Supplementary Materials: Sesamin inhibits hypoxia-stimulated angiogenesis via NF-κB p65/HIF-1α/VEGFA signaling pathway in human colorectal cancer

Yefei Huang, Zixuan Liu, Lingling Li, Min Jiang, Yu Tang, Li Zhou, Jing Li and Yansu Chen

Supplemental Experimental Procedure

Western blot analysis

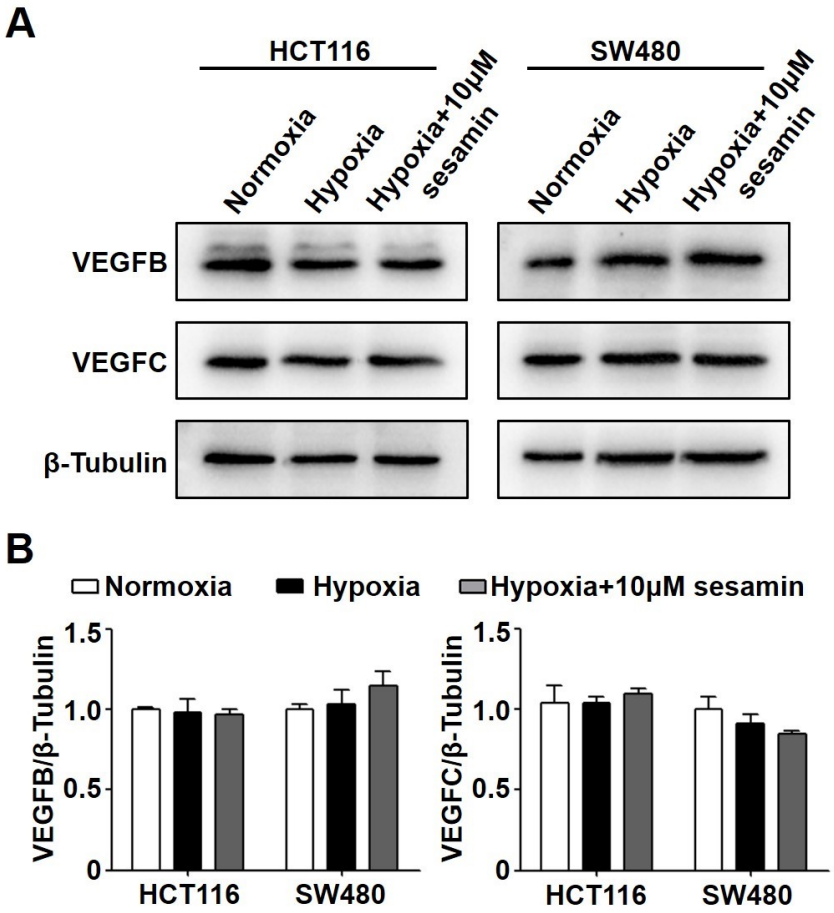
Total cell lysates were prepared with a detergent lysis buffer (Beyotime Biotechnology, China) on ice for 30 min and the supernatants were collected by centrifugation at 12,000 rpm for 15 min at 4 °C. The protein concentration was measured with BCA Protein Assay Kit (Beyotime Biotechnology, China). A total of 60 μg protein from cell lysates were separated by SDS-PAGE, and transferred by electroblotting to a polyvinylidene fluoride membrane. After blocked with 5% nonfat milk in TBST buffer for 1h, the membranes were cut according to the molecular weight of the antibody specification and the PageRuler™ Prestained Protein Ladder (Thermo Scientific, USA) on the membrane, then the membranes were incubated with primary antibodies, including anti-IκBα, anti-phospho-IκBα, anti-NF-κB p65, anti-phospho-NF-κB p65, anti-VEGFA, anti-HIF-1α, anti-Histone H3 and anti-β-tubulin (1:1000; CST, USA) overnight at 4°C. Then, the membranes were washed five times in TBST buffer (5 min for per time). After incubation with the appropriate secondary antibody (Beyotime Biotechnology, China) diluted in 5% nonfat milk for 1 h at room temperature, the membranes were washed ten times in TBST buffer (5 min for per time). The antigen-antibody complex was detected by a ChemiDoc XRS+ imaging system (Bio-Rad Laboratories, Inc., Hercules, CA, USA). The β-tubulin was used for the protein loading control. Moreover, we checked these same molecules in HCT116 and SW480 cells, and the results showed that the same molecule was displayed at the same location on the membrane and all the molecular weights were consistent with the antibody specifications. In addition, there were few or almost no non-specific bands in all blots. Western blot bands were quantified using the Image J software.

Immunohistochemistry (IHC)

The slides were dewaxed at 65 °C for 2 h and were dewaxed three times with xylene for 15 min each. The tissues were then rehydrated by washing the slides for 5-min each with 100%, 95%, 80%, 75%, 50% ethanol and finally with distilled water, and then put into boiling sodium citrate (pH 6.0) for 2 min to retrieve the antigen and washed with PBS (pH 7.4) three times for 5 min each. The endogenous peroxidase activity of the tissue was blocked by incubation in 3% hydrogen peroxide for 30 min and washed with PBS (pH 7.4) three times for 5 min each. The sections were blocked in 3% BSA for 30 min, and then incubated with polyclonal rabbit anti-Phospho-NF-kB p65 (Ser536) antibody (AF2006, 1:100 dilution; Affinity, USA), anti-HIF-1α antibody (48085S, 1:400 dilution; Cell Signaling Technology, USA), anti-VEGFA antibody (ER30607, 1:200 dilution; HUABIO, China) and anti-CD31 antibody (GB11063-2, 1:200 dilution; Servicebio, China) at 4°C overnight and washed with PBS (pH 7.4) three times for 5 min each. The sections were then incubated for 50 min each with a HPR-labeled secondary antibody (1:200 dilution; Servicebio, China) and washed with PBS (pH 7.4) three times for 5 min each. 3,3'-diaminobenzidine (DAB; GB1211, Servicebio, China) was used to produce a brown precipitate and counterstained with hematoxylin. The slides were then dehydrated according to the standard procedures and sealed with coverslips.

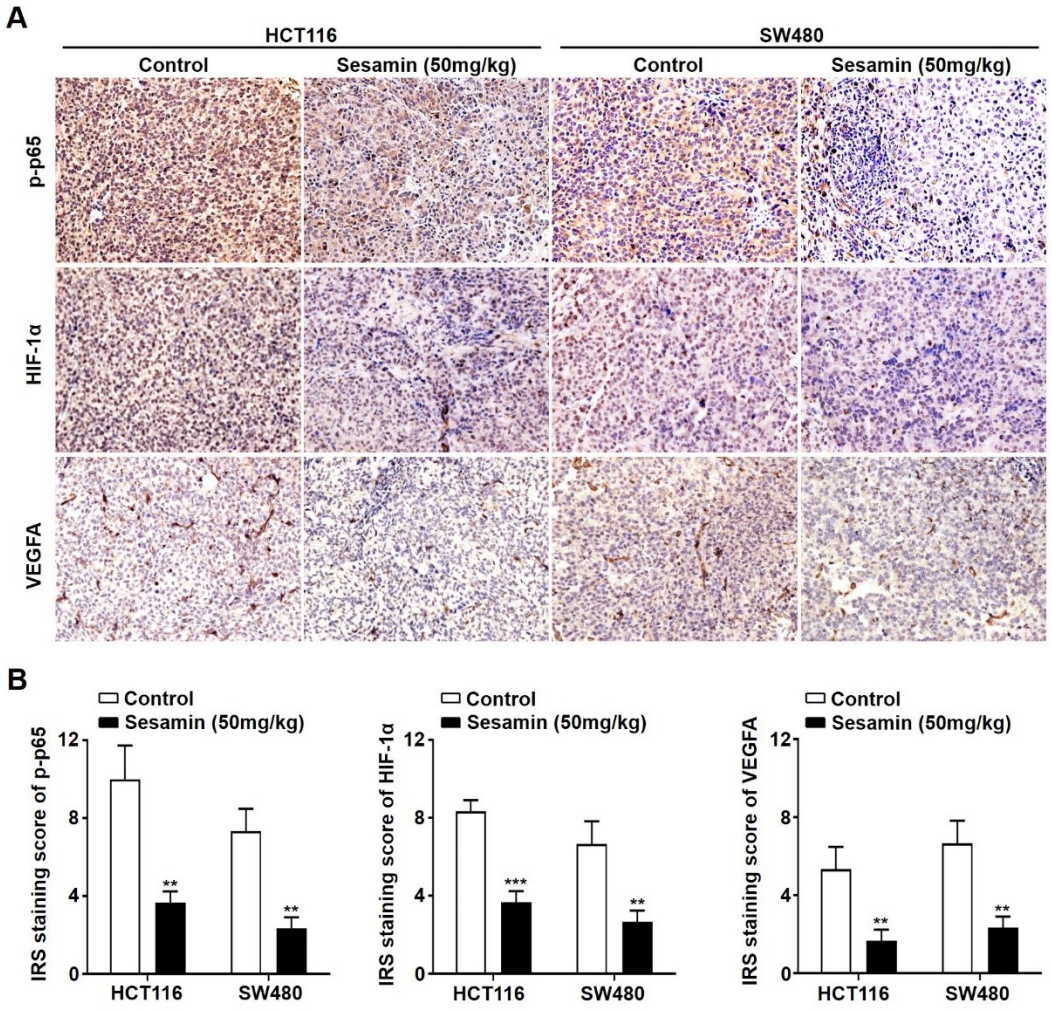
Supplemental Experimental Data

Supplemental Figure 1



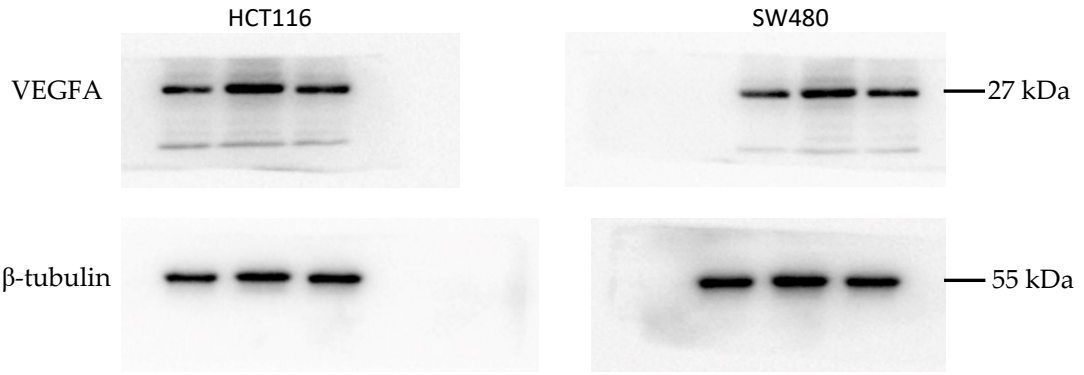
Supplemental Figure 1 Effects of sesamin on VEGFB and VEGFC protein expression in CRC cells under hypoxia. (A) The protein expression levels of VEGFB and VEGFC in HCT116 and SW480 cells under normoxia, hypoxia or hypoxia+10μM sesamin, and relative protein expression of VEGFB and VEGFC was normalized to respective β-tubulin (B). Note: Data were presented as mean ± SD.

Supplemental Figure 2

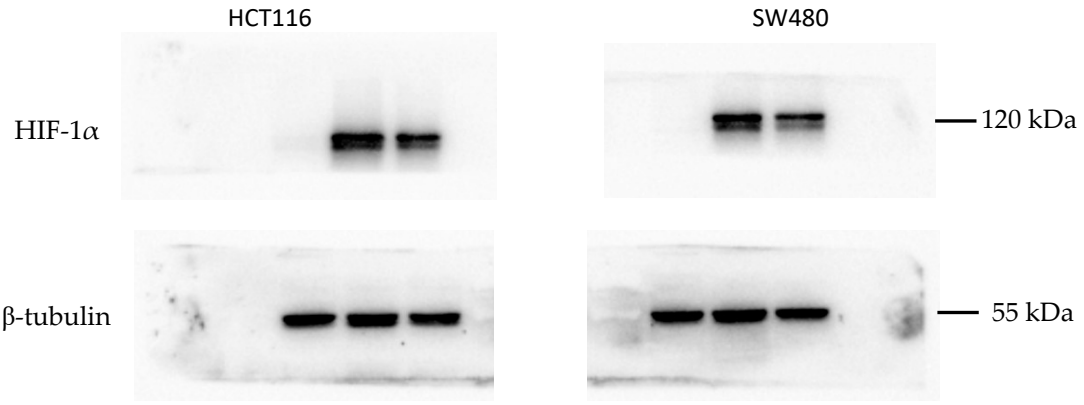


Supplemental Figure 2 Sesamin inhibited NF-κB/HIF-1α/VEGFA signaling pathway in vivo. (A) The matrigel plugs of HCT116 and SW480 cells excised from mice were performed immunochemistry staining by antibody against p-p65, HIF-1α and VEGFA (n = 5); representative images were shown (×200 magnification). (B) The IRS staining scores of p-p65, HIF-1α and VEGFA. Note: Data were presented as mean ± SD; *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

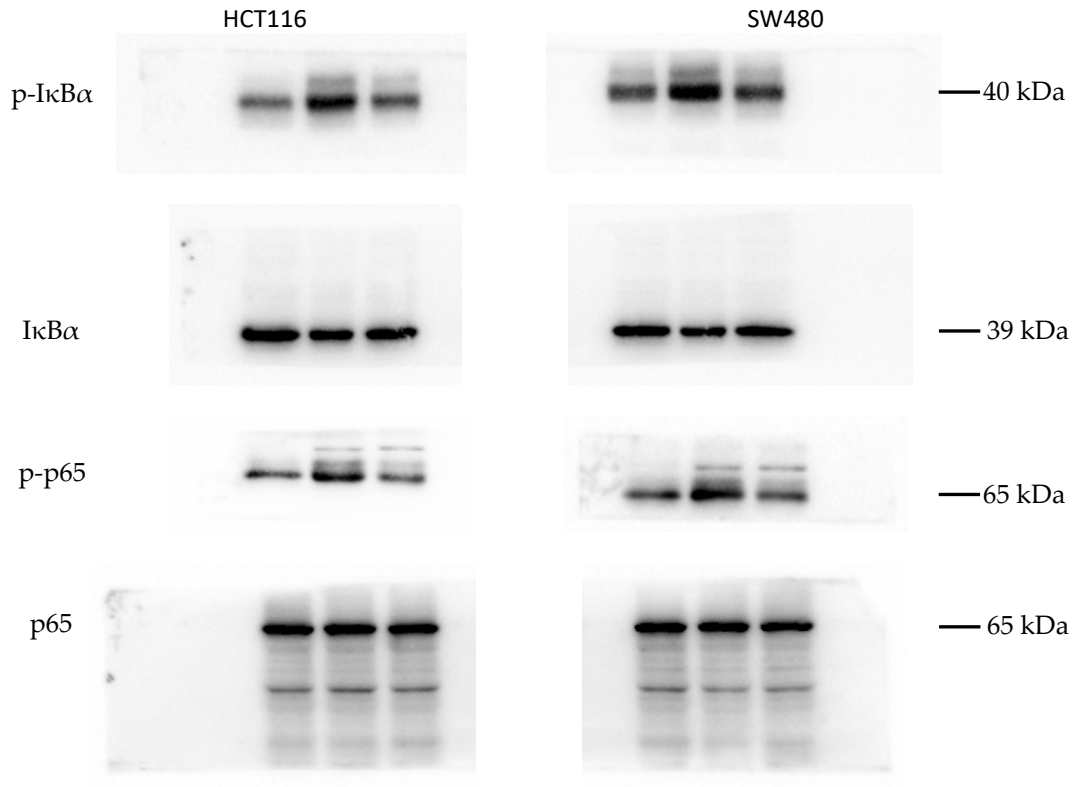
Original Images for Blots and Gels of Figure 3B



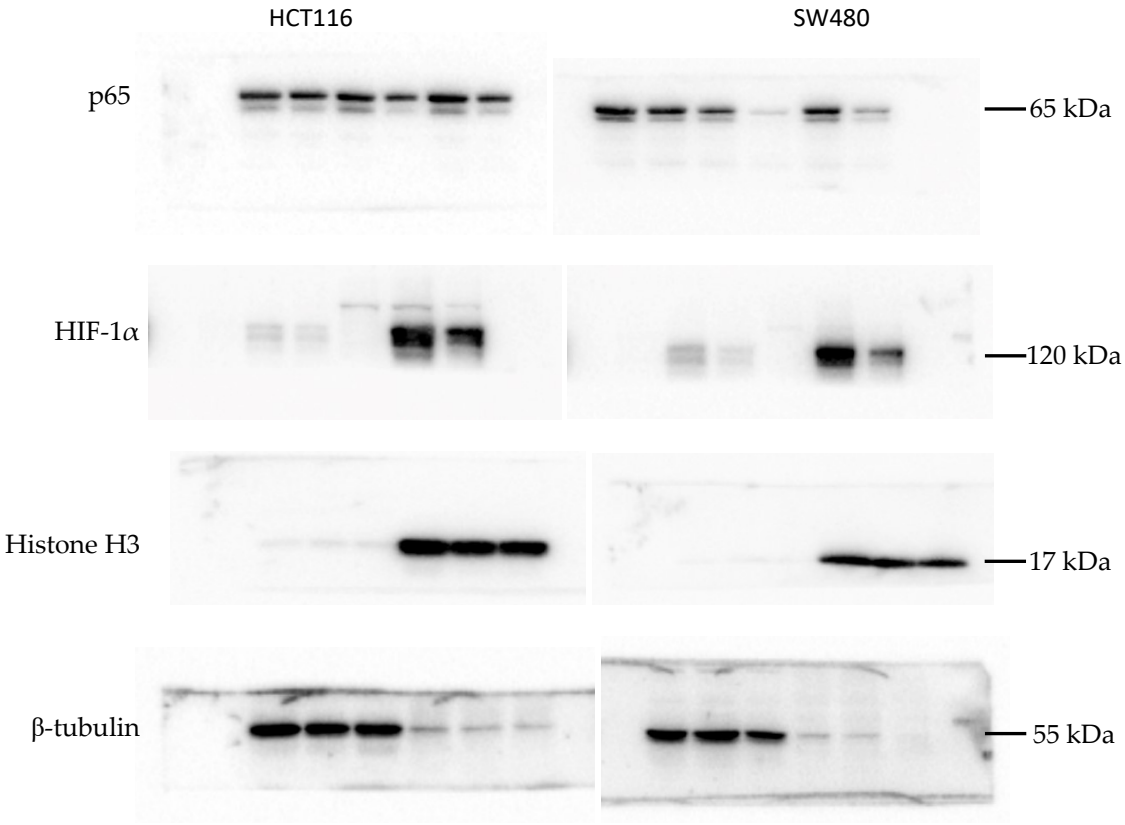
Original Images for Blots and Gels of Figure 4B



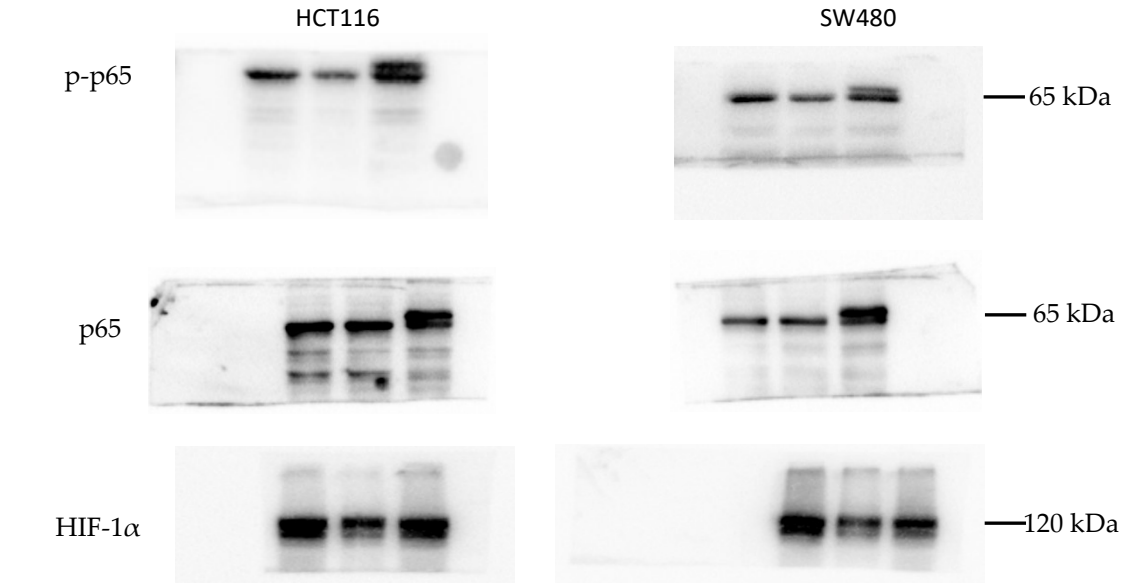
Original Images for Blots and Gels of Figure 4D

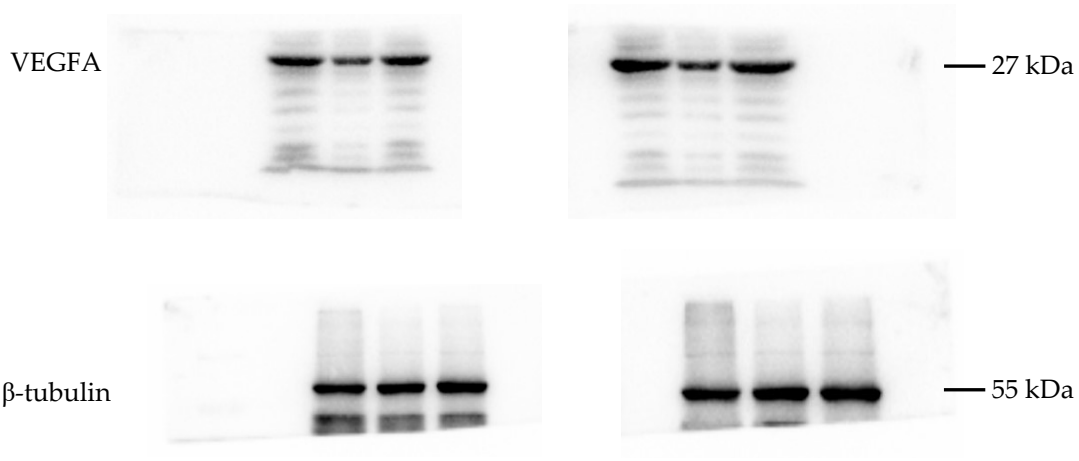


Original Images for Blots and Gels of Figure 5B



Original Images for Blots and Gels of Figure 6A





Original Images for Blots and Gels of Supplemental Figure 1A