ANXA1-derived peptides suppress gastric and colon cancer cell growth by targeting EphA2 degradation

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Abstract. EphA2 (EPH receptor A2) (erythropoietin-producing hepatocellular receptor tyrosine kinase subtype A2) plays a crucial role in human cancers, and is a promising target for the development of new anticancer drugs. In this study, we showed that the interaction of Annexin A1 (ANXA1) and EphA2 increased EphA2 stability by inhibiting its proteasome degradation in gastric cancer (GC) and colon cancer (CC) cells, and the amino acid residues 20-30 and 28-30 of ANXA1 N terminal were responsible for binding and stabilizing EphA2. Based on the amino acid residues of ANXA1 responsible for binding EphA2, we developed ANXA1-derived 3 amino acid-long (SKG) and 11 amino acid-long peptides (EYVQTVKSSKG) in fusion to cell-penetrating peptide, named as A1(28-30) and A1(20-30) respectively, and found that A1(28-30) and A1(20-30) blocked the binding of ANXA1 with EphA2, targeted EphA2 degradation, and suppressed the growth of GC and CC cells *in vitro* and in mice. Our data demonstrated that ANXA1 was able to bind and stabilize EphA2 in GC and CC cells, and disruption of ANXA1-EphA2 interaction by the two ANXA1-derived peptides inhibited the growth of GC and CC cells by targeting EphA2 degradation, presenting a potential strategy for treating GC and CC with these peptides.

Introduction

Gastric cancer (GC) and colon cancer (CC) are the most common gastrointestinal malignancies worldwide (1), accounting for 10 and 6% of all cancer diagnoses, and also being the second and third leading cause of cancer mortality, respectively (2,3). The prevalence and poor prognosis of GC and CC as well as limited treatment options necessitate the search for novel treatments.

Eph receptors belong to a large family of receptor tyrosine kinases (RTKs), and are key regulators of both normal development and disease (4). Perturbation of the Eph receptor and Ephrin ligand system has been observed in various human cancers. Particularly, EphA2 (EPH receptor A2) (erythropoietin-producing hepatocellular receptor tyrosine kinase subtype A2) is the most frequently affected Eph receptor in human cancers (5). EphA2 is overexpressed in various types of cancers, and promotes tumor growth, metastasis, and cancer stem properties through a ligand-independent mechanism, and high EphA2 expression is also associated with an aggressive phenotype and poor patient prognosis (6-11). Thus, overexpression of EphA2 has been considered as a promising target for the treatment of cancers. Various approaches for downregulating EphA2, such as EphA2 antibody, Ephrin-A1 ligand, Ephrin-A1 mimic peptides and RNA interference, have attracted considerable interest as anticancer strategies (12,13).

We recently used immunoprecipitation and mass spectrometry analysis (IP-MS) to search for proteins that interact with EphA2 in nasopharyngeal carcinoma cells, and found that Annexin A1 (ANXA1) is one of the proteins that interact with EphA2, proteomic data of which are available via ProteomeXchange with identifier PXD015242 (https://www.ebi.ac.uk/pride/archive/projects/PXD015242/). ANXA1 is the first identified member of the annexin family of Ca^{2+} and phospholipid-binding proteins (14). It plays a role in the inflammatory and immune response, cell proliferation, apoptosis and differentiation (15,16). ANXA1 expression is deregulated in cancers, and has been linked to tumor development and metastasis (17). Accumulated studies have found that both ANXA1 and EphA2 are overexpressed, and promote tumor growth and progression in GC(18-24) and CC(25-29). However, the physiological and pathological significances of ANXA1 and EphA2 interaction in GC and CC are completely unclear.

Protein-protein interaction (PPI) controls various cellular functions by modulating protein stability, post-translational

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modification, and subcellular location. Previous studies indicate that interaction of ANXA1 N-terminal with the epidermal growth factor (EGF) receptor (EGFR) regulates the abundance of the EGFR (30,31), and promotes the oncogenicity of the EGFR (32). Interestingly, our present study found that interaction of ANXA1 and EphA2 increased EphA2 stability and tumor growth in GC and CC cells. Accumulative studies indicate that abnormal PPI is associated with cancers, representing a pivotal target for chemico-biological interventions (33-35). Numerous studies have indicated that inhibition of PPI by peptides is an efficient anticancer approach (36-38). Moreover, peptides possess various advantages with respect to chemotherapeutic drugs such as low toxicity, ease of synthesis, high target specificity, feasibility of chemical modification, and biocompatibility, making them suitable drug candidates (39,40).

In the present study, based on the fact that the interaction of ANXA1 and EphA2 increase EphA2 stability, we investigated the anti-GC and anti-CC effects of ANXA1-derived peptides, and found that ANXA1 N-terminal-derived 3 and 11 amino acid-long peptides disturbed EphA2-ANXA1 interaction, reduced EphA2 protein stability, and suppressed GC and CC cell growth *in vitro* and *in vivo*. Our findings provide an important basis to use the two ANXA1-derived peptides for the treatment of GC and CC.

Materials and methods

Clinical specimens. Formalin-fixed and paraffin-embedded archival tissue specimens from 30 GC, 30 CC, and 30 paired paracancerous tissues were obtained from Xiangya Hospital, Central South University between January 2019 and June 2019. All specimens were subjected to hematoxylin and eosin (H&E) staining, and the diagnosis was confirmed by two pathologists. The clinicopathological data of the patients are presented in Tables SI and SII.

Cell lines and culture. Human GC cell line AGS and human colon cancer cell lines HCT116 and SW620, and 293 cells were purchased from the American Type Culture Collection (ATCC). All cell lines were grown in RPMI-1640 medium supplemented with 10% fetal bovine serum (FBS; Thermo Fisher Scientific, Inc.) at 37˚C in 5% CO_2. The presence of mycoplasma was detected by staining with 4,6-dimethylindole-2-phenylindole in the cell lines, and no mycoplasma was detected.

Antibodies and reagents. The following antibodies were used in the present study: EphA2 (sc-398832; Santa Cruz Biotechnology, Inc.), ANXA1 (ab137745; Abcam), Flag-tag (F1804; Sigma-Aldrich; Merck KGaA), tubulin (E-AB-20036; Elabscience), goat anti-rabbit IgG-HRP (ab6721; Abcam), and goat anti-mouse IgG-HRP (ab6789; Abcam). Protein G/A-Sepharose™ 4B (82085), streptavidin agarose (20357) and Lipofectamine 2000 (11668019) were purchased from Thermo Fischer Scientific, Inc. pBabepuro-EphA2 expression plasmid, and lentiviral vector GV101 expressing EphA2 shRNA have been previously described by us (41). Lentiviral vector GV112 expressing ANXA1 shRNA was constructed, and its target sequence located in the 3'UTR of ANAX1 mRNA is 5'-AACCCTATACAAGTTGTTCTA-3'. The full-length and N-terminal deletion mutant ANXA1 with Flag tag were constructed, the vector of which was pcDNA3.1. All constructs were established by Genechem (Shanghai, China), and were verified by DNA sequencing.

Peptides. Cell-penetrating peptide (YGRKKRRQRRR) (CPP), CPP-ANXA1-derived 3-mer peptide (28-30aa) (SKG), CPP-ANXA1-derived 11-mer peptide (20-30aa) (EYVQTVKSSKG), FITC-labeled CPP, FITC-labeled CPP-ANXA1-derived 3-mer peptide, FITC-labeled CPP-ANXA1-derived 11-mer peptide, biotin-labeled ANXA1-derived 3-mer peptide, and biotin-labeled ANXA1-derived 11-mer peptide were synthesized by ChinaPeptides (Suzhou, China).

Immunoblotting. Immunoblotting was performed to detect the expression of proteins in the indicated cells as described previously by us (41,42). Briefly, proteins were exacted from cells using RIPA lysis buffer. An equal amount of protein in each sample was subjected to SDS-PAGE separation, followed by blotting onto a PVDF membrane. After blocking, blots were incubated with primary antibodies overnight at 4˚C, followed by incubation with HRP-conjugated secondary antibody for 2 h at room temperature. The signal was visualized with an enhanced chemiluminescence detection reagent (Roche).

Immunoprecipitation and immunoblotting (co-IP). Co-IP was performed to detect protein and protein interaction. In brief, whole cell lysates were incubated with indicated antibodies and Protein G/A-Sepharose 4B overnight at 4˚C. After 5 times wash with RIPA buffer, beads were boiled in 2X SDS-PAGE loading buffer for 5 min to elute protein complexes, followed by SDS-PAGE separation and immunoblotting with specific antibodies.

Biotin pull-down assay. Biotin pull-down assay was performed to detect the interaction of ANXA1-derived 3-mer or 11-mer peptide and EphA2 as previously described (43). In brief, 1 mg of whole cell lysates was incubated with 30 nM peptide overnight at 4˚C, and then incubated with 30 μl streptavidin agarose beads for 4 h at 4˚C. After 5 times wash with PBS buffer, the beads were boiled in 2X SDS-PAGE loading buffer for 6 min, followed by SDS-PAGE separation and immunoblotting with the EphA2 antibody.

Immunohistochemistry. Immunohistochemical staining of ANXA1 and EphA2 was performed on the formalin-fixed and paraffin-embedded tissue sections as described previously by us (44). Briefly, tissue sections were incubated with ANXA1 antibody (1:1,000 dilution) or EphA2 antibody (1:100 dilution) overnight at 4˚C, and then incubated with a biotinylated secondary antibody at room temperature for 15 min, and stained with DAB (3,3-diaminobenzidine). Finally, tissue sections were counterstained with hematoxylin. In negative controls, primary antibodies were replaced with a mouse or rabbit IgG.

Immunohistochemical staining was assessed and scored by two independent pathologists who were blinded to the clinicopathological data; discrepancies were resolved by

consensus. Positive reactions were defined as brown signals in the cytoplasm and/or cell membrane. Staining intensity was categorized: Absent staining as 0, weak as 1, moderate as 2, and strong as 3. The percentage of stained cells (examined in at least 500 cells) was categorized as no staining=0, <30% of stained cells=1, 30~60%=2, and >60%=3. The staining score (ranging from 0-6) for each tissue was calculated by adding the area score and the intensity score. A combined staining score of ≤3 was considered to be low expression, and >3 was considered to be high expression.

Quantitative PCR. qPCR was performed to detect the expression of ANXA1 and EphA2 in the indicated cells as described previously by us (41,42). The primers are presented in the Table SIII.

Molecular docking. The human ANXA1 structure was modelled on the available porcine full-length ANXA1 structure (PDB code: 1hm6) using the SWISS-MODEL server (45). The modelled ANXA1 structure was then docked to the EphA2 structure (PDB code: 5ia2) using ClusPro (46). The best docking result was selected based on the related experimental results, which show that the three amino acids (S28, K29 and G30) of ANXA1 are critical to the binding. To further refine the binding model, molecular dynamics simulations were conducted on the ANXA1 and EphA2 complex. The modelled structure was firstly prepared with the Protein Preparation Wizard implemented in the Schrödinger suite 2015 (47). This procedure added hydrogen atoms of residues and optimized the orientation of polar hydrogens and the protonated states of the proteins. The simulation system was then built with AmberTools14. The prepared simulation box contains 3,340 TIP3P water molecules and 8 chloride ions, resulting in a total of 80,119 atoms. The proteins, water molecules, and ions were modelled using the ff14SB force field (48). The system was minimized and equilibrated using Amber 14 in an NPT ensemble at 300 K and 1 bar. The production run lasted for 10 nsec.

MTT assay. Cells were treated with the treatment peptides or control CPP for 24 h, and cell viability was tested by MTT assay as previously described by us (49). The cytotoxicity of peptides was calculated using the formula: % viability=(A570-A630) treated/(A570-A630) control x100%. The assay was performed three times in triplicate.

Cell Counting Kit-8 (CCK-8) assay. Cells were treated with the treatment peptides or control CPP, and the peptides were replenished every day. Cell proliferation was measured using a CCK-8 kit as previously described by us (50). The assay was performed three times in triplicate.

Plate colony formation assay. Cells were treated with the treatment peptides or control CPP, and the peptides were replenished every day. Cell proliferation was measured by plate colony formation assay as previously described by us (50). The assay was performed three times in triplicate.

Soft agar colony formation assay. Cells were treated with the treatment peptides or control CPP, and the peptides were replenished every day. Soft agar colony formation assay was performed to detect cell anchorage independent growth as previously described by us (50). Cells were allowed to grow in the soft agar cultures for 12 days and colonies consisting of >50 cells were counted under a microscope (LEICA, x50 magnification). The assay was performed three times in triplicate.

Animal experiment. Thirty-six nude male mice (BALB/c nu/nu) (initial weight 16~18 g; 4 weeks old) were obtained from the Laboratory Animal Center of Central South University and maintained in pathogen-free conditions. Mice were randomly divided into three groups before inoculation, cancer cells ($2x10^6$) were inoculated into the flank of mice by subcutaneous injection. After xenografts grew for 6 days, CPP-ANXA1-derived 3-mer peptide, CPP-ANXA1-derived 11-mer peptide or control CPP was intraperitoneally injected into the mice at a dose of 10 mg/kg once daily, tumors were measured using an electronic caliper daily, and tumor volume was calculated using the formula (length x $width^2/2$). The mice were sacrificed by cervical dislocation at a time-defined endpoint, and their tumor and organs (heart, liver, spleen, lung and kidney) were removed and measured using double-blinded evaluation. The tissues were fixed with 4% paraformaldehyde and embedding in paraffin, and subjected to H&E and/or immunohistochemistry.

Statistical analysis. Statistical analysis was performed using IBM SPSS statistical software package 22 (IBM Corp.). Data are presented as means ± SD. For comparisons between two groups, a Student's t test was used, and for analysis with multiple comparisons, one-way ANOVA test followed by Turkey's post-hoc analysis was used. Classification variables were compared by the Chi-square test. Pearson test was used for correlation analysis. P-values <0.05 were considered statistically significant.

Results

ANXA1 is a protein that interacts with EphA2, and expression of both proteins is positively correlated in GC and CC. We recently used immunoprecipitation and mass spectrometry analysis (IP-MS) to search for proteins that interact with EphA2 in nasopharyngeal carcinoma cells, and found that ANXA1 is one of the proteins that interacts with EphA2, proteomic data of which are available via ProteomeXchange with identifier PXD015242. As both ANXA1 and EphA2 promote tumor growth and progression in GC (18-24) and CC (25-29), we aimed to ascertain whether ANXA1 interacts with EphA2 in GC and CC cells. Co-IP showed that ANXA1 interacted with EphA2 in the GC (AGS) and CC (HCT116 and SW620) cell lines (Fig. 1A). Next, we detected the expression levels of ANXA1 and EphA2 in the 30 GC, 30 CC, and 30 paired paracancerous tissues by immunohistochemistry, and observed that the expression levels of both ANXA1 and EphA2 were significantly higher in the GC and CC tissues than those in the paracancerous tissues (Fig. 1B), and were positively correlated in the GC and CC tissues (Fig. 1C). The interaction of ANXA and EphA2, and positive correlation of their expression levels, prompted us to investigate the function and significance of the ANXA-EphA2 interaction in GC and CC.

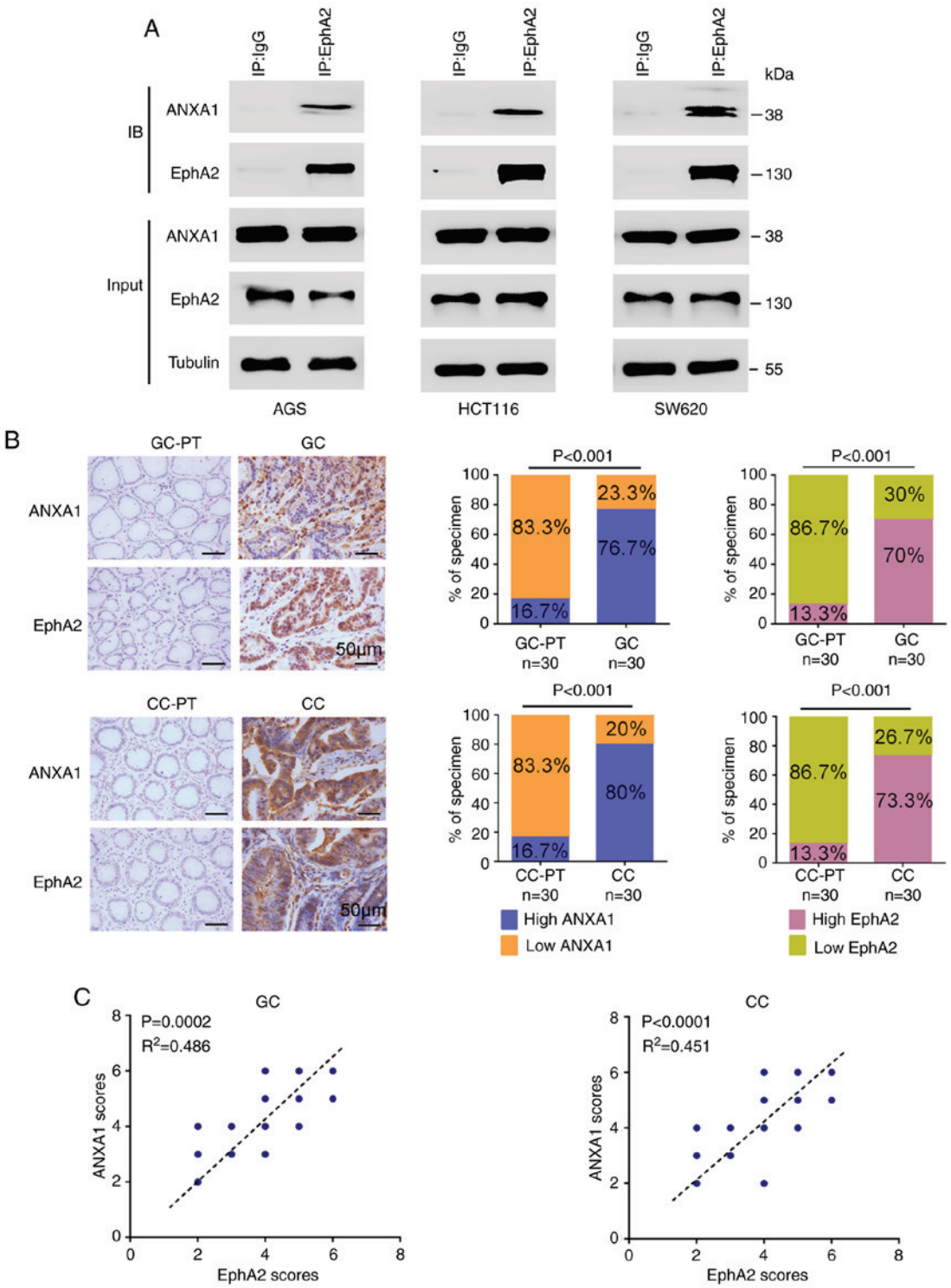

Figure 1. The interaction and expression correlation of ANXA1 and EphA2 in GC and CC. (A) Co-IP showing the interaction of endogenous ANXA1 and EphA2 in the GC (AGS) and CC (HCT116 and SW620) cell lines. Total proteins from the cells were prepared, and subjected to immunoprecipitation (IP) with anti-EphA2 antibody or control IgG followed by immunoblotting (IB) with antibodies against ANXA1 or EphA2. (B) Immunohistochemistry (IHC) showing the expression levels of ANXA1 and EphA2 in the 30 GC, 30 CC, and their paracancerous tissues (PT). Representative IHC images are shown on the left, and quantitative data are presented on the right. P<0.001, Chi-squared test. Scale bars, 50 μm. (C) Positive correlation between ANXA1 and EphA2 expression in the 30 GC and 30 CC tissues. P<0.001, Pearson's correlation test. GC, gastric cancer; CC, colon cancer; ANXA1, Annexin 1.

ANXA1 increases EphA2 stability in the GC and CC cells. It has been reported that ANXA1 regulates the stability of the EGFR (30,31). Therefore, we analyzed the effect of ANXA1 on EphA2 protein stability after blocking protein synthesis with cycloheximide (CHX). The result showed that knockdown of ANXA1 by shRNA dramatically decreased EphA2 levels in the AGS and HCT116 cells (Fig. 2A), but had no effect on its mRNA levels (Fig. 2B), indicating that ANXA1 increased EphA2 protein stability. We also observed that the decrease in EphA2 protein in the ANXA1-knockdown AGS and HCT116

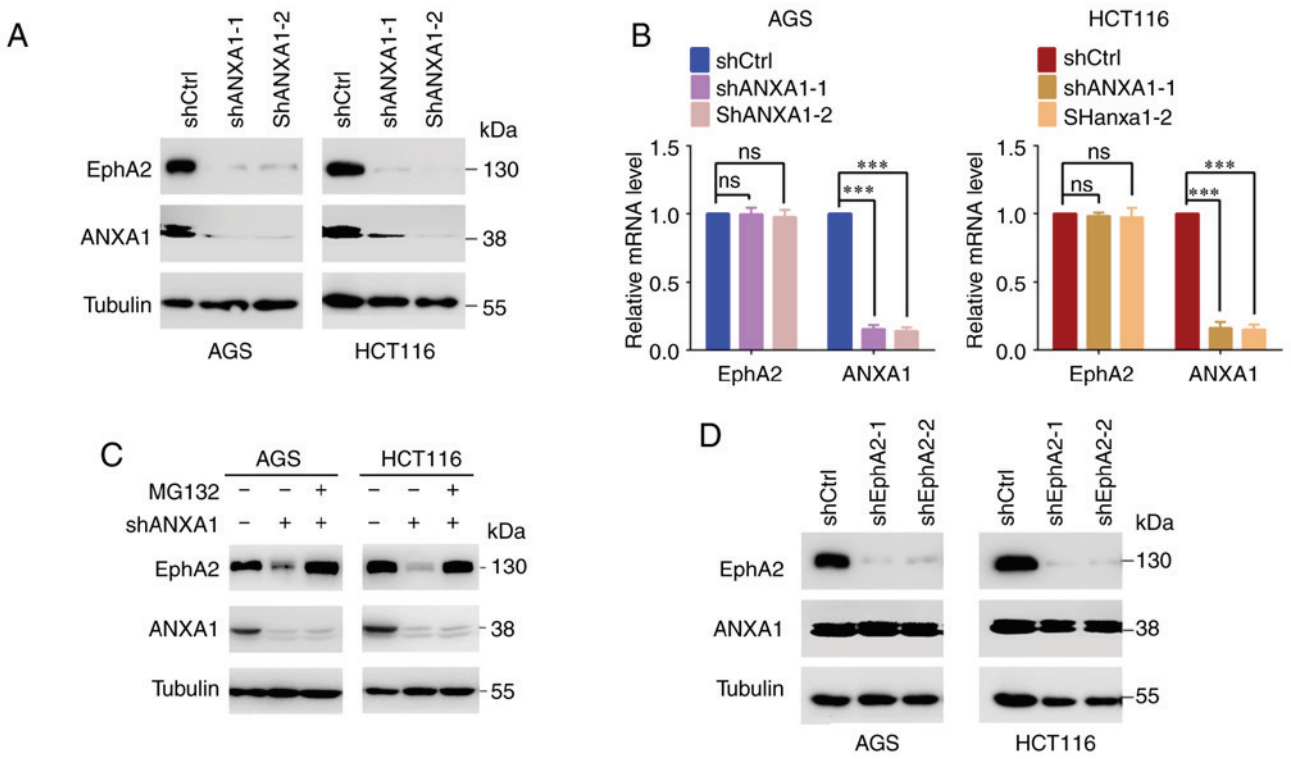

Figure 2. ANXA1 stabilizes EphA2 in GC and CC cells. (A) Immunoblotting showing the protein levels of EphA2 in the AGS and HCT116 cells with ANXA1 knockdown and their control cells. (B) qPCR showing the mRNA levels of EphA2 in the AGS and HCT116 cells with ANXA1 knockdown and their control cells. Error bars indicate means ± SD; ***P<0.001; ns, not significant as determined by Student's t-test. (C) Immunoblotting showing the protein levels of EphA2 in the ANXA1-knockdown AGS and HCT116 cells treated with 10 mM MG132 for 6 h, and their control cells. (D) Immunoblotting showing the protein levels of ANXA1 in the AGS and HCT116 cells with EphA2 knockdown, and their control cells. shANXA1-1 and shANXA1-2, ANXA1 knockdown by shRNA; shEphA2, EphA2 knockdown by shRNA; shCrtl, scramble non-target shRNA; GC, gastric cancer; CC, colon cancer; ANXA1, Annexin 1.

cells was reversed by treatment with the proteasome inhibitor MG132 (Fig. 2C), indicating that ANXA1 increases EphA2 stability by a proteasome-dependent mechanism. However, knockdown of EphA2 had no impact on ANXA1 protein stability in the AGS and HCT116 cells (Fig. 2D).

Identification of the ANXA1 region that binds to EphA2. To map ANXA1 N-terminal responsible for binding EphA2, we constructed a series of ANXA1 N-terminal deletion mutants (Fig. 3A), and cotransfected each of ANXA1 deletion mutants with EphA2 into 293 cells following co-IP analysis. The results showed that N-terminal deletion ANXA1 (D1-40) could not bind to EphA2, indicating that ANXA1 N-terminal was responsible for binding EphA2 (Fig. 3B). We further mapped the ANXA1 N-terminal region responsible for binding EphA2, and observed that D1-19 and D31-40 but not D20-40 and D20-30 were able to bind to EphA2, indicating the 11 amino acid residues (20-30aa) responsible for binding EphA2 (Fig. 3B). Moreover, D28-30 could not bind to EphA2, indicating the 3 amino acid residues (28-30aa) responsible for binding EphA2 (Fig. 3B). Modeling of the structure of the ANXA1-EphA2 complex showed that the three amino acid residues 28-30 (S28, K29 and G30) of ANXA1 are critical to binding EphA2 (Fig. 3C), supporting our experimental result. Next, we analyzed whether the amino acid residues (28-30aa) and (20-30aa) of ANXA1 have functional relevance with EphA2 stability. We transfected the plasmid expressing shRNA-resistant ANXA1, D28-30 or D20-30 into AGS and HCT116 cells with knockdown of endogenous ANXA1 by shRNA, and observed that ANXA1 but not D28-30 and D20-30 could rescue EphA2 levels (Fig. 3D), indicating that the amino acid residues (28-30aa) and (20-30aa) of ANXA1 are important for EphA2 stability.

ANXA1-dirived peptides block EphA2-ANXA1 interaction and target EphA2 degradation. To explore the effects of ANXA1-derived peptides on EphA2-ANXA1 interaction and EphA2 stability, ANXA1-derived 3-mer (28-30aa) (SKG) and 11-mer (20-30aa) (EYVQTVKSSKG) peptides were synthesized in fusion to previously characterized cell-penetrating peptide (CPP) (YGRKKRRQRRR) respectively (51), thereafter named as A1(28-30) and A1(20-30) respectively, and CPP was used as control. Efficient cellular uptake of the three peptides was confirmed by immunofluorescent labeling with fluorescein isothiocyanate (FITC) (Fig. 4A). As compared to CPP, both A1(28-30) and A1(20-30) dramatically decreased ANXA1 bound to EphA2 (Fig. 4B), and efficiently decreased EphA2 protein levels in the GC and CC cells (Fig. 4C). Moreover, biotin pull-down assay, a method for detecting peptide-protein interaction, showed that both A1(28-30) and A1(20-30) could efficiently pull down EphA2 in the GC and CC cells (Fig. 4D), indicating that A1(28-30) and A1(20-30) bind EphA2. Collectively, these results demonstrate that both A1(28-30) and A1(20-30) block ANXA1 binding EphA2, and target EphA2 degradation.

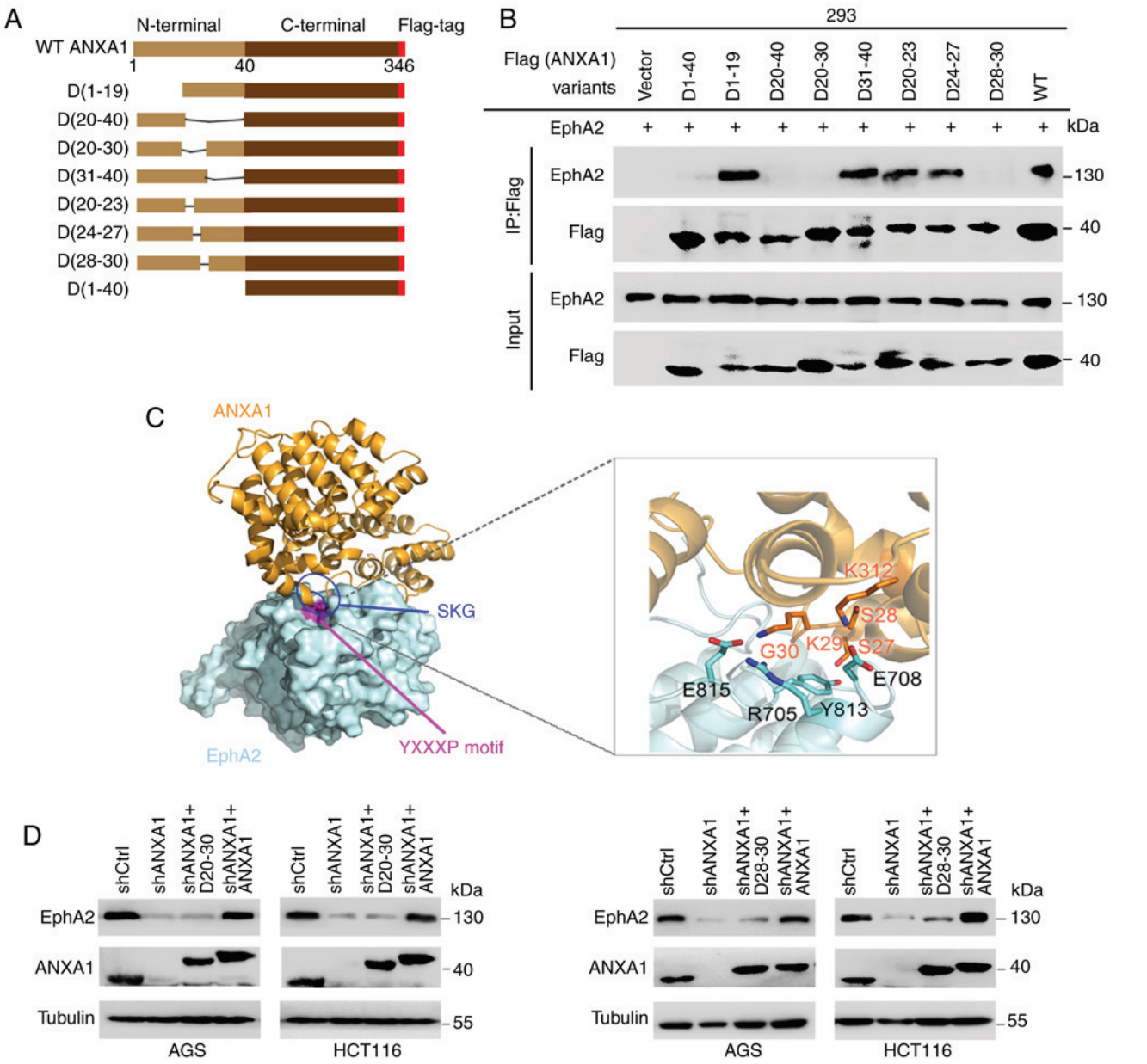

Figure 3. Mapping of ANXA1 region for binding EphA2. (A) Diagrammatic representation of ANXA1 and its N-terminal deleted mutants. The main regions of ANXA1 protein are indicated. Numbers indicate amino acid position within the sequence. D, deletion mutant. (B) Mapping of the region of ANXA1 that binds to EphA2. Total proteins from 293 cells transfected with the indicated constructs were subjected to immunoprecipitation (IP) with anti-Flag (ANXA1) antibody followed by immunoblotting with antibodies against EphA2 or Flag (ANXA1). (C) Overall structure of the ANXA1-EphA2 complex. (Left) ANXA1 is colored orange with important residues (S28, K29 and G30) colored blue, and EphA2 is colored cyan with the Y813XXXP motif colored magenta. The binding interface is located in the residues (S28, K29 and G30) of ANXA1 and Y813XXXP motif of EphA2. (Right) Detailed interface between ANXA1 (orange) and EphA2 (cyan). (D) Immunoblotting showing the levels of EphA2 in the AGS and HCT116 cells transfected with shCtrl or shANXA1, and endogenous ANXA1-knockdown AGS and HCT116 cells transfected with D20-30, D28-30 or ANXA1 expression plasmid. ANXA1, Annexin 1.

A1(28-30) and A1(20-30) possess anti-GC and-CC effects in vitro and in vivo. Overexpression of EphA2 has been considered as a promising target for the treatment of cancers. Therefore, we tested the tumor-suppression function of A1(28-30) and A1(20-30). MTT assay showed that A1(28-30) and A1(20-30) dramatically decreased the viability of CC and GC cells (Fig. 5A), CCK-8 and plate colony formation assay showed that A1(28-30) and A1(20-30) dramatically inhibited CC and GC cell proliferation (Fig. 5B and C), and soft agar colony formation assay showed that A1(28-30) and A1(20-30) dramatically inhibited CC and GC cell anchorage-independent growth (Fig. 5D). Moreover, EphA2 overexpression was able to rescue the proliferation and anchorage-independent growth of CC and GC cells treated with the two peptides (Fig. 5B-D).

Next, we further tested the *in vivo* tumor suppression function of A1(28-30) and A1(20-30) via peritoneal injection into mice carrying the xenograft tumors of GC and CC cells. Consistent with our *in vitro* observations, not only the sizes and weights of tumors (Fig. 6A), but also EphA2 expression in the tumors was markedly decreased in the mice received A1(28-30) or A1(20-30) (Fig. 6B). Moreover, H&E staining showed that the morphology and structure of the heart, lung, liver and kidney of mice receiving peptide A1(28-30)

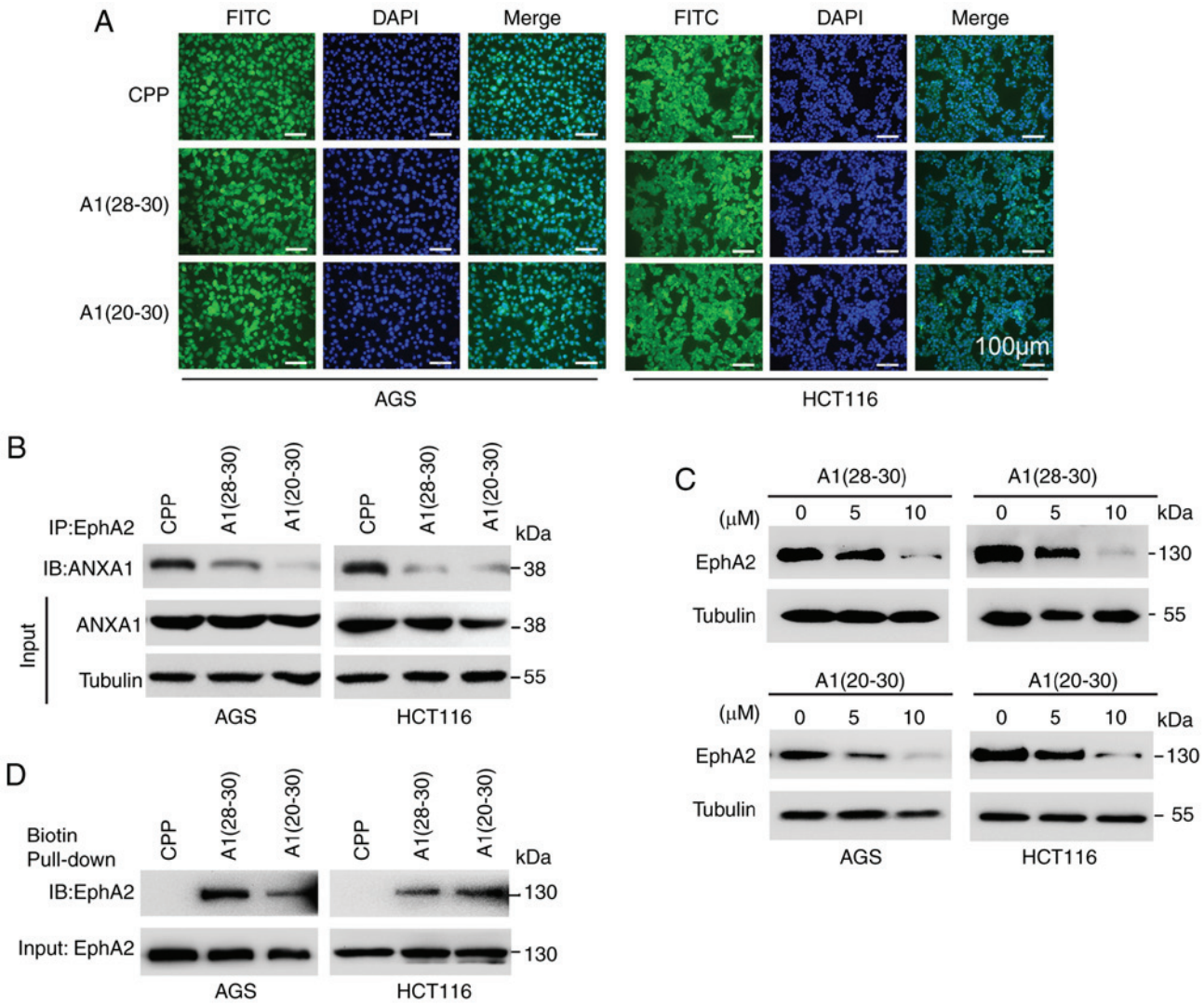

Figure 4. ANXA1-dirived peptides block EphA2-ANXA1 interaction and target EphA2 for degradation in GC and CC cells. (A) The subcellular distribution of FITC-labeled CPP-A1(28-30), FITC-labeled CPP-A1(20-30) and control FITC-labeled CPP in the AGS and HCT116 cells. Cells were incubated with 5 μM FITC-labeled peptides for 1 h, then observed by fluorescence microscopy. Cell nuclei were stained by DAPI. Scale bars, 100 μm. (B) Co-IP showing that the effects of A1(28-30) and A1(20-30) on ANXA1 bound to EphA2 in the AGS and HCT116 cells. Total proteins were prepared from the cells incubated with 10 μM peptides for 24 h, and subjected to immunoprecipitation (IP) with anti-EphA2 antibody followed by immunoblotting with anti-ANXA1 antibody. (C) Immunoblotting showing that the effects of A1(28-30) and A1(20-30) on the protein levels of EphA2 in the AGS and HCT116 cells. The cells were incubated with 5 and 10 μM peptides for 24 h respectively, and total cell proteins were subjected to immunoblotting with anti-EphA2 antibody. (D) Biotin pull-down showing A1(28-30) and A1(20-30) binding endogenous EphA2. Total proteins from AGS and HCT116 cells were incubated with the biotin-labeled peptides and streptavidin-conjugated agarose. Samples were electrophoresed and immunoblotted with against EphA2 antibody. A1(28-30), CPP-ANXA1-derived 3-mer (28-30aa) (SKG); A1(20-30), CPP-11-mer (20-30aa) (EYVQTVKSSKG) peptides; CPP, cell-penetrating peptide. GC, gastric cancer; CC, colon cancer; ANXA1, Annexin 1.

or A1(20-30) were normal, indicating that A1(28-30) and A1(20-30) are not toxicity to mice (Fig. 6C).

Discussion

EphA2 is overexpressed, promotes tumor growth and progression, and correlates with poor patient prognosis in gastric cancer (GC) (18-20) and colon cancer (CC) (25,26). Therefore, overexpression of EphA2 is a promising target for the treatment of GC and CC. Abnormal protein-protein interaction (PPI) is associated with cancer, representing a pivotal target for chemico-biological interventions (33-35).

In the present study, we identified the interaction of ANXA and EphA2, and demonstrated a positive correlation of the expression levels of both proteins in CC and GC, which prompted us to investigate the function and significance of the ANXA-EphA2 interaction. Our results revealed that ANXA1 obviously increased EphA2 protein stability, and proteasome inhibitor MG132 was able to reverse the decrease in EphA2 protein in the ANXA1-knockdown GC and CC cells, indicating that ANXA1 stabilizes EphA2 possibly by inhibiting its proteasomal degradation.

It has been reported that ANXA1 N-terminal regulates the level and activity of the epidermal growth factor receptor (EGFR) (30-32). Therefore, we analyzed whether ANXA1 N-terminal is responsible for binding EphA2, and observed that ANXA1 N-terminal bound to EphA2. We further mapped the region of ANXA1 binding to EphA2, and found the amino

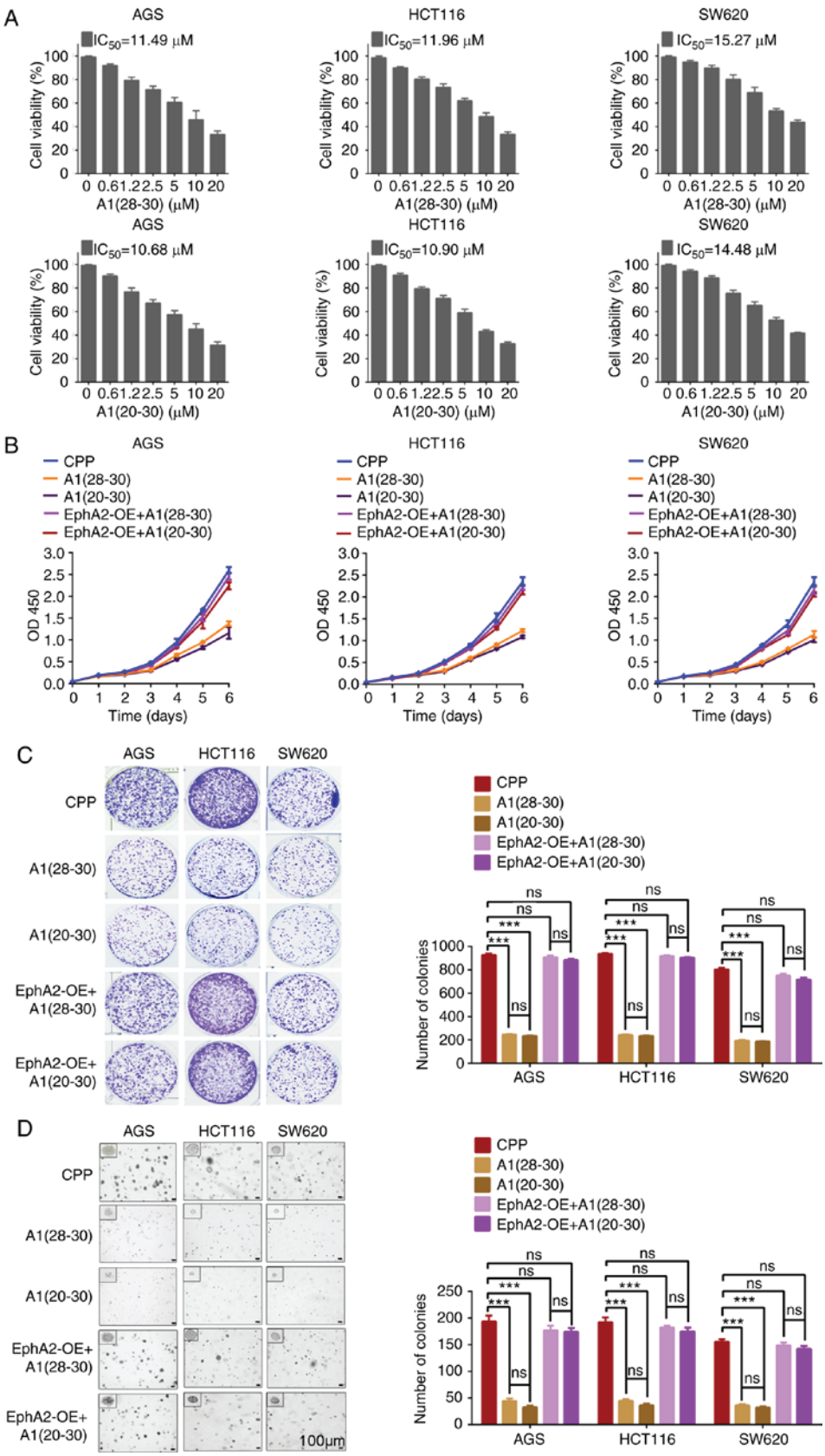

Figure 5. A1(28-30) and A1(20-30) possess anti-GC and anti-CC effect *in vitro*. (A) A1(28-30) and A1(20-30) decrease the viability of GC (AGS) and CC (HCT116 and SW620) cells. The cells were incubated with 0-20 μM peptides for 48 h, and cell viability was measured by MTT assay. (B and C) A1(28-30) and A1(20-30) decrease the proliferation of GC (AGS) and CC (HCT116 and SW620) cells, and EphA2 overexpression rescues the effect of both peptides on the proliferation of GC and CC cells. The cells were incubated with 10 mM peptides that was replenished every 24 h, and cell proliferation was detected by CCK-8 (B) and plate colony formation (C) assay. (D) A1(28-30) and A1(20-30) decrease the anchorage-independent growth of GC (AGS) and CC (HCT116 and SW620) cells, and EphA2 overexpression rescues the effect of both peptides on the anchorage-independent growth of GC and CC cells. The cells were incubated with 10 μM peptides that was replenished every 24 h, and cell anchorage-independent growth was detected by soft agar colony formation assay. Representative images are shown on the left, and quantitative data are presented on the right. Scale bars, 100 μm. Error bars indicate means ± SD. ***P<0.001; ns, not significant as determined by Student's t-test. EphA2-OE, EphA2 overexpression; GC, gastric cancer; CC, colon cancer.

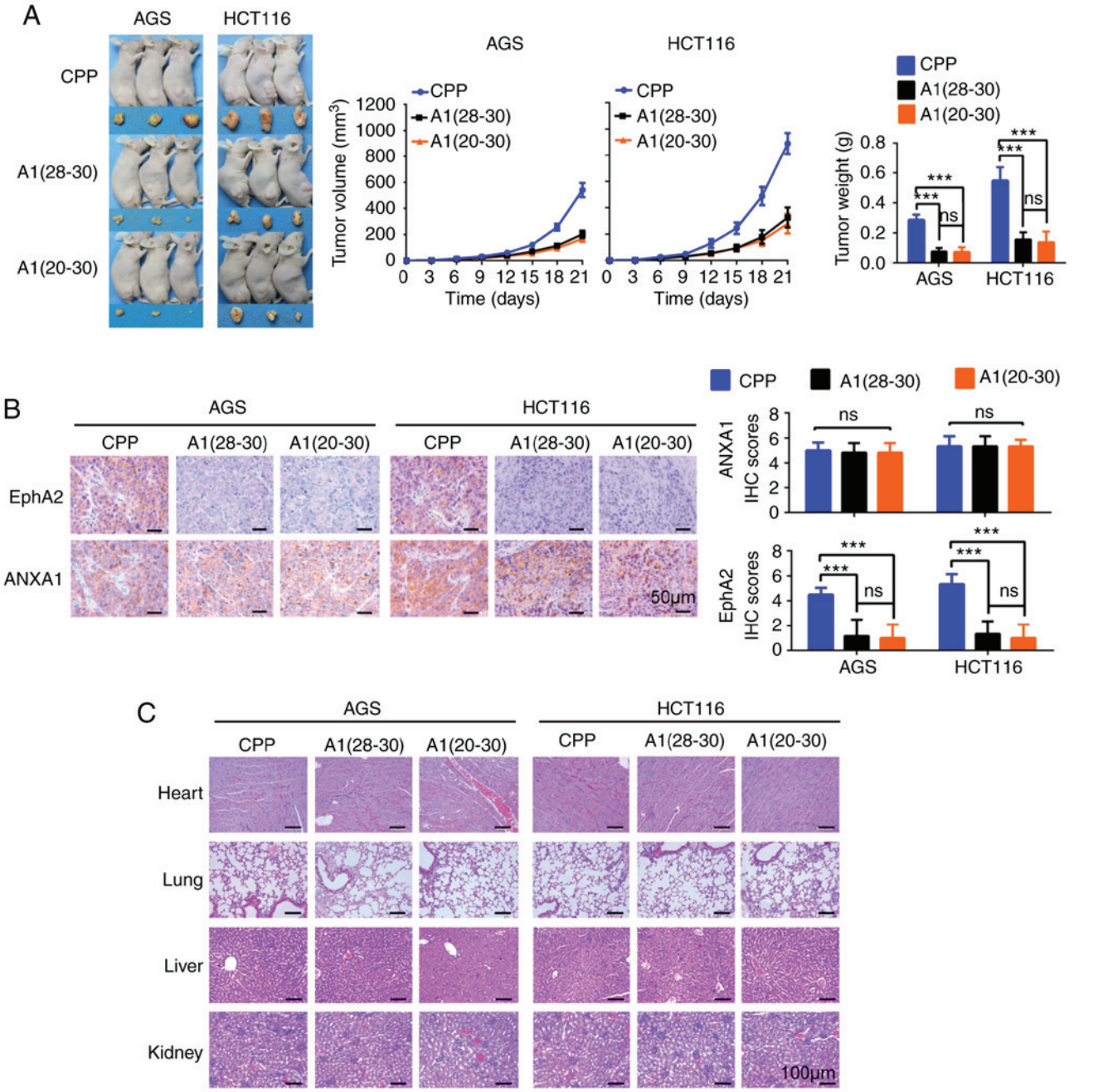

Figure 6. A1(28-30) and A1(20-30) possess anti-GC and anti-CC effect *in vivo*. (A) Tumor formation assay evaluating the effect of A1(28-30) and A1(20-30) on the oncogenicity of GC and CC cells in mice. (Left) The subcutaneous xenografts harvested from each mouse intraperitoneally injected with A1(28-30), A1(20-30) or CPP were imaged before further processing. (Middle and right) Tumor volume was periodically monitored, and tumor volume and weight for each group (six mice) were plotted. ***P<0.001, ns, not significant as determined by Student's t-test. (B) Immunohistochemistry (IHC) showing the expression of EphA2 and ANXA1 in the xenograft tumors. Representative IHC images are shown on the left, and quantitative data are presented on the right. Scale bars, 50 μm. Error bars indicate means ± SD. ***P<0.001, ns, not significant as determined by one-way ANOVA test. (C) H&E staining showing the morphology and structure of heart, lung, liver and kidney from the mice received A1(28-30), A1(20-30) or CPP. Scale bars, 100 μm. GC, gastric cancer; CC, colon cancer; ANXA1, Annexin 1; CPP, cell-penetrating peptide.

acid residues 20-30 and 28-30 of ANXA1 N-terminal were responsible for binding EphA2, which functionally was correlated with EphA2 stability.

Protein-derived peptides and peptidomimetics have the propensity to bind targeted protein surfaces and interfere with PPIs (52,53). Numerous studies have indicated that inhibition of PPI by peptides is an efficient anticancer approach (36-38), possessing many advantages compared with chemotherapeutic drug (39,40). Since the EphA2-ANXA1 interaction stabilized EphA2, one potentially effective approach for targeting EphA2 degradation is to disturb EphA2-ANXA1 interaction. Based on the amino acid residues 20-30 and 28-30 of ANXA1 N-terminal responsible for binding EphA2, we synthesized ANXA1-derived 3-mer (28-30aa) and 11-mer (20-30aa) peptides, named A1(28-30) and A1(20-30), respectively. With the help of cell-penetrating peptide (CPP), A1(28-30) and A1(20-30) could be uptaken by GC and CC cells, bound with EphA2, blocked ANXA1 binding EphA2, and targeted EphA2 degradation.

Next, we tested the effect of A1(28-30) and A1(20-30) on the oncogenicity of GC and CC cells. The results showed that A1(28-30) and A1(20-30) dramatically decreased the viability of GC and CC cells, inhibited the proliferation and anchorage-independent growth of GC and CC cells, and decreased the growth of xenografts from GC and CC cells without toxicity. Our results indicate that inhibition of ANXA1-EphA2 interaction by A1(28-30) and A1(20-30) may represent a promising strategy to impair the oncogenicity of EphA2, and antagonize GC and CC growth.

Why do A1(28-30) and A1(20-30) decrease EphA2 stability in the GC and CC cells? It is reported that the levels of many RTKs are regulated by ubiquitination degradation, and ubiquitination represents a key mechanism driving proteasomal degradation of EphA2 (54-56). We believe that A1(28-30) and A1(20-30) decrease EphA2 stability possibly by inhibiting its ubiquitination degradation, the detailed mechanism of which needs further study.

In summary, in the present study we demonstrated that the interaction of ANXA1 and EphA2 stabilized EphA2 in GC and CC cells, and we developed two ANXA1-derived peptides, which blocked the interaction of ANXA1 and EphA2, and downregulated EphA2 with anti-GC and -CC effects *in vitro* and *in vivo*.

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Availability of data and materials

The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE partner repository with the dataset identifier PXD015242 (https://www.ebi.ac.uk/pride/archive/projects/PXD015242/).

Authors' contributions

JF conceived the study, performed the experiments and analyzed and interpreted the data. TX and SSL performed the experiments and analyzed and interpreted data. XPH, HY and QYH performed the experiments. WH and YYT collected and provided the resources and supervised the study. ZQX designed and supervised this project, and wrote the manuscript. All authors read and approved the final manuscript.

Ethics approval and consent to participate

The use of human tissues was approved by the Ethics Committee, Xiangya Hospital of Central South University. As only archived tumor specimens were included in this study, the ethics committee waived the need for consent. All animal experimental procedures were performed in accordance with the Guide for the Care and Use of Laboratory Animals of Xiangya Hospital of Central South University, with the approval of the Institutional Animal Ethics Committee.

Patient consent for publication

Not applicable.

Competing interests

The authors state that they have no competing interests.

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