***) in brain capillaries. Mean values for control group were normalized to 1.0. One-way ANOVA followed by Student–Newman–Keulspost hoctests were used. Data are means ± SEM. * $p < 0.05$,* * $p < 0.01$ vs. control group, $^{\#}$ $p < 0.05$, n = 3.

**Supplementary Figure.3**




**Supplementary Figure.3:** Immunoprecipitation (IP) of cell lysates with ANXA1 antibody. Western blots of IP with indicated antibodies showed the phosphorylation of ANXA1 at the threonine residues and serine residues. Mean values for control group were normalized to 1.0. One-way ANOVA followed by Student–Newman–Keulspost hoctests were used. Data are means ± SEM. * * $p < 0.01$ vs. control group, $^{\#}$ $p < 0.05$ vs. oxyHb group, n = 3.

**Supplementary Figure.4**




**Supplementary Figure.4:** Immunoprecipitation (IP) of cultured supernatant of hCMEC/D3 cells with GFP antibody. Western blots of IP with ANXA1 antibody showed the cultured supernatant content of wild type GFP-ANXA1. The relative protein levels was shown below. Mean values for control + wild

type GFP-ANXA1 group were normalized to 1.0. One-way ANOVA followed by Student–Newman–Keulspost hoctests were used. Data are means ± SEM. * $p < 0.05$ vs. control + wild type GFP-ANXA1 group, $^{\#\#}$ $p < 0.01$ vs. oxyHb + wild type GFP-ANXA1 group, n = 3.