



A Noncanonical RNA-Binding Function of NQO1 Drives Angiogenesis in Esophageal Squamous Cell Carcinoma via Extracellular Vesicle-Mediated AGRN Transfer

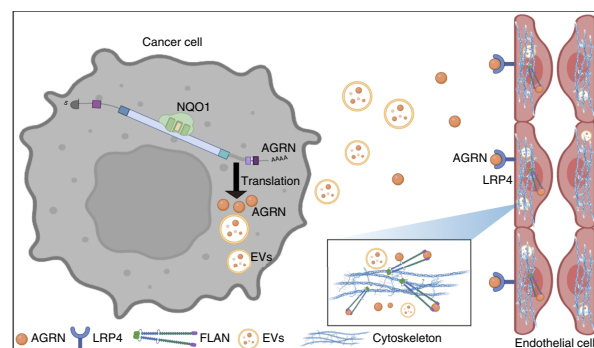
Jun Wu^{1,2}, Chen Chen³, Xiaoxia Lv⁴, Qiang Wang³, Zhangding Wang³, Yong Chen⁵, Jiaqi Hou⁶, Qingting Fan^{1,2}, Qinglin Ren⁶, Chao Sun^{1,2}, Shichun Lu^{1,2}, Yusheng Shu⁴, Shouyu Wang³, and Xiaolin Wang^{1,2}

ABSTRACT

Esophageal squamous cell carcinoma (ESCC) remains a highly aggressive malignancy with dismal clinical outcomes and limited treatment options. NAD(P)H quinone oxidoreductase 1 (NQO1) is classically characterized as a cytosolic oxidoreductase that prevents the formation of reactive oxygen species. In this study, we demonstrated that NQO1 promoted ESCC progression and lung colonization via an enzymatic activity-independent mechanism. Integrated transcriptomic and direct RNA-binding analyses revealed that NQO1 acted as an RNA-binding protein to stabilize the mRNA encoding agrin (AGRN), thereby increasing AGRN expression. Upregulation of AGRN enhanced endothelial cytoskeletal organization by interacting with filamin A and stimulated angiogenesis through selective extracellular vesicle-mediated transfer. Structure-based screening identified the clinically approved agent panobinostat as a direct NQO1-binding compound that destabilized NQO1 and suppressed AGRN-dependent angiogenic signaling. Importantly, combined treatment with panobinostat and the antiangiogenic agent anlotinib resulted in superior inhibition of tumor growth and vascularization compared with either monotherapy in patient-derived organoid xenograft models. Together, these findings uncover an enzymatic activity-independent RNA-regulatory function of

NQO1 in ESCC and provide a mechanistic rationale for targeting the NQO1/AGRN axis.

Significance: The enzyme-independent RNA regulatory function of NQO1 enhances extracellular vesicle-mediated AGRN signaling to drive angiogenesis in esophageal cancer, which induces a therapeutic vulnerability to combinatorial anti-angiogenic treatment with panobinostat and anlotinib.



Created in BioRender. Wu, J. (2026) <https://BioRender.com/adxmx32>

Introduction

Esophageal squamous cell carcinoma (ESCC) is the most prevalent histologic subtype of esophageal cancer, accounting for nearly 90% of all esophageal cancer cases, and is characterized by high malignancy, rapid progression, and a poor prognosis. China bears a disproportionate burden, contributing to more than half of the global ESCC incidence and mortality of ESCC (1). Despite advances in diagnostic and therapeutic strategies, the 5-year survival rate of patients with ESCC remains unsatisfactory because of the late

diagnosis and high rates of recurrence and metastasis. Understanding the molecular mechanisms underlying ESCC progression is therefore essential for the development of novel therapeutic strategies.

Recent attention in cancer biology has turned to proteins that perform multiple mechanistically distinct functions independent of alternative splicing or posttranslational modifications. These proteins often exhibit functions beyond their canonical biochemical roles, and many are implicated in tumorigenesis, metastasis, and

¹Department of Thoracic Surgery, Northern Jiangsu People's Hospital Affiliated to Yangzhou University, Yangzhou, China. ²Yangzhou Key Laboratory of Thoracic and Cardiac Surgery, Yangzhou, China. ³Department of Hepatobiliary Surgery, The First Affiliated Hospital of Anhui Medical University, MOE Innovation Center for Basic Research in Tumor Immunotherapy, Anhui Province Key Laboratory of Tumor Immune Microenvironment and Immunotherapy, Hefei, China. ⁴The Yangzhou School of Clinical Medicine of Nanjing Medical University, Yangzhou, China. ⁵Department of Thoracic Surgery, Sichuan Cancer Hospital and Institute, Sichuan Cancer Center, School of Medicine, University of Electronic Science and Technology of China, Chengdu, China. ⁶First College of Clinical Medicine, Dalian Medical University, Dalian, China.

J. Wu, C. Chen, X. Lv, and Q. Wang contributed equally to this article.

Corresponding Authors: Xiaolin Wang, Department of Thoracic Surgery, Northern Jiangsu People's Hospital Affiliated to Yangzhou University, 98 Nantong West Road, Yangzhou 225000, China. E-mail: 18051063909@yzu.edu.cn; Shouyu Wang, Department of Hepatobiliary Surgery, The First Affiliated Hospital of Anhui Medical University, Hefei 230022, China. E-mail: shouyuwang@ahmu.edu.cn; Yusheng Shu, The Yangzhou School of Clinical Medicine of Nanjing Medical University, 98 Nantong West Road, Yangzhou 225000, China. E-mail: 18051061999@yzu.edu.cn

Cancer Res 2026;86:2951-69

doi: 10.1158/0008-5472.CAN-25-4415

This open access article is distributed under the Creative Commons Attribution-NonCommercial-NoDerivatives 4.0 International (CC BY-NC-ND 4.0) license.

©2026 The Authors; Published by the American Association for Cancer Research

therapeutic resistance. For example, alanyl-tRNA synthetase 1, traditionally known for its role in aminoacylation, has been identified as a lactyltransferase that directly uses lactate and ATP to catalyze protein lactylation, thereby activating the YAP-TEAD complex and promoting gastric cancer cell proliferation (2). Similarly, in addition to its metabolic function, creatine kinase (CK) B acts as a protein kinase that phosphorylates and stabilizes glutathione peroxidase 4, suppressing ferroptosis and facilitating tumor growth in hepatocellular carcinoma (3). Additionally, phosphofructokinase, liver type, a key glycolytic enzyme, functions as a protein kinase under energy stress, phosphorylating perilipin 2 to promote lipid droplet-mitochondria tethering, increasing β -oxidation and supporting tumor cell proliferation (4).

NAD(P)H quinone oxidoreductase 1 (NQO1) is a cytosolic flavoprotein that is traditionally recognized for its antioxidant function and catalyzes the two-electron reduction of quinones to hydroquinones and thereby prevents the formation of reactive oxygen species (5). Recent evidence has revealed its oncogenic role in various cancers, including lung (6), pancreatic (7), and breast cancers (8). However, the potential functions independent of enzymatic activity of NQO1 in ESCC remain unexplored.

In this study, we demonstrated that NQO1 drives ESCC progression and angiogenesis not only through its canonical enzymatic function but also through a newly identified mechanism independent of its enzymatic activity. Specifically, we showed that NQO1 directly binds to and stabilizes the mRNA encoding agrin (AGRN), a key extracellular matrix (ECM) protein involved in tumor growth and neovascularization. Stabilized AGRN, in turn, interacts with filamin A (FLNA) to orchestrate cytoskeletal remodeling and potentiate angiogenic activity in endothelial cells. Furthermore, we identified the FDA-approved drug panobinostat as a small molecule that directly binds to and destabilizes NQO1, thereby suppressing its oncogenic functions. Our findings reveal a previously unrecognized NQO1-AGRN-FLNA regulatory axis underlying ESCC malignancy and highlight the therapeutic potential of targeting the enzymatic activity-independent function of NQO1.

Materials and Methods

Patients and specimens

All ESCC specimens and matched adjacent nontumor tissues were provided by the Department of Biobank, Northern Jiangsu People's Hospital. Clinicopathologic data, including gender, mortality status, overall survival (OS) time, recurrence status, disease-free survival (DFS) time, T/N/M classification, stage, and serum levels of tumor markers (CEA and CA199), were collected for all patients with ESCC ($n = 60$). These data are summarized in Supplementary Tables S1 and S2. Written informed consent was obtained from all patients prior to sample collection. All experiments involving human specimens were approved by the Ethics Committee of Northern Jiangsu People's Hospital. The study is conducted in accordance with the Declaration of Helsinki and is compliant with all relevant ethical regulations involving human subjects.

Cell lines and cell culture

Six human ESCC cell lines [ECA109 (RRID: CVCL_6898), TE1 (RRID: CVCL_1759), KYSE30 (RRID: CVCL_1351), KYSE140 (RRID: CVCL_1347), KYSE150 (RRID: CVCL_1348), and KYSE410 (RRID: CVCL_1352)], a normal human esophageal epithelial cell line (HEpIC), human umbilical vein endothelial cells (HUVEC,

RRID: CVCL_2959), and human HEK293T cells (RRID: CVCL_0063) were purchased from the American Type Culture Collection (ATCC) and cultured according to the instructions provided by the ATCC. Aliquots were cryopreserved at early passages (P3–P4) and freshly thawed every 2 to 3 months for experiments. Cell lines were authenticated by short tandem repeat profiling and routinely tested for *Mycoplasma* contamination using a PCR-based method and were used within 10 passages.

Colony formation assay

For colony formation assays, cells were seeded in six-well plates at a density of 500 to 1,000 cells per well and allowed to adhere overnight. Cells were then treated with the indicated compounds or left untreated and cultured in complete medium for 10 to 14 days, with the medium changed every 3 to 4 days. Colonies were fixed with 4% paraformaldehyde for 15 minutes, stained with 0.1% crystal violet for 30 minutes at room temperature, washed with phosphate-buffered saline (PBS), and counted under a light microscope. Colonies containing more than 50 cells were considered positive.

Cell viability assay

Cell viability was assessed using a CCK-8 assay (Dojindo Laboratories, Kumamoto, Japan). Cells were seeded in 96-well plates at a density of 5×10^3 cells per well and incubated overnight to allow attachment. Cells were then treated with various concentrations of the compounds for 24, 48, or 72 hours. After treatment, 10 μ L of CCK-8 reagent was added to each well, followed by incubation at 37°C for 1 to 2 hours. The absorbance was measured at 450 nm using a microplate reader, and the cell viability is reported as a percentage relative to that of the control cells.

Cell invasion assay

For invasion assays, chambers were precoated with Matrigel and incubated at 37°C for 4 hours. The cells were serum-starved in medium containing 0.5% fetal bovine serum (FBS) for 24 hours prior to detachment. Subsequently, 1×10^5 cells in 100 μ L of serum-free medium were seeded into the upper chamber. The lower chamber contained 600 μ L of medium supplemented with 20% FBS as a chemoattractant. After 24 hours of incubation (37°C, 5% CO₂), nonmigrated cells on the upper membrane surface were removed using a preloosened cotton swab. Invaded cells on the lower surface were fixed with 4% paraformaldehyde (15 minutes), stained with 0.1% crystal violet (20 minutes), and rinsed three times with PBS.

Cell adhesion assay

ESCC cells were added to the culture medium of HUVECs and incubated for 4 hours to assess the adhesion ability of ESCC cells to HUVECs. The cells were subsequently washed 5 times with PBS, after which the adherent cells were imaged using a Leica DMI8 system.

UID RNA sequencing and bioinformatics analysis

Total RNA was extracted using TRIzol reagent (cat. #15596026, Thermo Fisher Scientific) according to the manufacturer's instructions. Genomic DNA contamination was removed by treating the samples with DNase I (cat. #M0303L, NEB). RNA purity was assessed using a NanoDrop OneC spectrophotometer (Thermo Fisher Scientific), and RNA integrity was verified with the LabChip GX Touch system (Revvity). RNA concentrations were quantified using a Qubit 3.0 fluorometer using Qubit RNA Broad Range Assay Kit (cat. #Q10210, Thermo Fisher Scientific). For library

preparation, high-quality total RNA was used as input for stranded mRNA library construction using the KC Digital mRNA Library Prep Kit (Seqhealth Technology, Co., Ltd.) according to the manufacturer's protocol. This kit uses unique molecular identifiers (UMI; 12 random bases) to eliminate PCR and sequencing duplication bias. PCR-enriched fragments of 200 to 500 bp were selected, and the resulting libraries were quantified and sequenced on the DNBSEQ-T7 platform (MGI) in paired-end 150 bp (PE150) mode. UID mRNA sequencing (mRNA-seq) experiments, library preparation, high-throughput sequencing, and data analysis were conducted by Seqhealth Technology, Co., Ltd. Raw sequencing reads were subjected to quality control using FastQC (v0.11.9), followed by adapter and low-quality base trimming using Trimmomatic (v0.39). Clean reads were aligned to the human reference genome (hg38) or the mouse reference genome (mm10) using STAR (v2.7.3a). Gene-level read counts were quantified with featureCounts (v2.0.2), and differential gene expression analysis was performed using DESeq2 (v1.38.0). Genes with an absolute $\log_2$ fold change >1 and an adjusted P value <0.05 were considered differentially expressed. A functional enrichment analysis of differentially expressed genes (DEG) was conducted using clusterProfiler (v4.14.4), with a focus on Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway annotations.

Western blot and co-immunoprecipitation assays

Western blot and co-immunoprecipitation (co-IP) assays were performed as previously reported (9). The antibodies used were as follows: NQO1 (11451-1-AP, Proteintech, RRID: AB_2298729); AGRN (20692-1-AP, Proteintech, RRID: AB_3085612); AGRN (sc-374117, RRID: AB_10947251); FLNA (67133-1-Ig, Proteintech, RRID: AB_2882432); CD63 (ab315108, Abcam, RRID: AB_3099512); CD81 (ab109201, Abcam, RRID: AB_2333083); calnexin (ab22595, Abcam, RRID: AB_2069006); histone H1.0 (ab125027, Abcam, RRID: AB_11000797); β -actin (ab8226, Abcam, RRID: AB_306371); and GAPDH (ab1289151, Abcam, RRID: AB_1067077).

Animal models

A 4-NQO-induced primary ESCC mouse model was established as previously described. Briefly, 6-week-old C57BL/6 mice (RRID: IMSR_JAX:000664) were housed under controlled conditions and administered 4-NQO (100 μ g/mL; Sigma-Aldrich) in their drinking water for 16 weeks. From week 17 onward, the mice were switched to sterile water. At week 26, the mice were sacrificed, and esophageal tissues were collected for analysis.

For xenograft studies, male BALB/c nude mice (5–6 weeks old; GemPharmatech, RRID: MGI:2683685) were maintained under specific pathogen-free conditions. A total of 2×10^6 ESCC cells were subcutaneously injected into the right axilla. The tumor volume was measured every 3 days and calculated using the following formula: volume = (length $\times$ width²)/2. After 5 weeks, the mice were sacrificed, and the tumors were excised, weighed, and imaged.

For the lung colonization model, 1×10^6 ESCC cells suspended in 100 μ L of PBS were injected into the tail vein of BALB/c nude mice. Six weeks later, pulmonary metastases were evaluated by performing *in vivo* bioluminescence imaging and confirmed using hematoxylin and eosin (H&E) staining.

For the combined treatment model, after subcutaneous implantation of 2×10^5 ESCC organoids, the mice were treated with PBS, panobinostat [20 mg/kg, twice a day, intraperitoneal injection (10, 11)], anlotinib [2 mg/kg per day, oral gavage (12, 13)], or a combination of the two drugs, when the tumor volume was 20 mm³.

The tumor weight and tumor size were recorded every 3 days, and the tumor volume was calculated as previously described. The xenograft tumors were harvested after 6 weeks.

All animal experiments were approved by the Ethics Committee of Northern Jiangsu People's Hospital (Yangzhou, China).

Serum biochemistry analysis

For comprehensive assessment of treatment-related systemic toxicity, blood samples were collected from mice at the experimental endpoint. Whole blood was clotted at room temperature and centrifuged at $3,000 \times g$ for 10 minutes to obtain serum. Serum biochemical parameters were measured using an automated clinical chemistry analyzer (Hitachi 7180, Hitachi High-Technologies) according to the manufacturer's instructions. The following parameters were evaluated: liver function—alanine aminotransferase (ALT), aspartate aminotransferase (AST), alkaline phosphatase (ALP), total bilirubin (TBIL), and albumin (ALB); renal function—creatinine (CRE) and urea (UREA); and cardiac injury—CK and CK-MB. All measurements were performed in a blinded manner.

Establishment of patient-derived organoids

Tissues from patients with ESCC were enzymatically digested into single-cell suspensions, which were then mixed with growth factor-reduced Matrigel (0827555, Mogenel) and seeded into 24-well plates. After the Matrigel solidified, esophageal organoid medium consisting of advanced DMEM/F12 supplemented with $1 \times$ Glutamax, 10 mmol/L HEPES, $1 \times$ N2 supplement, $1 \times$ B27 supplement, 40 ng/mL human EGF, 10 μ mol/L ROCK inhibitor Y-27632, 500 nmol/L TGF- β inhibitor A8301, and 3% conditioned medium from L-WRN cells containing Wnt3a, Noggin, and R-spondin was added (14). Organoids were established and maintained under these conditions.

RNA stability assay

RNA stability was assessed by treating cells with actinomycin D (5 μ g/mL; MCE) to inhibit transcription. Cells were collected at the indicated time points after treatment. Total RNA was then extracted and analyzed using quantitative real-time reverse transcription-PCR (qRT-PCR). The remaining RNA levels at each time point were normalized to the level detected at 0 hours.

qRT-PCR

RNA was isolated from cells or tissues using TRIzol reagent (Invitrogen) according to a previously described method (9). RT was performed using HiScript Q RT SuperMix for qPCR (Vazyme Biotech, Co., Ltd.). qRT-PCR was conducted in triplicate using SYBR Green PCR Kit (Vazyme Biotech, Co., Ltd.) on an Applied Biosystems 7900HT system (Applied Biosystems). The primer sequences are provided in Supplementary Table S3.

siRNA and plasmid transfection and lentiviral transduction

siRNAs targeting LRP4 were designed and synthesized by Ribo-Bio. The sequences are listed in Supplementary Table S4. sgRNAs targeting NQO1 (U6-Luc-Puro) and FLNA (U6-Luc-BSD) and shRNAs (U6-Luc-BSD) targeting AGRN were constructed by Corues Biotechnology using GV358 vectors. The sequences are listed in Supplementary Table S4. NQO1-OE [wild-type (WT)] and NQO1-mutant (P187S) lentiviruses were constructed by Corues Biotechnology using pLV4ltr vectors (CMV-C3 $\times$ flag-PGK-Luc-BSD).

NQO1, FLNA, and AGRN constructs as well as the corresponding mutants were subcloned and inserted into the pcDNA3.1 vector (YouBio). The AGRN (exons 4–7) plasmid used in the RNA pulldown assays was subsequently cloned and inserted into a pGEM-3Z vector at the Xba I/BamH I sites (YouBio). siRNAs were transfected into cells with DharmaFECT4 (Dharmacon). All of the plasmids were transfected into cells with Lipofectamine 3000 (Invitrogen). The lentiviruses and empty vectors were added to the ESCC cells. Eight to 12 hours later, the lentiviruses were removed, and fresh culture medium was added. After 72 hours, infected ESCC cells were selected with 1 µg/mL puromycin or 10 µg/mL blasticidin (Sigma).

Linear amplification of complementary DNA ends and sequencing assay and bioinformatics analysis

Briefly, KYSE30 cells stably overexpressing Flag-tagged NQO1 were used for the assay. Cells were UV-crosslinked at 254 nm with 400 mJ/cm² energy twice, lysed using wash buffer (1× PBS, 0.1% SDS, 0.5% NP-40, and 0.5% sodium deoxycholate), and treated with DNase I (M6101, Promega) and RNase inhibitor (AM2696, Ambion). The lysates were incubated with streptavidin magnetic beads (26162, Thermo Fisher Scientific) conjugated to anti-Flag antibodies (2 µg per sample) at 4°C for 2 to 4 hours. After immunoprecipitation, the beads were extensively washed with wash buffer, high-salt buffer, and PNK buffer. RNA was fragmented to ~70 bp using diluted MNase (EN0181, Thermo Fisher Scientific) at 37°C for 3 minutes, and reactions were terminated with EGTA-containing buffer. RNA was dephosphorylated with FastAP (EF0651, Thermo Fisher Scientific) and ligated to a preadenylated 3' linker using T4 RNA ligase 2 (NEB). After RT (SuperScript II, Life Technologies) with linear amplification of complementary DNA ends (LACE)-specific RT primers, RNA templates were digested with RNase H and exonuclease I. First-strand cDNAs were captured by streptavidin beads (65001, Invitrogen) and ligated with a 5' cDNA linker using T4 RNA ligase 1 (NEB). Following second-strand cDNA synthesis, the libraries were amplified by pre-PCR (KAPA HiFi HotStart ReadyMix, KAPA Biosystems) for 12 to 20 cycles depending on the sample input. PCR products were purified using AMPure XP beads (A63881, Beckman Coulter) and size-selected by 2% agarose gel electrophoresis to retain fragments between 250 and 500 bp. Purified cDNAs were subjected to *in vitro* transcription with T7 RNA polymerase (NEB) overnight at 37°C to produce amplified RNA (aRNA). After DNase I treatment, aRNA was purified using Agencourt RNAClean XP beads (A63987, Beckman Coulter). First-strand cDNA was synthesized again from aRNA, followed by PCR amplification with indexed primers to generate the final sequencing libraries. The libraries were quantified and sequenced on an Illumina platform.

LACE-seq data were processed according to the published LACE-seq pipeline (15). Adapter sequences and 3' poly(A) tails were removed using Cutadapt (v.1.18). Clean reads were first aligned to pre-rRNA sequences, and the remaining unmapped reads were subsequently aligned to the human reference genome (hg38) using Bowtie (v2.3.5.1), allowing up to two mismatches. LACE-seq peaks were identified using Piranha, followed by peak annotation with HOMER and visualization using IGV (v2.14.0). To calculate the count, BAM files were used to quantify the reads using FeatureCounts (v2.0.2). Only peaks that were enriched in samples immunoprecipitated with the NQO1 antibody compared with control samples immunoprecipitated with IgG antibody were defined as NQO1-specific RNA-binding sites (absolute log₂ fold change >1).

RNA pulldown assay

Biotinylated AGRN exon 4 to 7 RNA fragments were generated by enzymatic digestion with XbaI or BamHI (NEB), followed by *in vitro* transcription using T7 or SP6 RNA polymerase (Invitrogen) and a Biotin RNA Labeling Mix (Roche). The transcription products were verified by agarose gel electrophoresis and purified using RNA Clean & Concentrator Kit (Zymo Research). Streptavidin beads (Invitrogen) were prepared according to the manufacturer's instructions. Lysates from 293T or KYSE30 cells pretreated with RNase inhibitors and protease/phosphatase inhibitors (Beyotime) were incubated with RNA-bound beads at 4°C for 4 hours. RNA-binding proteins were detected by Western blotting. RNA pulldown assays with recombinant WT and P187S-mutant NQO1 proteins were performed similarly.

RNA fluorescence *in situ* hybridization

Specific Cy3-labeled probes were designed and synthesized for the detection of AGRN (exons 4–7) by RiboBio. After fixation, permeabilization (1× PBS/0.5% Triton X-100), and prehybridization, the cells were hybridized with the Cy3-labeled probes in hybridization buffer at 37°C overnight. The hybridization buffer was then gradually removed by washes with 4× SSC (including 0.1% Tween-20), 2× SSC, and 1× SSC at 42°C. Nuclei were counterstained with 4,6-diamidino-2-phenylindole (DAPI) (Beyotime). Confocal images of the cells were captured using Zeiss AIM software and a Zeiss LSM 700 confocal microscope (Carl Zeiss Jena, RRID: SCR_015695).

Immunofluorescence staining

After being fixed with 4% paraformaldehyde for 15 minutes at room temperature, the cells were permeabilized with 0.2% Triton X-100 for 10 minutes. Nonspecific binding was blocked by incubating the cells with 5% bovine serum albumin (BSA) in PBS for 1 hour. The cells were then incubated overnight at 4°C with primary antibodies diluted in 1% BSA-PBS. After washes with PBS, the cells were incubated with the appropriate fluorescently labeled secondary antibodies for 1 hour at room temperature. The nuclei were stained with DAPI (1 µg/mL) for 5 minutes. Images were captured using a confocal microscope (Zeiss LSM 880).

Co-IP and mass spectrometry

The purified His-tagged AGRN protein was added to HUVECs and incubated for 24 hours. Then, a co-IP assay was performed as previously reported (9). For the mass spectrometry (MS) analysis, the eluted proteins were separated by SDS-PAGE and stained with Coomassie blue. The protein bands of interest were excised from the gel and digested with trypsin. The resulting peptides were analyzed by liquid chromatography–tandem mass spectrometry (LC-MS/MS) using a Q Exactive mass spectrometer (Thermo Fisher Scientific). The acquired data were analyzed using MaxQuant software, and proteins were identified by searching the results against the UniProt database.

Extracellular vesicle isolation and characterization

Extracellular vesicles (EV) were isolated from the conditioned medium of ESCC cells. Cells were cultured in medium supplemented with EV-depleted FBS for 48 hours. Conditioned medium was collected and sequentially centrifuged to remove cells and debris (300 × g for 10 minutes, 2,000 × g for 20 minutes, and 10,000 × g for 30 minutes). The supernatant was then subjected to ultracentrifugation at 100,000 × g for 70 minutes at 4°C (Beckman Coulter). The EV

pellet was subsequently washed once with sterile PBS and centrifuged again at $100,000 \times g$ for 70 minutes. The purified EVs were resuspended in PBS for downstream analyses. For EV characterization, EV marker proteins were examined by immunoblotting using antibodies against CD63, CD81, and TSG101. Calnexin and histone H1.0 were used as negative controls to confirm the absence of endoplasmic reticulum and nuclear contamination, respectively, and to assess the purity of the sample. EV morphology was further examined by transmission electron microscopy (TEM). Purified EVs were fixed with 2% paraformaldehyde, placed onto formvar/carbon-coated copper grids, negatively stained with uranyl acetate, and imaged using a transmission electron microscope. Representative images showed round, membrane-bound vesicles with a typical EV morphology.

Nanoparticle tracking analysis

The EV size distribution and particle concentration were determined by performing a nanoparticle tracking analysis (NTA). Briefly, EVs were isolated from the conditioned medium of ESCC cells as described above and resuspended in sterile PBS. The samples were appropriately diluted with PBS to achieve a particle concentration within the optimal detection range of the instrument. NTA was performed using a nanoparticle tracking analyzer (NanoSight NS300, Malvern Panalytical) equipped with a 488-nm laser. For each sample, three independent videos of 60 seconds were recorded at room temperature with identical camera settings. Data acquisition and analysis were conducted using the manufacturer's software, and the particle size distribution and concentration were calculated based on Brownian motion using the Stokes–Einstein equation.

Prediction of the potential allosteric sites of NQO1 and molecular docking

The structure of the NQO1_HUMAN protein was optimized using the Protein Preparation Wizard panel (Schrödinger 2021) by correcting the bond order, adding hydrogen atoms, distributing charges, and predicting the protonation states (pH 7.0). The OPLS4 force field was used for constrained energy optimization to eliminate the atomic conflict on the structure with the RMSD of heavy atoms converging to 0.3 Å, and the side chain position was optimized to obtain a reasonable side chain structure.

A total of 120,000 compounds from TargetMOL were processed with the LigPrep panel (Schrödinger 2021). First, the compounds were protonated and desalted with the Epik program at pH 7.0 ± 2.0 to generate tautomers and maintain the original atomic chirality. Each small molecule was generated in up to 32 conformations to ensure the conformational diversity of small molecules in the virtual screening process.

Glide HTVS (high-throughput virtual screening mode) → Glide SP (standard precision mode) → Glide XP (extra precision) was used for screening. The protein structure was set as rigid, the structures of the small molecules were set as flexible, and other parameters were set to the default values. Energy optimization was performed on the docking results, and the top 20% with docking scores were reserved for the next screening step. A visual inspection was performed after duplicates were removed and filtered for drug-like properties.

We utilized the protein–ligand interaction fingerprint module of MOE for the binding mode analysis to further analyze the compounds obtained from molecular docking. We subsequently analyzed compounds with docking scores lower than -10 kcal/mol.

Furthermore, we analyzed the pharmacologic properties of the compounds combined with the 1KBQ structure, including the

five rules of the properties of the compound class [relative molecular weight ($WM \leq 500$)], the donor and acceptor of hydrogen bonds (OH and NH groups ≤ 5 , numbers of Ns and Os ≤ 10 , and rotatable keys ≤ 11), polar surface area ($TPSA \leq 140$), water solubility ($-4 \leq \log S \leq 0.5$), and the logarithm of the octanol/water partition coefficient ($0 \leq \log P \leq 3$).

The authors are grateful to the TargetMol group for their technical assistance with the CADD job described in this work.

Surface plasmon resonance

A surface plasmon resonance (SPR) assay was performed using a Biacore T200 SPR instrument (Cytiva). Purified proteins (NQO1-WT and NQO1-P187S) were diluted to 50 $\mu\text{g/mL}$ with 10 mmol/L sodium acetate buffer (pH 4.0), after which the purified proteins were attached to the CM5 sensor chip. The indicated concentrations of the solutions of the compounds were diluted with 1.05×PBS-P+ buffer without DMSO, and then threefold dilutions were performed with running buffer containing 5% DMSO to obtain five concentrations of small-molecule samples. The indicated concentrations of the compounds were injected into calcium- and magnesium ion-free Dulbecco PBS buffer supplemented with 5% DMSO at a flow rate of 30 $\mu\text{L/minute}$, with a binding time of 60 seconds and a dissociation time of 120 seconds. The results were adjusted using the solvent correction method, and all the data were subtracted from the 0 concentration to obtain the final binding and dissociation curves.

Statistical analysis

Statistical analyses were conducted using SPSS 18.0 and Graph-Pad Prism 8 software. Differences in the IRs for NQO1 staining in primary tumor tissues were evaluated using the Wilcoxon test. Differences in OS were assessed using the Kaplan–Meier method with log-rank tests. A univariate Cox regression analysis was performed to estimate hazard ratios and 95% confidence intervals. The data are presented as the means $\pm$ SD. Paired two-tailed *t* tests were used for within-group comparisons, and unpaired two-tailed *t* tests were used for between-group comparisons. For comparisons among more than two groups, one-way ANOVA or two-way ANOVA were applied. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; ****, $P < 0.0001$; ns., not significant. All key experiments were independently repeated at least three times or validated with multiple biologically independent samples.

Results

NQO1 is upregulated in ESCC epithelial cells and predicts a poor prognosis for patients with ESCC

We systematically evaluated the expression of 46 metabolic enzymes with putative RNA-binding activity (16) in ESCC by performing RNA-seq on tumor and matched adjacent normal tissues from both human patients with ESCC ($n = 6$) and a primary murine ESCC model ($n = 6$; Fig. 1A; Supplementary Fig. S1A). Among these candidates, NQO1 was the only gene whose expression was consistently and significantly upregulated in both species (Fig. 1B and C). An analysis of The Cancer Genome Atlas ESCC dataset further confirmed that NQO1 is highly expressed in tumor tissues and is correlated with a poor prognosis (Fig. 1D and E).

As bulk RNA-seq data do not provide single-cell resolution, we investigated a published single-cell RNA-seq (scRNA-seq) dataset (GSE160269), and the results revealed the predominant expression of NQO1 in epithelial cells (Fig. 1F and G; Supplementary Fig. S1B). InferCNV and pseudotime trajectory analyses further indicated that

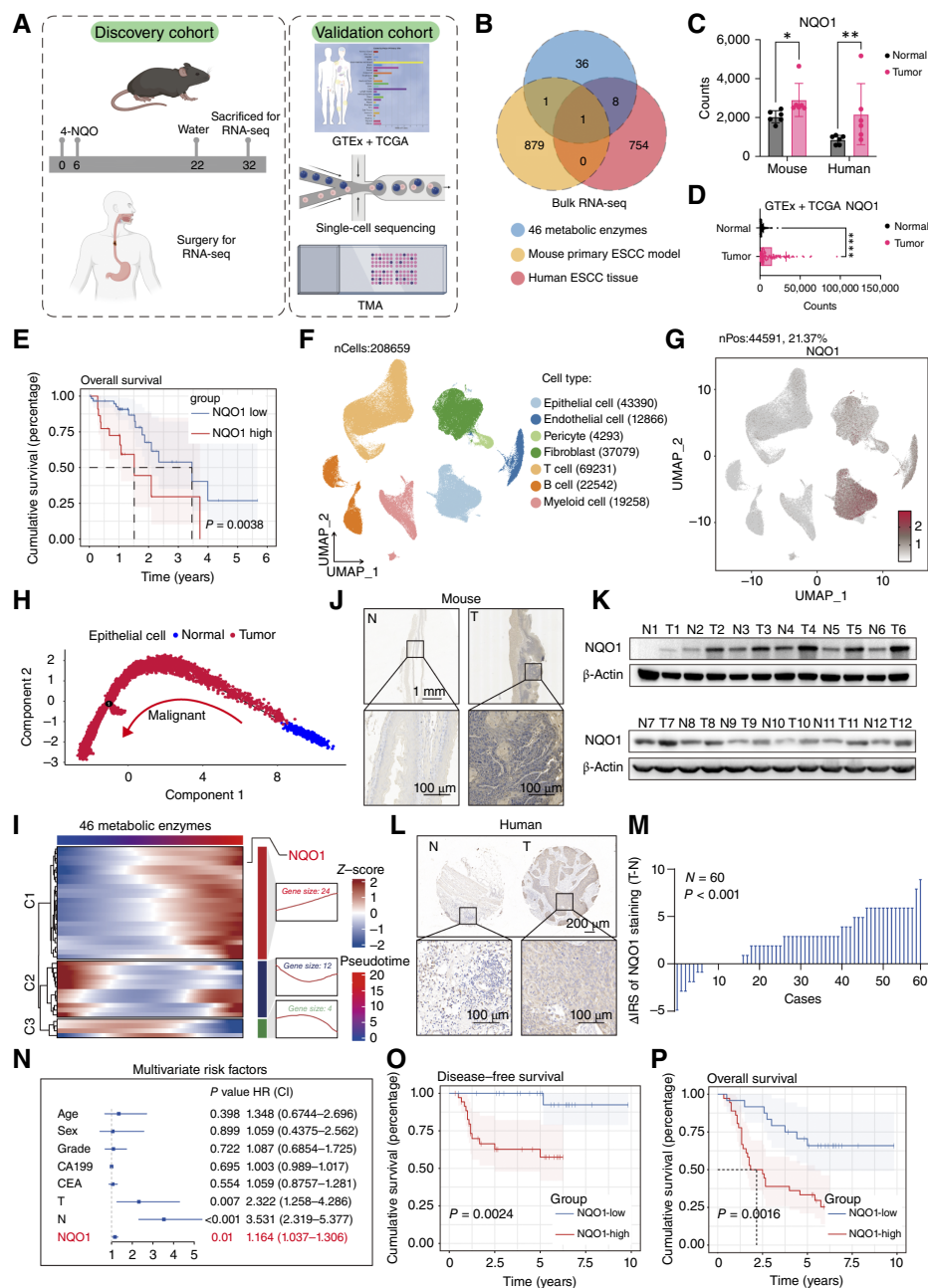


Figure 1.

Elevated NQO1 expression correlates with a poor prognosis for patients with ESCC. **A**, Schematic diagram illustrating the strategy used to screen 46 metabolic enzymes. **B**, Overlapping DEGs among the 46 metabolic enzymes identified in human ($n = 6$) and mouse ($n = 6$) ESCC RNA-seq datasets. **C**, NQO1 mRNA expression levels in ESCC tissues from mouse and human RNA-seq datasets ($n = 6$). **D**, NQO1 mRNA expression levels in ESCC samples from the GTEx and TCGA datasets. **E**, Kaplan-Meier OS curves stratified by NQO1 expression in patients with ESCC from the TCGA database. **F**, Uniform manifold approximation and projection (UMAP) plot of cell types in the ESCC scRNA-seq dataset (GSE138709). **G**, UMAP plot showing the distribution of NQO1 expression among different cell types. **H**, Pseudotime trajectory analysis of epithelial cells inferred by Monocle2, with each dot representing a single cell. **I**, Heatmap displaying the expression profiles of 46 metabolic enzymes associated with the malignant progression of ESCC. **J**, Representative images of IHC staining showing NQO1 expression in a murine primary ESCC model (scale bars, 1 mm). **K**, Western blot analysis of NQO1 protein levels in paired ESCC and adjacent normal tissues from 12 patients. **L**, Representative images of IHC staining for NQO1 in ESCC tissue microarrays (scale bars, 200 and 100 μ m). **M**, Distribution of differences in NQO1 immunoreactivity scores (IRS) between 60 paired ESCC and noncancerous tissues (Δ IRS = IRS_T - IRS_N). **N**, Multivariate Cox regression analysis of the ESCC cohort, with the 95% confidence intervals (CI) indicated. **O** and **P**, Kaplan-Meier DFS and OS curves stratified by NQO1 expression levels in patients with ESCC. The data are shown as the means $\pm$ SDs (**C** and **D**). The data from three independent experiments are presented, and the results from one of three representative experiments are shown (**J**, **K**, and **L**). Statistical analyses were performed using two-tailed Student *t* tests (**C** and **D**), Wilcoxon tests (grouped; **M**) or log-rank tests (**O** and **P**). *, $P < 0.05$; **, $P < 0.01$; ****, $P < 0.0001$; ns, not significant. **A**, Created in BioRender. Wu, J. (2026) <https://BioRender.com/adxmx32>.

NQO1 expression increases progressively during malignant transformation from normal epithelial cells to tumor cells, indicating that it is involved in early tumorigenesis (Fig. 1H and I; Supplementary Fig. S1C–S1G).

Consistent with the transcriptomic data, NQO1 protein levels were markedly increased in tumor tissues compared with those in adjacent normal tissues in the murine ESCC model (Fig. 1J). Similarly, elevated NQO1 protein levels were observed in human ESCC samples (Fig. 1K). We performed immunohistochemistry (IHC) on tissue microarrays comprising 60 ESCC samples to assess the clinical relevance of NQO1 in a larger cohort (Fig. 1L and M). The multivariate Cox regression analysis revealed that NQO1 expression was an independent prognostic factor (Fig. 1N), and the survival analysis showed that patients with high levels of NQO1 expression experienced significantly shorter DFS and OS (Fig. 1O and P).

Collectively, these results identify NQO1 as a clinically relevant protumorigenic regulator that contributes to tumor progression in ESCC, underscoring its potential functional and therapeutic relevance.

NQO1 facilitates the progression and lung colonization of ESCC *in vitro* and *in vivo*

We established stable NQO1-overexpressing or -knockout ESCC cell lines to determine the function of NQO1 in ESCC (Supplementary Fig. S2A, S2B, S2E, S2H, and S2K). NQO1 overexpression significantly increased colony formation, whereas NQO1 knockout had the opposite effect (Supplementary Fig. S2C, S2D, S2F, S2G, S2I, S2J, S2L, and S2M). We established a biobank of six patient-derived ESCC organoids to corroborate these findings in a more physiologically relevant model. Histologic features were confirmed by H&E staining (Supplementary Fig. S2N), and NQO1 protein levels were evaluated (Supplementary Fig. S2O). Lentivirus-mediated NQO1 overexpression or knockout was performed in the organoids according to their endogenous NQO1 expression level. NQO1 knockout substantially suppressed organoid growth (Fig. 2A and B) and reduced the proportion of Ki-67-positive proliferating cells, as shown by multiplex immunofluorescence (MIF) staining (Fig. 2C). Conversely, NQO1 overexpression promoted organoid expansion and increased Ki-67 levels, indicating a pro-proliferative role (Fig. 2D–F).

Consistent with the *in vitro* data, xenograft assays in nude mice showed that NQO1 knockout markedly inhibited tumor growth (Fig. 2G–I) and decreased Ki-67 staining (Fig. 2J). Conversely, NQO1 overexpression accelerated tumor growth and proliferation (Fig. 2K–N). A tail vein injection-based model was used to evaluate the effect of NQO1 on the lung colonization of tumor cells. NQO1 knockout significantly reduced the lung colonization burden, as assessed by bioluminescence imaging and a histopathologic analysis (Fig. 2O and P; Supplementary Fig. S2P). In contrast, NQO1 overexpression markedly increased the number and size of tumors colonizing the lung (Fig. 2Q and R; Supplementary Fig. S2Q).

Collectively, these results demonstrate that NQO1 facilitates tumor growth and lung colonization in multiple experimental models, including ESCC cell lines, patient-derived organoids, subcutaneous xenograft models, and *in vivo* lung colonization assays.

NQO1 promoted ESCC progression and lung colonization through a mechanism independent of its enzymatic activity

NQO1 is a cytosolic two-electron oxidoreductase known to catalyze the reduction of quinones, nitroaromatic compounds, imidazoles, and iron ions (5, 17, 18). To examine whether the tumor-promoting effects of NQO1 in ESCC depend on its

enzymatic activity, we established stable cell lines overexpressing either WT NQO1 or an enzymatically inactive mutant (P187S; ref. 19) on an NQO1-knockout background (Fig. 3A and B). Colony formation assays showed that NQO1 knockout markedly impaired the clonogenic capacity of ESCC cells. The reintroduction of WT NQO1 completely restored colony formation, whereas the P187S mutant only partially reversed this defect, indicating that the nonenzymatic activity of NQO1 significantly contributed to tumor development (Fig. 3C and D). Comparable patterns were observed in the Transwell assays (Fig. 3E and F). Importantly, these findings were independently reproduced in a second NQO1-knockout clone (KO#2), confirming the robustness of the enzymatic activity-independent effect of NQO1 (Supplementary Fig. S3A–S3F).

Consistent with the *in vitro* data, subcutaneous xenograft models indicated that NQO1 knockout significantly suppressed tumor volume and weight. Tumors derived from cells expressing the enzymatically inactive mutant exhibited an intermediate phenotype, further supporting a dual role for NQO1 (Fig. 3G–I). Ki-67 staining of tumor sections confirmed the differences in proliferative activity (Fig. 3J). We used a tail vein injection-based model to evaluate the role of NQO1 in lung colonization. NQO1 knockout markedly reduced the pulmonary tumor burden, whereas the expression of the enzymatically inactive mutant substantially restored the lung colonization capacity, which approached that observed with WT NQO1 (Fig. 3K–M). Notably, this trend was also consistently observed in the KO#2 clone, reinforcing the conclusion that NQO1 promotes lung colonization largely through an enzymatic activity-independent mechanism (Supplementary Fig. S3G–S3M).

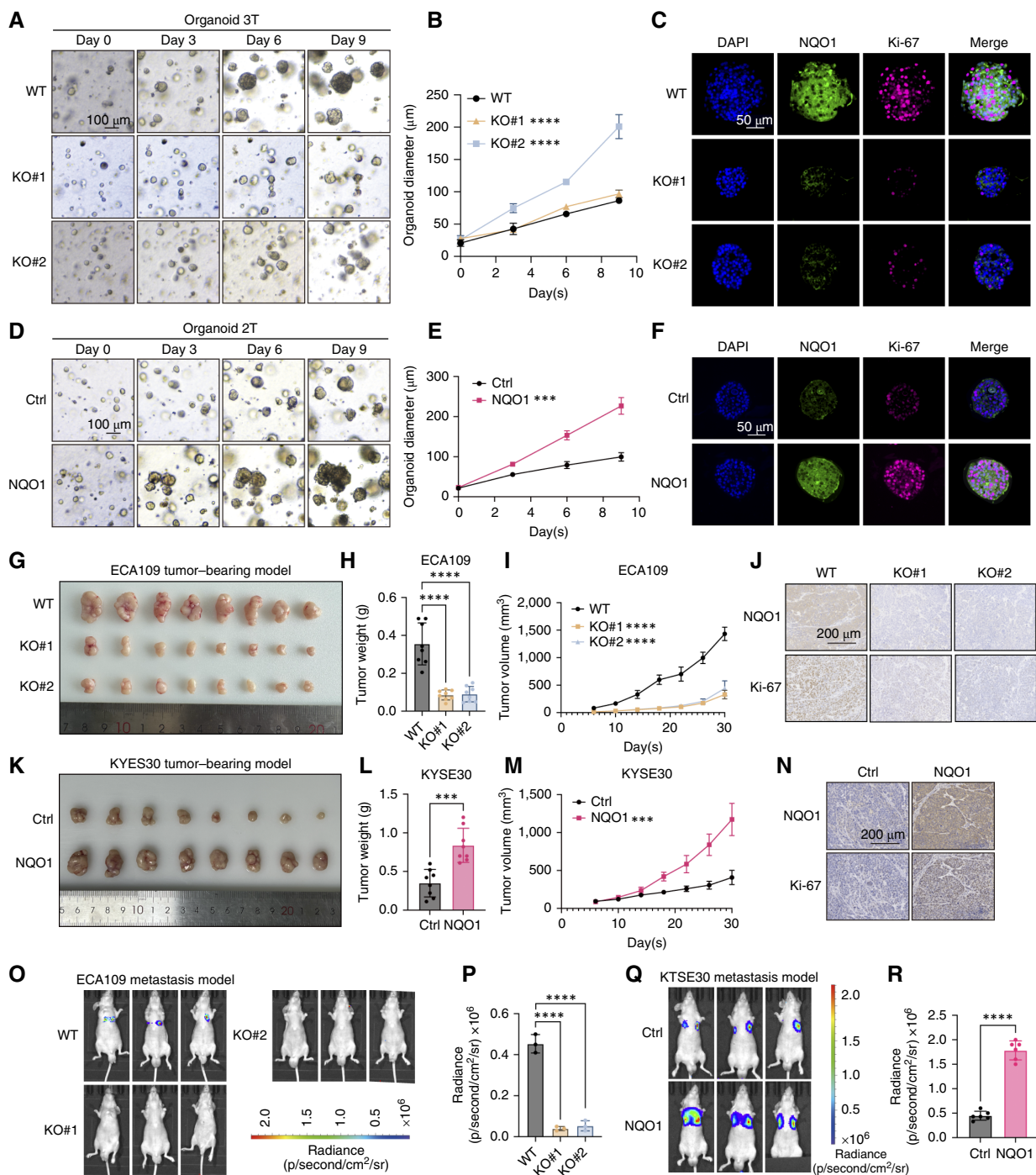
Taken together, these findings indicate that NQO1 promotes ESCC progression and lung colonization not only through its canonical enzymatic function but also through a previously unrecognized function independent of its enzymatic activity.

NQO1 directly binds to the *AGRN* mRNA and increases its stability

Previous studies have suggested that, in addition to its canonical oxidoreductase function, NQO1 may also act as an RNA-binding protein (16). To investigate this possibility, we performed LACE-seq in ESCC cells overexpressing NQO1 (15). LACE-seq profiling revealed that NQO1 primarily binds to the coding sequence regions of target mRNAs (Fig. 4A).

We next performed RNA-seq on ESCC cells with stable NQO1 knockout and conducted a differential expression analysis to identify functionally relevant RNA targets downstream of NQO1. A total of 452 DEGs were identified, including 242 upregulated genes and 210 downregulated genes ($|\log_2$ fold change| > 1 and adjusted *P* value < 0.05). The KEGG pathway enrichment analysis of these DEGs revealed that the ECM–receptor interaction pathway was the most significantly enriched pathway based on the adjusted *P* values (Fig. 4B).

We intersected the RNA-seq dataset with the LACE-seq results to further prioritize candidate genes that are not only transcriptionally regulated but also directly associated with NQO1. LACE-seq identified 865 mRNAs that were specifically enriched in NQO1 immunoprecipitates compared with those in IgG control immunoprecipitates ($\log_2$ fold change > 1). The intersection of these 865 transcripts with the eight DEGs enriched in the ECM–receptor interaction pathway revealed that *AGRN* was the sole overlapping gene (Fig. 4C). Peak calling of the LACE-seq data suggested

**Figure 2.**

NQO1 facilitates ESCC progression and metastasis *in vivo*. **A** and **D**, Representative brightfield images of ESCC organoids infected with an NQO1-knockout or -overexpressing lentivirus (scale bars, 100 μ m). **B** and **E**, Organoid diameters were measured every other day, and growth curves were generated. **C** and **F**, MIF staining of ESCC organoid sections with anti-NQO1 and anti-Ki-67 antibodies (scale bars, 50 μ m). **G**, NQO1 knockout inhibits subcutaneous ESCC tumor growth in nude mice ($n = 8$). **K**, Subcutaneous xenograft model showing that NQO1 overexpression promotes ESCC tumor growth in nude mice ($n = 8$). **H** and **L**, Weights of tumors harvested from mice bearing tumors derived from ESCC cells. **I** and **M**, Tumor growth curves showing the volumes measured every other day. **J** and **N**, IHC staining of tumor sections with anti-NQO1 and anti-Ki-67 antibodies (scale bars, 200 μ m). **O** and **Q**, Representative bioluminescence images of lung metastases 4 weeks after the tail vein injection of NQO1-overexpressing or -knockout ESCC cells, **P** and **R**, along with the quantification of lung bioluminescence signals. Representative gross images of lung metastatic nodules ($n = 6$). The data are shown as the means $\pm$ SDs of the indicated organoids or mice per group (**B**, **E**, **I**, **M**, **P**, and **R**). Statistical analyses were performed using two-tailed Student *t* test (**R**), one-way ANOVA (**P**), or two-way ANOVA (**B**, **E**, **I**, and **M**). ***, $P < 0.001$; ****, $P < 0.0001$; ns, not significant.

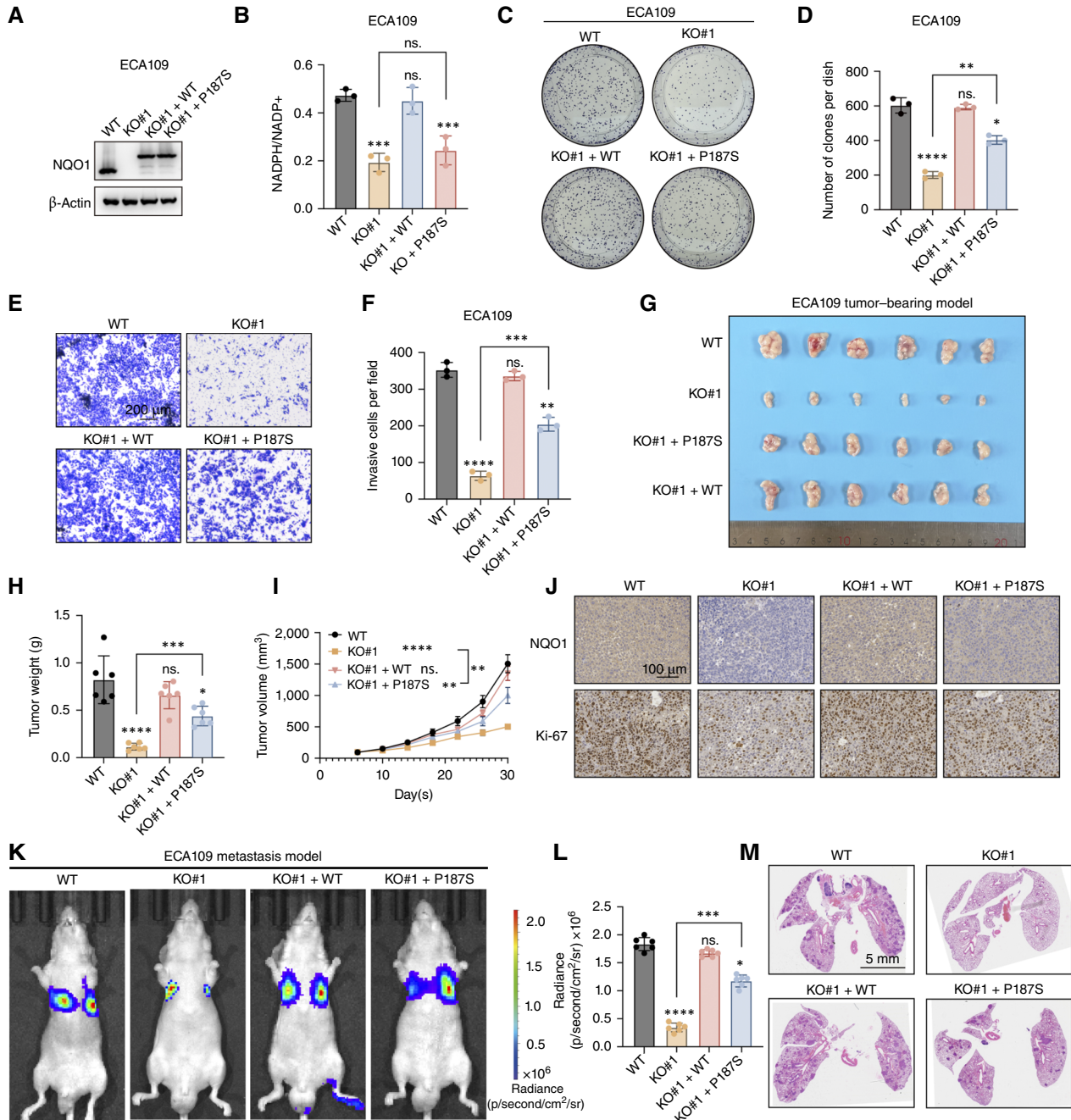


Figure 3.

NQO1 promotes ESCC progression and metastasis through a mechanism independent of its enzymatic activity. **A**, Western blot analysis of NQO1 protein levels in ESCC cells from the NQO1 WT, #1 knockout (KO#1), KO#1 + WT rescue, and KO#1 + mutant (P187S) rescue groups. **B**, NADPH/NADP⁺ levels in the ESCC cells in **A**. **C** and **D**, Representative images (**C**) and quantification (**D**) of the colony formation ability of ECA109 cells. **E** and **F**, Representative images (**E**) and quantification (**F**) of migration and invasion assays using ECA109 cells. **G**, Growth of subcutaneous tumors formed by ECA109 cells in nude mice ($n = 6$). **H**, Weights of tumors extracted from xenografted mice. **I**, Tumor volumes measured every other day and corresponding tumor growth curves. **J**, IHC staining of tumor sections for NQO1 and Ki-67 (scale bars, 100 μ m). **K** and **L**, Representative bioluminescence images of mice 4 weeks after the tail vein injection of ECA109 cells (**K**) and quantification of lung bioluminescence signals (**L**). **M**, H&E staining of lung sections (scale bars, 5 mm). The data are presented as the means $\pm$ SDs of three (**D** and **G**) or six (**H**, **I**, and **L**) independent experiments. The results from one of three representative experiments are shown in **C**, **E**, **J**, and **K**. Statistical analyses were performed using one-way ANOVA (**D**, **G**, **H**, and **L**) or two-way ANOVA (**I**). *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; ****, $P < 0.0001$; ns, not significant.

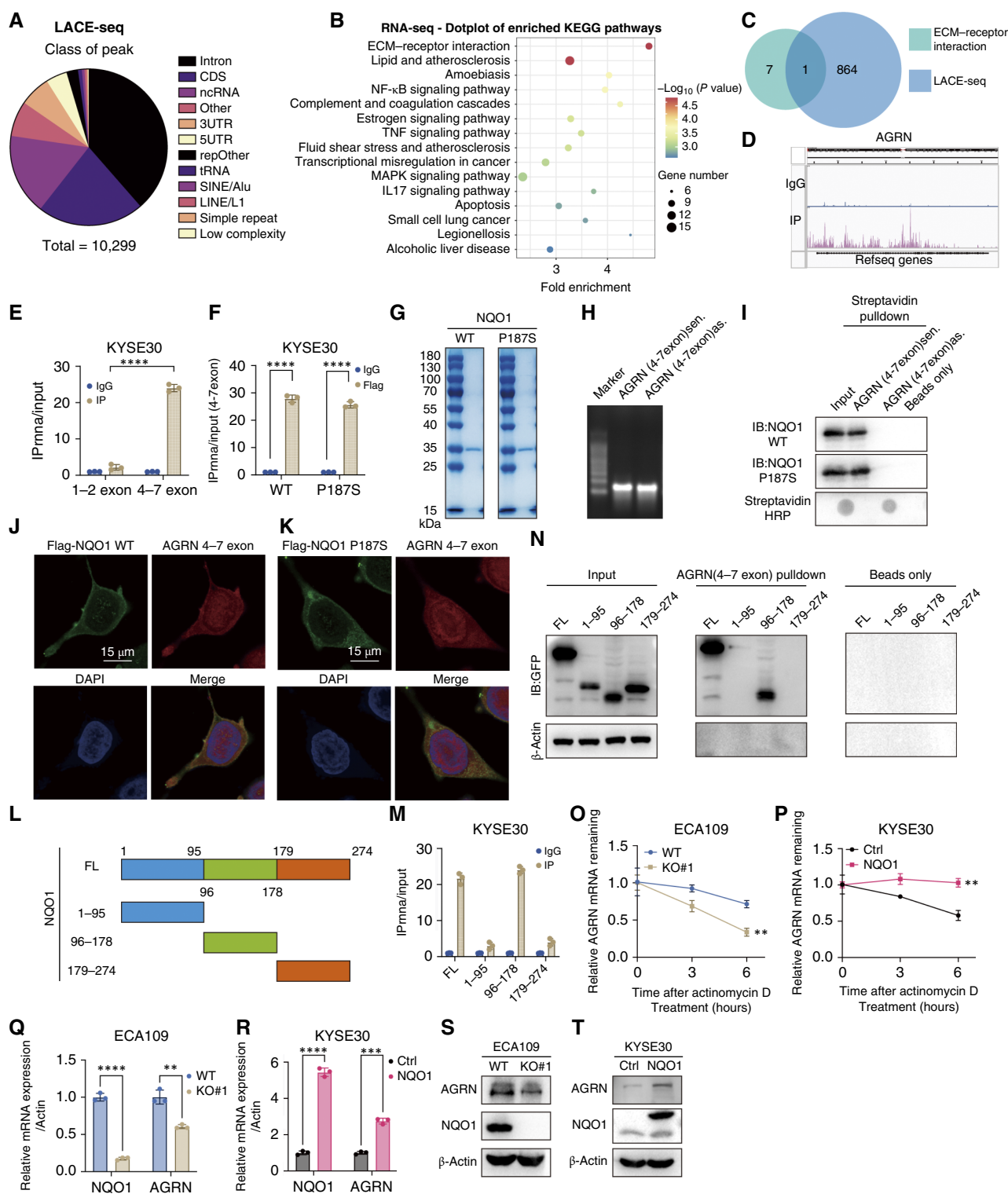


Figure 4.

NQO1 directly binds to the *AGRN* mRNA. **A**, Genomic distribution of NQO1-binding peaks identified by LACE-seq. **B**, KEGG pathway enrichment analysis of DEGs identified upon NQO1 knockout. **C**, Overlapping genes between the ECM-receptor interaction pathway (RNA-seq DEGs) and NQO1-bound targets (LACE-seq). **D**, LACE-seq profiling of NQO1 binding sites on the *AGRN* mRNA. **E** and **F**, RIP-qPCR analysis to determine the *AGRN* exons (1-2 or 4-7) bound by NQO1 in KYSE30 cells overexpressing WT or mutant (P187S) NQO1. **G**, SDS-PAGE and Coomassie Brilliant Blue staining of the purified WT and P187S NQO1 proteins. **H**, *In vitro* transcription and validation of sense and antisense *AGRN* exons 4-7 RNA fragments. **I**, Western blot analysis of (Continued on the following page.)

potential binding of NQO1 to exons 1 to 2 or 4 to 7 of the *AGRN* mRNA (Fig. 4D). RNA immunoprecipitation followed by qPCR (RIP-qPCR) confirmed the specific and robust interaction between NQO1 and *AGRN* exons 4 to 7 (Fig. 4E). Notably, this interaction was preserved in cells expressing the enzymatically inactive NQO1 mutant, indicating that *AGRN* binding occurs independently of the catalytic activity of NQO1 (Fig. 4F).

To confirm a direct physical interaction, we expressed and purified FLAG-tagged NQO1 from *Escherichia coli* and synthesized biotin-labeled RNA corresponding to *AGRN* exons 4 to 7. The quality of the purified protein and RNA was confirmed by Coomassie blue staining and RNA electrophoresis, respectively (Fig. 4G and H). RNA pulldown assays showed that both wild-type and catalytically inactive NQO1 directly bound to *AGRN* exons 4 to 7 (Fig. 4I). Furthermore, combined immunofluorescence staining and RNA fluorescence *in situ* hybridization experiments demonstrated the cytoplasmic colocalization of NQO1 and the *AGRN* mRNA, confirming their interaction in a cellular context (Fig. 4J and K). As NQO1 lacks known RNA-binding domains, we conducted domain mapping by dividing NQO1 into three fragments fused to GFP (Fig. 4L). RIP-qPCR and RNA pulldown assays revealed that the region encompassing amino acids 96 to 178 is responsible for binding to the *AGRN* mRNA (Fig. 4M and N).

We measured the decay rate of the *AGRN* mRNA following actinomycin D treatment to assess the functional impact of this interaction. *AGRN* mRNA expression decreased more rapidly in NQO1-knockout cells and was stabilized in NQO1-overexpressing cells (Fig. 4O and P). Consistently, qRT-PCR and Western blot assays confirmed that NQO1 upregulates *AGRN* expression at both the mRNA and protein levels (Fig. 4Q–T).

Together, these findings indicate that NQO1 directly binds to the *AGRN* mRNA via a previously unrecognized RNA-binding region (amino acids 96–178) and increases its stability in a manner independent of enzymatic activity, revealing a novel posttranscriptional mechanism regulating ESCC progression.

NQO1 facilitates ESCC progression and angiogenesis by upregulating *AGRN*

The ECM plays a crucial role in tumor development and metastasis via complex interactions with multiple cell types (20). To identify key cellular contributors to ECM-mediated tumor progression, we performed CellChat analysis based on the ECM receptor–ligand interaction module (Supplementary Fig. S4A). The analysis revealed that among stromal populations, endothelial cells showed the strongest interactions with tumor epithelial cells, whereas myeloid cells, fibroblasts, and lymphoid subsets displayed comparatively weaker signaling (Fig. 5A). To validate these findings, we performed MIF staining of ESCC tissues for NQO1 together with markers of endothelial cells (CD31), myeloid cells (CD68), fibroblasts (α SMA), T cells (CD4 and CD8), and B cells (CD20). Consistent with the scRNA-seq data, NQO1 was

predominantly expressed in tumor epithelial cells. Notably, NQO1-high tumors showed increased CD31⁺ endothelial cell density, whereas the abundance of CD68⁺ myeloid cells, α SMA⁺ fibroblasts, CD4⁺ T cells, CD8⁺ T cells, and CD20⁺ B cells remained largely unchanged (Fig. 5B; Supplementary Fig. S4B and S4C). Based on these observations, we subsequently focused our mechanistic and functional studies on elucidating how NQO1-regulated *AGRN* specifically modulates endothelial cell behavior and tumor progression.

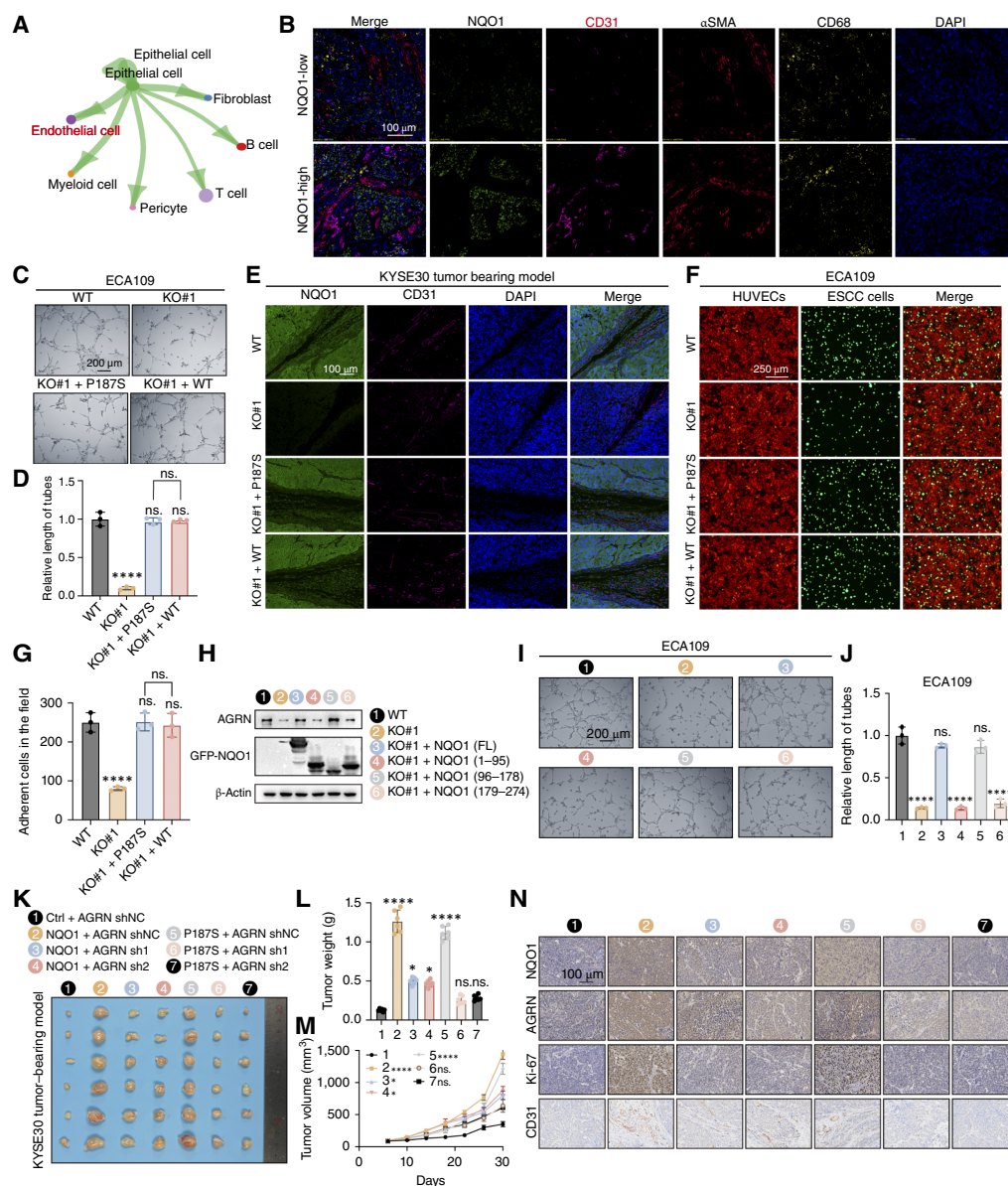
Functionally, compared with control media, conditioned media from NQO1-knockout ECA109 cells significantly impaired HUVEC tube formation (Supplementary Fig. S4D and S4E), whereas medium from KYSE30 cells overexpressing NQO1 increased tube formation (Supplementary Fig. S4F and S4G). Both WT and enzymatically inactive NQO1 (P187S) effectively restored tube formation in knockout cells to a similar extent (Fig. 5C and D). Adhesion assays revealed that NQO1 knockout reduced ESCC–HUVEC adhesion (Supplementary Fig. S4H and S4I), whereas NQO1 overexpression increased the interaction of these cells (Fig. S4J–K). Both WT and mutant NQO1 comparably rescued the adhesive capacity (Fig. 5F and G). MIF staining showed decreased expression of CD31, a marker of endothelial cells and angiogenesis, in NQO1-knockout tumors, whereas NQO1 overexpression increased CD31 levels (Supplementary Fig. S4L and S4M). Both WT and mutant NQO1 similarly restored CD31 expression in knockout tumors (Fig. 5E).

We generated a series of NQO1 truncation constructs and transfected them into NQO1-knockout ESCC cells to determine whether the NQO1 region, which is responsible for *AGRN* mRNA binding, is also functionally required for angiogenic activity. All truncation constructs were expressed at comparable levels, as confirmed by immunoblotting (Fig. 5H). As expected, the expression of full-length NQO1 robustly restored endothelial tube formation and angiogenic activity. Notably, among the truncated variants, only the fragment encompassing amino acids 96 to 178, which was previously mapped as the *AGRN* mRNA-binding region, was sufficient to rescue the impaired proangiogenic phenotype to an extent comparable with that observed with WT NQO1, whereas the remaining truncations failed to do so (Fig. 5I and J). These results indicate that the 96 to 178 region of NQO1 is not only necessary for *AGRN* binding but also functionally sufficient to mediate NQO1-dependent angiogenic activity.

We generated stable *AGRN*-knockdown ESCC cell lines to determine whether *AGRN* mediates the protumor and proangiogenic effects of NQO1 (Supplementary Fig. S5A). *AGRN* knockdown markedly inhibited colony formation and attenuated the angiogenic potential *in vitro* (Supplementary Fig. S5B–S5E). In subcutaneous xenograft models, *AGRN*-deficient tumors exhibited reduced growth and decreased expression of Ki-67 and CD31 (Supplementary Fig. S5F–S5I).

To further test whether NQO1 promotes ESCC progression and angiogenesis through *AGRN*, we knocked down *AGRN* in cells

(Continued.) WT and P187S NQO1 proteins pulled down by *in vitro*-transcribed biotinylated *AGRN* exons 4–7 RNA. J and K, Confocal microscopy showing the colocalization of NQO1 (WT or P187S, green) and *AGRN* exons 4–7 (red) in ECA109 cells (scale bars, 5 μ m). L, Schematic diagram of NQO1 deletion mutants. M and N, RIP-qPCR and RNA pulldown analyses identifying the *AGRN*-binding region within NQO1 (residues 1–95, 96–178, or 179–274) in KYSE30 cells overexpressing WT NQO1. O and P, qRT-PCR analysis of *AGRN* mRNA levels in ECA109 and KYSE30 cells treated with actinomycin D (5 μ g/mL) at the indicated time points. Q and R, Relative *AGRN* mRNA levels in NQO1-knockout ESCC cells measured by qPCR. S and T, *AGRN* protein levels in NQO1-knockout ESCC cells measured by Western blotting. The data are presented as the means $\pm$ SDs of three independent experiments (E, F, and M–R). The results from one of three representative experiments are shown in J. Statistical analyses were performed using two-tailed Student *t* test (E, F, Q, and R), one-way ANOVA (M), or two-way ANOVA (O and P). **, *P* < 0.01; ****, *P* < 0.0001; ns, not significant.

**Figure 5.**

NQO1 positively regulates AGRN expression to promote angiogenesis. **A**, A cell-cell communication analysis was performed using CellChat to evaluate the effects of components of the tumor microenvironment on ESCC cells. The node size represents the cell population abundance, and the line thickness indicates the strength of the interaction. **B**, Representative images of MIF staining showing CD31⁺ endothelial cells, CD68⁺ myeloid cells, and αSMA⁺ fibroblasts in tumors from NQO1-low and NQO1-high patients with ESCC. **C** and **D**, Tube formation assays of HUVECs cultured with conditioned media from ESCC cells. Representative images of the tubes (scale bars, 200 μm; **C**) and the quantitative results (**D**) are shown. **E**, MIF staining of tumor sections showing NQO1 expression and vascular invasion (CD31) in nude mice subcutaneously injected with control and NQO1-overexpressing ECA109 cells. **F** and **G**, Representative images showing the adhesion of NQO1-knockout ECA109 cells (GFP) to HUVECs (Cherry; scale bars, 100 μm). **H**, GFP-tagged full-length NQO1 and its deletion mutants were transfected into NQO1-KO cells. Afterward, the transfection efficiency was determined by WB. **I** and **J**, Tube formation assays of HUVECs cultured with conditioned media from the cells in **H**. Representative images of tubes (scale bars, 200 μm; **I**) and the quantitative results (**J**) are shown. **K**, Growth of subcutaneous ECA109 tumors in nude mice ($n = 6$). **L**, Weights of tumors collected at the endpoint. **M**, Tumor volumes measured every other day and corresponding growth curves. **N**, IHC staining of tumor sections for AGRN, Ki-67, and CD31 (scale bars, 100 μm). The data are presented as the means ± SDs of three independent experiments (**D**, **G**, **J**, **L**, and **M**). The results from one of three representative experiments are shown in **B**, **C**, **F**, and **I**. Statistical analyses were performed using one-way ANOVA (**D**, **G**, and **J**) or two-way ANOVA (**M**). *, $P < 0.05$; ****, $P < 0.0001$; ns, not significant.

overexpressing either WT or mutant NQO1. Colony formation assays showed that both NQO1 variants significantly increased the clonogenicity of ESCC cells, and this effect was abolished by AGRN

knockdown (Supplementary Fig. S5J and S5K). Similar results were observed in the HUVEC tube formation assays (Supplementary Fig. S5L and S5M). We validated these findings *in vivo* by establishing

subcutaneous xenografts with the aforementioned engineered cell lines. Tumor growth was monitored every 3 days, followed by the harvesting of the tumors and histologic analysis after 1 month (Fig. 5K). Tumors that formed from P187S-overexpressing cells were smaller than those expressing WT NQO1 but larger than the tumors that formed from control cells. AGRN knockdown completely abrogated the growth advantage in the P187S group and reduced the tumor size to some extent in the WT group (Fig. 5L and M). IHC staining confirmed that Ki-67 and CD31 expression levels correlated with the tumor burden across the groups, supporting the functional relevance of the NQO1-AGRN axis in regulating proliferation and angiogenesis (Fig. 5N).

Collectively, these findings demonstrate that NQO1 promotes ESCC progression and angiogenesis through a nonenzymatic, RNA-binding function by upregulating the expression of AGRN, which acts as a critical downstream effector.

EV-delivered AGRN interacts with FLNA to stabilize the cytoskeleton and promote angiogenesis

To elucidate how AGRN promotes angiogenesis, we segmented AGRN protein into two structure domains: T30–R1102 and A1260–P2045. Each fragment was fused with a Myc tag and expressed in *E. coli* (Supplementary Fig. S6A). Functional screening revealed that only the A1260–P2045 region exhibited proangiogenic activity (Supplementary Fig. S6B).

AGRN is traditionally known to reside in the synaptic basal lamina, where it signals through the extracellular domain of low-density lipoprotein receptor-related protein 4 (LRP4), a transmembrane receptor of the LDLR family (21, 22). We treated HUVECs with purified AGRN (A1260–P2045) to investigate the localization of AGRN in endothelial cells. Immunofluorescence staining showed AGRN localization at both the plasma membrane and in the intracellular compartment (Fig. 6A), suggesting that AGRN may act through both LRP4-dependent and -independent pathways. Intriguingly, we found that knocking down LRP4 in HUVECs significantly attenuated but did not completely abrogate the proangiogenic effect of AGRN (Fig. 6B and C), suggesting the involvement of additional intracellular mediators. We assessed this possibility by incubating Myc-tagged AGRN (A1260–P2045) with HUVEC lysates, followed by co-IP and LC-MS/MS analysis (Fig. 6D; Supplementary Table S5). Among the interacting proteins, FLNA was identified as the top candidate (Fig. 6E; Supplementary Fig. S6C), and this interaction was further confirmed by co-IP assays (Fig. 6F and G). Given that the FLNA binding region for AGRN is unknown, we divided FLNA into five structural domains (Fig. 6H). Co-IP-based mapping revealed that the D21–23 repeat domain of FLNA mediates its interaction with AGRN (Fig. 6I). Moreover, the dual knockdown of LRP4 and FLNA completely abolished AGRN-induced angiogenesis (Fig. 6J and K; Supplementary Fig. S6D), indicating that both LRP4 and FLNA are required for AGRN-mediated angiogenesis.

Given previous evidence that AGRN is secreted via EVs in pancreatic cancer (23), we next examined whether ESCC cells also release AGRN in EVs. Using ultracentrifugation followed by TEM and Western blot analyses, we confirmed that ESCC cells indeed secreted AGRN in EVs (Fig. 6L and M). To determine whether NQO1 affects global EV secretion or alters the abundance of AGRN carried by tumor-derived EVs, we assessed both intracellular AGRN expression and EV-associated AGRN levels following NQO1 depletion. Consistent with its role in stabilizing AGRN mRNA, AGRN protein levels were markedly reduced in

NQO1-knockout ESCC cells (Fig. 6N). In contrast, NTA revealed no significant differences in either the total number or size distribution of EVs between WT and NQO1-knockout cells (Fig. 6O), indicating that loss of NQO1 does not impair global EV biogenesis or secretion. Immunoblot analysis of purified EVs further demonstrated a substantial reduction in AGRN abundance in EVs derived from NQO1-deficient cells, with the EV marker CD63 serving as a loading control (Fig. 6P). Together, these data indicate that NQO1 regulates the intracellular availability of AGRN, thereby influencing the amount of AGRN carried by tumor-derived EVs, rather than broadly controlling EV production or cargo-sorting mechanisms.

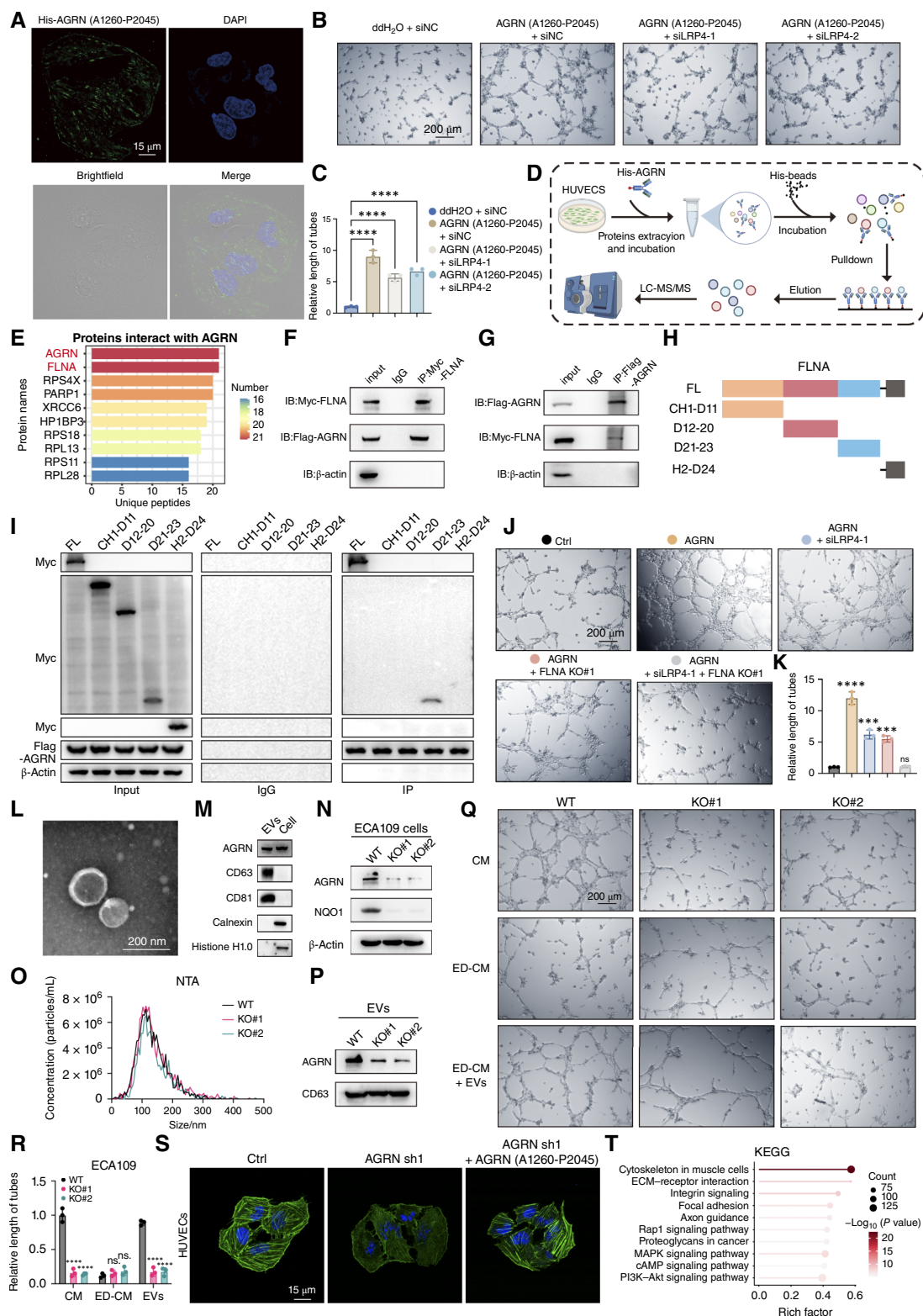
To determine whether the proangiogenic effects of NQO1 are mediated through EVs rather than EV-independent soluble factors, we performed EV depletion and rescue experiments using conditioned media (CM) from ESCC cells. Compared with control CM, CM derived from NQO1 knockout cells significantly reduced proangiogenic activity in HUVECs. Following ultracentrifugation to remove EVs, EV-depleted CM (ED-CM) from either control or NQO1-knockout cells induced comparable levels of endothelial tube formation, indicating that soluble factors alone are insufficient to mediate NQO1-dependent endothelial activation. In contrast, the supplementation of ED-CM with purified EVs restored endothelial angiogenic responses in an NQO1-dependent manner (Fig. 6Q and R). Together, these findings indicate that NQO1 promotes angiogenesis in endothelial cells predominantly through EV-mediated signaling.

FLNA is known to interact with actin filaments (F-actin) to organize and regulate cell morphology and motility (24, 25). Accordingly, AGRN knockdown in HUVECs resulted in pronounced cytoskeletal disassembly, which was reversed by the addition of the recombinant AGRN protein (Fig. 6S). RNA-seq was performed on HUVECs treated with exosomes derived from ESCC cells expressing control shRNA or two independent AGRN shRNAs to systematically identify the downstream signaling pathways in endothelial cells that were regulated by AGRN-positive exosomes (Supplementary Fig. S6E–S6G). DEGs ($P < 0.05$, $|\text{fold change}| > 1$) were subjected to a KEGG pathway enrichment analysis. The analysis revealed the significant enrichment of pathways related to ECM–receptor interaction, integrin signaling, focal adhesion, and cytoskeleton-associated processes, as well as canonical angiogenic signaling pathways, including the PI3K–Akt, MAPK, Rap1, and cAMP signaling pathways (Fig. 6T). These pathways are known to regulate endothelial cell migration, adhesion, cytoskeletal remodeling, and angiogenic responses (26, 27).

These results demonstrate that AGRN promotes angiogenesis through dual mechanisms involving LRP4-mediated extracellular signaling and a novel intracellular interaction with FLNA that preserves endothelial cytoskeletal integrity. Importantly, AGRN is selectively incorporated into tumor-derived EVs, which serve as key paracrine vehicles to transmit angiogenic signals from ESCC cells to endothelial cells.

Panobinostat directly targets NQO1 to suppress the malignant progression of ESCC

Given the oncogenic nonenzymatic function of NQO1 in ESCC and the current lack of inhibitors targeting it, we aimed to identify small molecules that disrupt this function. Based on the crystal structure of NQO1 (PDB ID: 1KBQ), we predicted potential allosteric binding sites and performed a virtual screen of a 120,000-compound library. The top 10 candidates selected based on their

**Figure 6.**

AGRN interacts with FLNA to stabilize the cytoskeleton. **A**, Confocal microscopy images showing the internalization of His-tagged rAGRN (A1260-P2045, GFP) in HUVECs (brightfield overlay). **B** and **C**, Tube formation assays using HUVECs treated with AGRN (A1260-P2045, GFP) with or without LRP4 knockdown. Representative images (**B**) and the quantification of the results (**C**) are shown. **D**, Diagram of the workflow for co-IP to (Continued on the following page.)

LibDock scores were further evaluated using SPR and cell viability assays (Fig. 7A).

Among these compounds, five exhibited direct binding to NQO1 (binding cutoff >30 RU in SPR; Fig. 7B), and four significantly reduced the viability of ESCC cells (Fig. 7C). Panobinostat emerged as the only compound common to both sets (Fig. 7D and E). Molecular docking predicted that panobinostat occupies the catalytic pocket of NQO1, overlapping with the NAD(P)H-binding site and engaging His161, a critical residue for enzymatic activity (Fig. 7F). SPR confirmed concentration-dependent binding to recombinant NQO1, with dissociation constants of $K_D = 29 \mu\text{mol/L}$ for WT NQO1 and $K_D = 35 \mu\text{mol/L}$ for the enzymatically inactive P187S mutant, indicating enzyme activity-independent binding (Fig. 7G and H).

Functionally, panobinostat induced the dose-dependent degradation of the NQO1 protein in ESCC cells (Fig. 7I) and significantly decreased ESCC cell viability (Fig. 7J). This antiproliferative effect was markedly attenuated in NQO1-knockout cells, confirming its NQO1-dependent activity (Fig. 7K and L; Supplementary Fig. S7A). At the mechanistic level, panobinostat treatment markedly reduced AGRN expression in ESCC cells without significantly affecting total EV release (Fig. 7M and O). However, the AGRN levels in the purified EVs were substantially decreased, indicating that panobinostat selectively impaired AGRN loading into EVs rather than global EV biogenesis (Fig. 7N). Functionally, EVs derived from panobinostat-treated ESCC cells exhibited a reduced capacity to promote HUVEC tube formation (Fig. 7P and Q).

Given the critical roles of NQO1 in angiogenesis and tumor progression, we further evaluated the therapeutic efficacy of panobinostat *in vivo* using patient-derived organoids with high endogenous NQO1 expression (org 3T) implanted subcutaneously into nude mice. Mice were treated with panobinostat, anlotinib [a multitarget VEGFR/PDGFR/FGFR inhibitor approved to treat lung cancer and under investigation as a treatment for ESCC (28, 29)], or their combination (Fig. 7R). Compared with monotherapy, combination therapy resulted in significantly reduced tumor weight (Fig. 7T) and slower tumor growth (Fig. 7S). IHC further showed markedly lower expression of Ki-67 and CD31 in the combination group, indicating the concurrent suppression of tumor growth and vascularization (Fig. S7B). Treatment with panobinostat, anlotinib, or their combination was well tolerated *in vivo*. The histologic examination of major organs, including the liver, kidney, lung, and spleen, revealed no overt pathologic abnormalities or metastatic burden (Supplementary Fig. S7C). To complement

histopathologic evaluation, we performed serum biochemical profiling in nude mice treated with vehicle, panobinostat, anlotinib, or their combination. As shown in Supplementary Table S6, serum levels of AST—along with other markers of liver (ALT, ALP, TBIL, and ALB), renal (CRE and UREA), and cardiac (CK and CK-MB) function—remained within normal physiologic ranges and did not differ significantly among treatment groups. These data confirm that panobinostat and anlotinib, administered alone or in combination, did not elicit detectable systemic toxicity under the experimental conditions used.

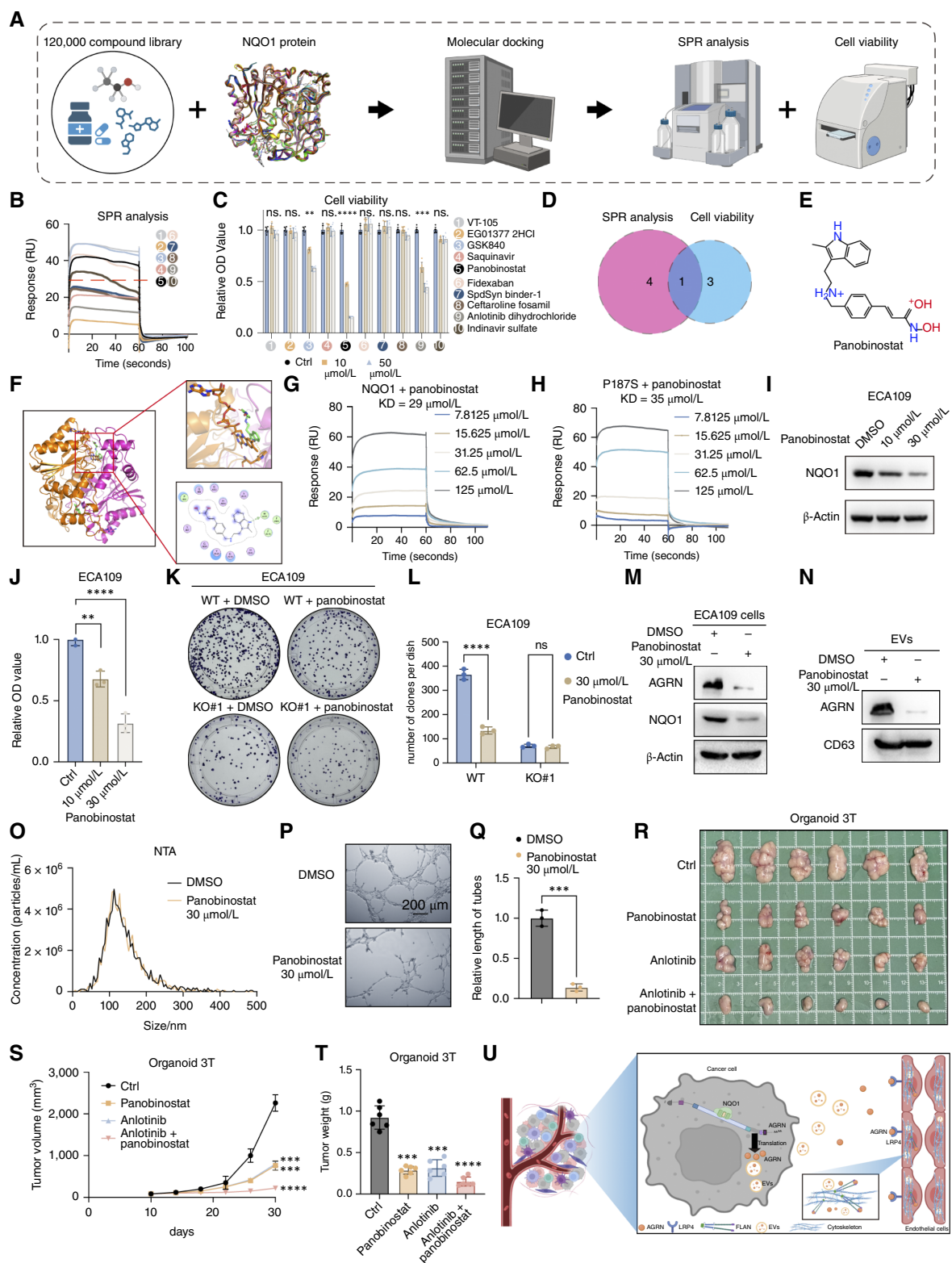
We further assessed the therapeutic effects of these treatments on tumor cell colonization in the lung using a lung colonization model established by a tail vein injection of ESCC cells. Treatment with either panobinostat or anlotinib alone significantly reduced the pulmonary tumor burden, whereas the combination therapy had a more pronounced inhibitory effect on lung colonization (Supplementary Fig. S7D–S7F). The histologic analysis confirmed marked reductions in both the number and size of lung tumor nodules in the combination group.

Discussion

ESCC remains a highly aggressive malignancy with limited therapeutic options and poor clinical outcomes (30). Although metabolic reprogramming is a recognized hallmark of ESCC (31, 32), the noncanonical roles of metabolic enzymes in tumor progression are still being elucidated. This study identifies a previously unrecognized, enzymatic activity-independent function of NQO1 in ESCC. We demonstrate that NQO1 promotes tumor progression and angiogenesis not primarily through its oxidoreductase activity but by acting as an RNA-binding protein (RBP) that post-transcriptionally regulates the ECM component AGRN. This mechanism coordinates tumor–endothelial communication via EVs, revealing a novel axis of microenvironmental cross-talk in ESCC.

NQO1 is classically characterized as a cytosolic oxidoreductase involved in quinone detoxification and redox homeostasis (5). Its frequent overexpression in malignancies has often been viewed as an adaptive response to oxidative stress rather than a direct driver of oncogenesis (6, 33). Consistent with this, our data do not support NQO1 as a classic, enzyme-dependent oncogenic driver. Instead, our findings indicate that elevated NQO1 expression confers a selective advantage by enabling a nonenzymatic regulatory program that foster angiogenesis and metastatic colonization. Crucially, genetic rescue experiments with the catalytically inactive P187S

(Continued.) identify AGRN-binding proteins in HUVECs. **E**, Bar plot showing candidate AGRN-interacting proteins identified by LC-MS/MS. **F** and **G**, Co-IP assays confirming the interaction between AGRN and FLNA in 293T cells coexpressing Flag-tagged AGRN and Myc-tagged FLNA. **H**, Schematic diagram of the FLNA domain structure. **I**, *In vitro* Co-IP assay validating the interaction between Flag-tagged AGRN and specific Myc-tagged FLNA fragments. **J** and **K**, Tube formation assays using HUVECs under the indicated conditions. Representative images (**J**) and the quantification of the results (**K**) are shown. **L**, TEM of EVs isolated from ESCC cells (scale bars, 200 nm). **M**, Western blot analysis of EV-associated proteins, including AGRN. **N**, Western blot analysis of AGRN expression in NQO1-knockout cells. **O**, NTA of the size distribution and concentration of EVs secreted by NQO1-knockout ECA109 cells. **P**, Western blot analysis of AGRN abundance in EVs derived from NQO1-deficient ECA109 cells. **Q** and **R**, CM from control or NQO1-knockout ESCC cells was added to HUVECs for tube formation assays. CM were subjected to ultracentrifugation to generate ED-CM, followed by supplementation with purified EVs where indicated. Representative images of HUVEC tube formation (**Q**) and the corresponding quantitative results (**R**) are shown. **S**, Phalloidin staining of the cytoskeleton in HUVECs following AGRN knockdown and rescue with the purified AGRN (A1260–P2045) protein. **T**, RNA-seq was performed on HUVECs treated with exosomes derived from ESCC cells expressing a control shRNA (NC) or two independent AGRN shRNAs (sh1 and sh2). DEGs ($P < 0.05$, |fold change| > 1) were subjected to a KEGG pathway enrichment analysis. The data are presented as the means $\pm$ SDs of three independent experiments (**C**, **E**, **G**, **H**, **K**, and **M**). The results from one of three representative experiments are shown in **C**, **O**, and **R**. Statistical analyses were performed using one-way ANOVA (**C**), two-way ANOVA (**O**), and two-tailed Student *t* test (**R**). ***, $P < 0.001$; ****, $P < 0.0001$; ns, not significant. **D**, Created in BioRender. Wu, J. (2026) <https://BioRender.com/admxm32>.

**Figure 7.**

Panobinostat directly targets NQO1 to suppress the malignant progression of ESCC. **A**, Diagram showing the workflow of the molecular docking of new pharmacologic inhibitors targeting NQO1. **B**, SPR analysis showing the binding kinetics between the top 10 compounds and the recombinant NQO1 protein. **C**, CCK-8 assays were performed following treatment with the compounds to evaluate cell viability. **D**, A Venn diagram (Continued on the following page.)

mutant demonstrate that a substantial portion of NQO1's protumorigenic activity is preserved independently of its oxidoreductase function. This provides a mechanistic basis for the long-observed correlation between high NQO1 expression and poor clinical outcome, suggesting NQO1 operates as a context-dependent regulator that shapes the tumor microenvironment rather than as a cell-intrinsic metabolic driver.

A central finding of this study is the identification of NQO1 as a functional RNA-binding protein. Although it lacks canonical RNA-binding domains, integrative LACE-seq profiling and biochemical mapping delineate a discrete RNA-interacting region (amino acids 96–178) that is structurally and functionally separable from the catalytic core. This region displays conformational flexibility—a feature often associated with adaptable macromolecular interactions—and is both necessary and sufficient for restoring angiogenic activity in NQO1-deficient cells. This aligns with emerging paradigms wherein proteins engage RNA through noncanonical, structurally plastic regions (34). Among its RNA targets, AGRN emerged as a key effector based on three criteria: direct physical binding to NQO1, posttranscriptional stabilization upon NQO1 modulation, and functional linkage to ECM–receptor signaling pathways. By stabilizing AGRN mRNA, NQO1 elevates AGRN protein levels, highlighting posttranscriptional control of ECM-associated signaling as a critical mechanism for microenvironmental reprogramming in ESCC.

Notably, AGRN is enriched in tumor-derived EVs in an NQO1-dependent manner. Whereas EVs are established mediators of tumor–stroma communication (35), how specific cargoes are selected remains poorly defined. Our data indicate that NQO1 does not directly regulate EV biogenesis, secretion, or cargo-sorting machinery. Instead, by stabilizing AGRN mRNA and increasing its intracellular pool, NQO1 elevates the amount of AGRN available to be carried by tumor-derived EVs. This availability-driven model explains why loss of NQO1 leads to a proportional decrease in EV-associated AGRN without affecting total EV output or size distribution. Functionally, EV-delivered AGRN is required for proangiogenic signaling, as EV-depleted conditioned media fails to stimulate endothelial responses. Vesicle-mediated transfer likely ensures localized, sustained delivery of AGRN to endothelial cells, protecting it from degradation and facilitating efficient paracrine communication within the vascular niche. Thus, NQO1 shapes tumor–endothelial cross-talk primarily by controlling the cellular availability of AGRN for subsequent EV-mediated release, rather than by actively directing cargo-sorting.

Mechanistically, AGRN promotes angiogenesis through a dual-pathway model in endothelial cells. Extracellularly, it engages LRP4 receptor, activating proangiogenic signaling (36). Intracellularly, following EV uptake, AGRN binds directly to FLNA, a key cytoskeletal scaffold protein. This interaction stabilizes the actin cytoskeleton, promoting endothelial cell sprouting and morphogenesis. The finding that simultaneous disruption of LRP4 and FLNA completely abrogates AGRN-driven angiogenesis underscores the cooperative integration of receptor-mediated signaling and intracellular cytoskeletal reinforcement.

From a therapeutic perspective, we identified the clinically approved HDAC inhibitor panobinostat as a direct binder of NQO1 that induces its destabilization. Panobinostat suppressed the NQO1–AGRN axis, reducing AGRN loading into EVs and diminishing their proangiogenic capacity, without broadly affecting EV release. This demonstrates pharmacologic tractability of targeting nonenzymatic functions of metabolic enzymes. Furthermore, combining panobinostat with the multi-targeted antiangiogenic agent anlotinib resulted in superior suppression of tumor growth and vascularization *in vivo*. This combination embodies a “vertical blockade” strategy, simultaneously targeting the tumor-derived production of angiogenic signals and their reception by endothelial cells, thereby potentially mitigating adaptive resistance.

Several limitations of this study should be noted. First, whereas we mapped the AGRN mRNA-binding region to amino acids 96 to 178 of NQO1, high-resolution structural analyses are needed to define the precise molecular interface and determine whether this region engages additional RNA or protein partners. Second, the tail vein injection model used in this study assesses lung colonization and vascular adaptation but does not capture the full metastatic cascade from a primary tumor. Orthotopic or spontaneous metastasis models would be valuable for future work. Finally, it remains to be determined whether this RNA-regulatory function of NQO1 is operational in other cancer types in which NQO1 is overexpressed. Addressing these questions may reveal how metabolic enzymes engage RNA regulation to shape intercellular communication and vascular remodeling in cancer.

In summary, our study uncovers an enzymatic activity-independent role for NQO1 as an RBP that stabilizes AGRN mRNA, thereby promoting EV-mediated angiogenic signaling in ESCC. This work expands the functional repertoire of metabolic enzymes in cancer, illustrates how posttranscriptional regulation can sculpt the tumor microenvironment, and provides a rationale for combination therapies targeting both the source and the reception of proangiogenic signals.

(Continued.) was constructed to identify the overlapping hits between the results of the CCK-8 and SPR assays. **E**, The structure of panobinostat. **F**, Superposition of the structure of the NQO1 protein with panobinostat. **G** and **H**, SPR analysis revealed the binding of panobinostat to the NQO1 protein (WT and P187S). **I**, Western blot analysis was performed to assess NQO1 protein expression in cells treated with panobinostat. **J**, Results of the CCK-8 assay of ESCC cells treated with increasing concentrations of panobinostat for 48 hours. **K** and **L**, Representative images and quantification of colony formation in WT and NQO1-knockout ESCC cells treated with panobinostat. **M**, Western blot analysis of NQO1 and AGRN expression in ECA109 cells treated with panobinostat. **N**, Western blot analysis of AGRN abundance in EVs derived from ECA109 cells treated with panobinostat. **O**, NTA of the size distribution and concentration of EVs secreted by ECA109 cells treated with panobinostat. **P** and **Q**, Tube formation assays using HUVECs cultured with CM from ECA109 cells treated with panobinostat. **R**, Representative images of tumors harvested at the endpoint. **S** and **T**, Tumor growth curves and final tumor weights in each treatment group ($n = 6$ mice per group). **U**, Graphical illustration of an enzymatic activity-independent RNA-binding function of NQO1 that promotes angiogenesis through EV-mediated stabilization of AGRN in ESCC. The data are presented as the means $\pm$ SDs of three independent experiments (**B**, **C**, **G–J**, **L**, **O**, **Q**, and **S**). The results from one of three representative experiments are shown in **K** and **P**. Statistical analyses were performed using one-way ANOVA (**C**, **J**, and **T**), two-way ANOVA (**G**, **H**, **O**, and **S**), and two-tailed Student *t* test (**L** and **Q**). **, $P < 0.01$; ***, $P < 0.001$; ****, $P < 0.0001$; ns, not significant. **U**, Created in BioRender. Wu, J. (2026) <https://BioRender.com/adxmx32>.

Data Availability

The UID RNA-seq data and LACE-seq data generated in this study are publicly available in the Genome Sequence Archive in National Genomics Data Center, China National Center for Bioinformation/Beijing Institute of Genomics, Chinese Academy of Sciences with accession number CRA032860 (<https://ngdc.cncb.ac.cn/gsa/>); HRA015904, HRA015789, HRA015771 (<https://ngdc.cncb.ac.cn/gsa-human/>). All data requests will be granted by the Data Access Committee. The public ESCC single-cell data set analyzed in this study were obtained from the Gene Expression Omnibus (RRID: SCR_005012) at accession number GSE160269 (<https://www.ncbi.nlm.nih.gov/geo/>). All other raw data are available from the corresponding author upon request.

Authors' Disclosures

No disclosures were reported.

Authors' Contributions

J. Wu: Conceptualization, data curation, investigation, writing—original draft. **C. Chen:** Data curation, software. **X. Lv:** Data curation. **Q. Wang:** Methodology.

Z. Wang: Visualization. **Y. Chen:** Data curation. **J. Hou:** Data curation. **Q. Fan:** Formal analysis. **Q. Ren:** Methodology. **C. Sun:** Methodology. **S. Lu:** Data curation. **Y. Shu:** Supervision. **S. Wang:** Conceptualization. **X. Wang:** Conceptualization, supervision, writing—review and editing.

Acknowledgments

This work was supported in part by the National Natural Science Foundation of China (U24A20720 and 82273157 to S. Wang; 82372755 to Q. Wang; 82503474 to C. Chen), China Postdoctoral Science Foundation (2024M760032 to C. Chen), Natural Science Foundation of Anhui Province (2408085QH235 to C. Chen).

Note

Supplementary data for this article are available at Cancer Research Online (<http://cancerres.aacrjournals.org/>).

Received September 29, 2025; revised January 20, 2026; accepted March 13, 2026; posted first March 16, 2026.

References

- Li J, Xu J, Zheng Y, Gao Y, He S, Li H, et al. Esophageal cancer: epidemiology, risk factors and screening. *Chin J Cancer Res* 2021;33:535–47.
- Ju J, Zhang H, Lin M, Yan Z, An L, Cao Z, et al. The alanyl-tRNA synthetase AARS1 moonlights as a lactyltransferase to promote YAP signaling in gastric cancer. *J Clin Invest* 2024;134:e174587.
- Wu K, Yan M, Liu T, Wang Z, Duan Y, Xia Y, et al. Creatine kinase B suppresses ferroptosis by phosphorylating GPX4 through a moonlighting function. *Nat Cell Biol* 2023;25:714–25.
- Meng Y, Guo D, Lin L, Zhao H, Xu W, Luo S, et al. Glycolytic enzyme PFKFB3 governs lipolysis by promoting lipid droplet-mitochondria tethering to enhance β -oxidation and tumor cell proliferation. *Nat Metab* 2024;6:1092–107.
- Ross D, Siegel D. The diverse functionality of NQO1 and its roles in redox control. *Redox Biol* 2021;41:101950.
- Yu J, Zhong B, Zhao L, Hou Y, Ai N, Lu J-J, et al. Fighting drug-resistant lung cancer by induction of NAD(P)H:quinone oxidoreductase 1 (NQO1)-mediated ferroptosis. *Drug Resist Updat* 2023;70:100977.
- Dou L, Liu H, Wang K, Liu J, Liu L, Ye J, et al. Albumin binding revitalizes NQO1 bioactivatable drugs as novel therapeutics for pancreatic cancer. *J Control Release* 2022;349:876–89.
- Luo M, Shen N, Shang L, Fang Z, Xin Y, Ma Y, et al. Simultaneous targeting of NQO1 and SOD1 eradicates breast cancer stem cells via mitochondrial futile redox cycling. *Cancer Res* 2024;84:4264–82.
- Wu J, Chen Y, Zou H, Xu K, Hou J, Wang M, et al. 6-Phosphogluconate dehydrogenase promotes glycolysis and fatty acid synthesis by inhibiting the AMPK pathway in lung adenocarcinoma cells. *Cancer Lett* 2024;601:217177.
- Ocio EM, Vilanova D, Atadja P, Maiso P, Crusoe E, Fernández-Lázaro D, et al. In vitro and in vivo rationale for the triple combination of panobinostat (LBH589) and dexamethasone with either bortezomib or lenalidomide in multiple myeloma. *Haematologica* 2010;95:794–803.
- Ma G, Gao A, Chen J, Liu P, Sarda R, Gulliver J, et al. Modeling high-risk wilms tumors enables the discovery of therapeutic vulnerability. *Cell Rep Med* 2024;5:101770.
- Tang H, You T, Ge H, Bai C, Wang Y, Sun Z, et al. Autophagy inhibition improves the efficacy of anlotinib and PD-1 inhibitors in the treatment of NSCLC. *J Immunother Cancer* 2025;13:e010812.
- Liu H, Zhou C, Lu J, Liu Y, Zou P, Zhu L, et al. Anlotinib enhances the efficacy of KRAS-G12C inhibitors through c-Myc/ORC2 axis inhibition in non-small cell lung cancer. *Cell Death Dis* 2025;16:356.
- Chen Y, Zhu S, Liu T, Zhang S, Lu J, Fan W, et al. Epithelial cells activate fibroblasts to promote esophageal cancer development. *Cancer Cell* 2023;41:903–18.e8.
- Su R, Fan L-H, Cao C, Wang L, Du Z, Cai Z, et al. Global profiling of RNA-binding protein target sites by LACE-seq. *Nat Cell Biol* 2021;23:664–75.
- Castello A, Fischer B, Eichelbaum K, Horos R, Beckmann BM, Strein C, et al. Insights into RNA biology from an atlas of mammalian mRNA-binding proteins. *Cell* 2012;149:1393–406.
- Chesis PL, Levin DE, Smith MT, Ernster L, Ames BN. Mutagenicity of quinones: pathways of metabolic activation and detoxification. *Proc Natl Acad Sci U S A* 1984;81:1696–700.
- Iyanagi T, Yamazaki I. One-electron-transfer reactions in biochemical systems. V. Difference in the mechanism of quinone reduction by the NADH dehydrogenase and the NAD(P)H dehydrogenase (DT-diaphorase). *Biochim Biophys Acta* 1970;216:282–94.
- Nebert DW, Roe AL, Vandale SE, Bingham E, Oakley GG. NAD(P)H:quinone oxidoreductase (NQO1) polymorphism, exposure to benzene, and predisposition to disease: a HuGE review. *Genet Med* 2002;4:62–70.
- Goenka A, Khan F, Verma B, Sinha P, Dmello CC, Jogalekar MP, et al. Tumor microenvironment signaling and therapeutics in cancer progression. *Cancer Commun (Lond)* 2023;43:525–61.
- Zhang B, Luo S, Wang Q, Suzuki T, Xiong WC, Mei L. LRP4 serves as a coreceptor of agrin. *Neuron* 2008;60:285–97.
- Kim N, Stiegler AL, Cameron TO, Hallock PT, Gomez AM, Huang JH, et al. Lrp4 is a receptor for agrin and forms a complex with MuSK. *Cell* 2008;135:334–42.
- Ruivo CF, Bastos N, Adem B, Batista I, Duraes C, Melo CA, et al. Extracellular vesicles from pancreatic cancer stem cells lead an intratumor communication network (EVNet) to fuel tumour progression. *Gut* 2022;71:2043–68.
- Falet H, Pollitt AY, Begonja AJ, Weber SE, Duerschmied D, Wagner DD, et al. A novel interaction between FlnA and Syk regulates platelet ITAM-mediated receptor signaling and function. *J Exp Med* 2010;207:1967–79.
- Rosa J-P, Raslova H, Bryckaert M. Filamin A: key actor in platelet biology. *Blood* 2019;134:1279–88.
- Hu Z, Cano I, Lei F, Liu J, Bossardi Ramos R, Gordon H, et al. Loss of the endothelial glycocalyx component EMCN leads to glomerular impairment. *Circ Res* 2025;136:59–74.
- Kobayashi H, Terkawi MA, Ota M, Hasegawa T, Yamamoto T, Shimizu T, et al. Involvement of Siglec-15 in regulating RAP1/RAC signaling in cytoskeletal remodeling in osteoclasts mediated by macrophage colony-stimulating factor. *Bone Res* 2024;12:35.
- Li N, Xia J, Gao X, Zhou J, Hong Y, Cui D, et al. First-line benmelstobart plus anlotinib and chemotherapy in advanced or metastatic/recurrent esophageal squamous cell carcinoma: a multi-center phase 2 study. *Sig Transduct Target Ther* 2024;9:303.
- Shen G, Zheng F, Ren D, Du F, Dong Q, Wang Z, et al. Anlotinib: a novel multi-targeting tyrosine kinase inhibitor in clinical development. *J Hematol Oncol* 2018;11:120.
- Abnet CC, Arnold M, Wei W-Q. Epidemiology of esophageal squamous cell carcinoma. *Gastroenterology* 2018;154:360–73.

31. Jin X, Liu L, Liu D, Wu J, Wang C, Wang S, et al. Unveiling the methionine cycle: a key metabolic signature and NR4A2 as a methionine-responsive oncogene in esophageal squamous cell carcinoma. *Cell Death Differ* 2024;31:558–73.
32. Zheng C, Yu X, Liang Y, Zhu Y, He Y, Liao L, et al. Targeting PFKL with penfluridol inhibits glycolysis and suppresses esophageal cancer tumorigenesis in an AMPK/FOXO3a/BIM-dependent manner. *Acta Pharm Sin B* 2022;12: 1271–87.
33. Ma N, Zhang M, Hu J, Wei Z, Zhang S. Daphnetin induces ferroptosis in ovarian cancer by inhibiting NAD(P)H:Quinone oxidoreductase 1 (NQO1). *Phytomedicine* 2024;132:155876.
34. Harrison-Oakes L, Igiogbe W, Nguyen DTT. Functional plasticity of RNA-binding proteins in cancer: both friend and foe. *Trends Cancer* 2026;12: 80–94.
35. Maacha S, Bhat AA, Jimenez L, Raza A, Haris M, Uddin S, et al. Extracellular vesicles-mediated intercellular communication: roles in the tumor microenvironment and anti-cancer drug resistance. *Mol Cancer* 2019;18:55.
36. Lin Y-L, Nhieu J, Liu P-Y, Le G, Lee DJ, Wei C-W, et al. CRABP1-CaMKII-*Agrn* regulates the maintenance of neuromuscular junction in spinal motor neuron. *Cell Death Differ* 2022;29:1744–56.