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Quadruple-editing of the MAPK and PI3K pathways effectively blocks the progression of KRAS-mutated colorectal cancer cells

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Abstract

Mutated KRAS promotes the activation of the MAPK pathway and the progression of colorectal cancer (CRC) cells. Aberrant activation of the PI3K pathway strongly attenuates the efficacy of MAPK suppression in KRAS-mutated CRC. The development of a novel strategy targeting a dual pathway is therefore highly essential for the therapy of KRAS-mutated CRC. In this study, a quadruple-depleting system for the KRAS, MEK1, PIK3CA, and MTOR genes based on CRISPR/SaCas9 was developed. Adenovirus serotype 5 (ADV5) was integrated with two engineered proteins, an adaptor and a protector, to form ADV-protein complex (APC) for systemic delivery of the CRISPR system. Quadruple-editing could significantly inhibit the MAPK and PI3K pathways in CRC cells with oncogenic mutations of KRAS and PIK3CA or with KRAS mutation and compensated PI3K activation. Compared with MEK and PI3K/MTOR inhibitors, quadruple-editing induced more significant survival inhibition on primary CRC cells with oncogenic mutations of KRAS and PIK3CA. The adaptor specifically targeting EpCAM and the hexon-shielding protector could dramatically enhance ADV5 infection efficiency to CRC cells and significantly reduce off-targeting tropisms to many organs except the colon. Moreover, quadruple-editing intravenously delivered by APC significantly blocked the dual pathway and tumor growth of KRAS-mutated CRC cells, without influencing normal tissues in cell- and patient-derived xenograft models. Therefore, APC-delivered quadruple-editing of the MAPK and PI3K pathways shows a promising therapeutic potential for KRAS-mutated CRC.

Zaozao Wang, Bin Kang, and Qianqian Gao have equally contributed to this article.

1 | BACKGROUND

Colorectal cancer (CRC) is one of the most commonly diagnosed malignancies, accounting for nearly 10% of the total incidence and mortality worldwide.[1] KRAS is the most frequently mutated oncogene in CRC; its diverse mutations promote the activation of the MAPK pathway and cause spontaneous tumor development.[2] Significant progress has been made to explore new treatments targeting mutated KRAS or other components of the MAPK pathway in the past decades.[3,4] However, aberrant activation of the PI3K/AKT/MTOR pathway either through mutant PIK3CA or compensatory activation of RTK signaling could remarkably reduce the therapeutic efficacy of inhibiting MAPK signaling in KRAS-mutated CRC cells.[5-7] Dual inhibition of the MAPK and PI3K pathways is urgently required for the complete inhibition of KRAS signaling and tumor progression. However, overlapping toxicities limit clinical activities of combined therapy with currently available inhibitors, such as Pimasertib and Voxtalisib[8] or AZD6244 together with MK2206.[9] Therefore, development of a novel strategy targeting the MAPK and PI3K pathways is highly essential for the treatment of KRAS-mutated CRC cells.

The clustered regularly interspaced short palindromic repeats (CRISPR) system has been reported as a potent strategy for efficient gene depletion in vitro and in vivo.[10] Moreover, multiplex genome engineering enables simultaneous editing of several sites within the mammalian genome by encoding multiple guide sequences into a single CRISPR array.[11,12] Recent studies demonstrated that therapeutic strategies based upon multiplex genome editing was powerful to fight against hematopoietic malignancies, which indicated its broad applications in cancer treatment.[13,14] Furthermore, selective targeting of oncogenic mutations of KRAS has been reported to inhibit tumor cell proliferation.[15,16] However, to date, no study has assessed the effects of multiple targeting on KRAS and its downstream signaling pathways.

One bottleneck undermining the application of genome-editing techniques to treat cancer is the lack of an efficient and safe delivery method in vivo. The human adenovirus serotype 5 (ADV5) is widely used in cancer gene therapy due to its high infection efficiency and strong expression of therapeutic genes.[17] However, ADV5 is only administered by local injection currently because of the nonspecific tissue tropism under a systemic delivery, especially a high risk of hepatotoxicity.[18] Engineered proteins composed of adaptors and protectors might be promising to overcome this challenge. An adaptor protein, which could interact with the knob protein of ADV5 fiber at one terminal and redirect the virus to target cells expressing a specific cell surface marker by a single-chain variable fragment (scFv) antibody at the other terminal, could not only remarkably increase

the ADV infection efficiency of the objective cells but also protect nontarget cells expressing only a little amount of the selected cell surface protein.[19-22] Besides, a hexon-shielding scFv, namely a protector, could generally reduce ADV5 tropism throughout the body by preventing the interaction between the hexon of ADV and corresponding receptors on target cells on the one hand[20] and blocking soluble blood factors which could bind to the hexon and enhance ADV infection on the other hand.[23,24]

In the present study, we constructed a quadruple-editing vector by CRISPR/SaCas9 simultaneously depleting KRAS, MEK1, PIK3CA, and MTOR and developed an adaptor against human epithelial cell adhesion molecule (EpCAM) as well as a protector. By detecting the antitumor effect, viral infection efficiency, and toxicity of the gene-editing ADV-protein complex (APC) in vitro and in vivo, we demonstrated that quadruple-depletion of the MAPK and PI3K pathways through systemic delivery of APC could effectively inhibit the progression of KRAS-mutated CRC cells without vital organ injury and might be a novel therapeutic strategy for CRC under clinical settings.

2 | MATERIALS AND METHODS

2.1 | CRC tumor samples

Seven tumor tissues were collected from CRC patients who underwent radical surgery with signed informed consent at Peking University Cancer Hospital. The specimens were used to isolate primary tumor cells and establish patient-derived xenograft (PDX) models. The current study was approved by the Ethics Committee of Peking University Cancer Hospital (Approval No. 2015KT71).

2.2 | Vector construction and virus packaging

The construction and description of lentiviral and adenoviral vectors for single and multiplex gene depletion are presented in Figure S1A,B. The most efficient sgRNA was chosen from Benchling tool (https://benchling.com). The sequences of single-guide RNAs (sgRNAs) and matched protospacer adjacent motifs are summarized in Table S1. The scrambled gRNA, which does not exist in human and mouse genome, was cloned into a nontargeting control vector (NT) (5′ GGCACTACCAGAGCTACTCA 3′). The coding sequences of adaptor and protector proteins were cloned into pENTER plasmid using restriction sites of Asis I and Xho I, respectively. The cloning primers are presented in Table S2. The lentivirus was packaged according to

the manufacturer's instructions (Addgene). The ADV5 was packaged and supplied by Obio Technology.

2.3 | Sanger and whole-exome sequencing (WES)

The mutation status of KRAS and PIK3CA in related cells was detected by Sanger sequencing. The PCR primers are listed in Table S2. The on-target and off-target mutations in tumor cells and tissues induced by quadruple-depletion APC intravenously were analyzed by WES as described in a previous study.[25] The sequencing data are available in the China National Genebank Nucleotide Sequence Archive (https://db.cngb.org/cnsa, accession number CNP0000324).

2.4 | T7E1 assay

The DNA fragment containing a breakpoint was PCR amplified and digested by T7 endonuclease I after annealing (primers in Table S2). The editing efficiencies of sgRNAs were determined by quantifying gel bands via ImageJ software.

2.5 | Establishment of cell-line-derived xenograft (CDX) and PDX mouse models of CRC

Here, 5×10^6 HCT116 or SW620 cells were subcutaneously injected into nude mice to establish the CDX model. The frozen specimens of CRC-PDX01 were thawed and subcutaneously injected into NOD-SCID mice to establish the PDX models. Once the tumor volume reached 100 mm^3, mice were used for genome editing experiments. All the experiments were approved by the Animal Ethics Committee of Peking University Cancer Hospital and performed in full compliance with the Experimental Animal Management Ordinance.

2.6 | In vivo genome editing

The ADV was preincubated with the adaptor and protector proteins (1.0×10^{-7} pmol) at room temperature for 2 hours and injected into the tail veins of CDX and PDX mouse models for two rounds with a 12-day interval. The number of viral particles was 7.0×10^9. The tumor size and weight were measured every 3 days.

2.7 | Tissue digestion and cell isolation

The primary CRC and PDX tumor tissues were cut into small pieces and digested by incubation for 40 min at 37°C in DMEM, containing collagenase type II (50 μg/ml; Sigma-Aldrich), DNase I (20 U/ml; Roche), and penicillin-streptomycin (1:500). After dissociation, the cell suspensions were filtered through a 70-μm cell strainer, and erythrocytes were removed using 2 ml red blood cell lysis buffer.

The isolated tumor cells were stored in liquid nitrogen for testing cell viability.

2.8 | Cell lines, cell viability, proliferation, transwell assays, and flow cytometry

Human CRC cells SW620 and HCT116, human normal colonic epithelial cells NCM460 and HCoEpiC, mouse EpCAM-positive Lewis lung cancer (LLC) cell line, and mouse CRC cell line CT26 with little EpCAM expression were maintained in corresponding culture media and incubated at 37°C.[26,27] Cell viability was assessed by CellTiter-Glo® 2.0 kit (Promega) according to the manufacturer's instructions. The IC$_{50}$ of AZD6244 or BEZ235 in CRC cells was calculated using GraphPad software. Cell proliferation was assessed using CCK-8 (Dojindo Molecular Technologies). Cell migration was performed by using a Boyden chamber that contained a polycarbonate filter with 8-μm pore size (Costar Inc). When conducted cell invasion assay, matrigel (5 mg/ml) was precoated on the surface of boyden chambers before cells were seeded. The GFP expression efficiency in cells infected by GFP-expressing ADV5 and the apoptotic rate of cells stained by PI/FITC-conjugated Annexin V were analyzed with the CytExpert 2.3 software (Beckman). All experiments were performed in triplicate.

2.9 | Western blotting analysis

Western blotting analysis was performed as described previously.[28] Primary antibodies used were as follows: KRAS (#12063-1; Proteintech), MEK (#9126; CST), PIK3CA (#4249; CST), MTOR (#2983; CST), AKT (#2920; CST), phospho-AKT (#4060; CST), S6K (#9202; CST), phospho-S6K (#9205; CST), ERK (#4695; CST), phospho-ERK (#4370; CST), EpCAM (#ab71916; Abcam), and β-actin (#66009-1; Proteintech). ImageJ software was used for analyzing band intensity, and relative protein expression levels were normalized to β-actin.

2.10 | Immunohistochemistry (IHC) and H&E staining

Formalin-fixed paraffin-embedded (FFPE) CDX and PDX tumor tissues were cut into 4-μm sections. After deparaffinization, rehydration, antigen retrieval, and blockage of endogenous peroxidase activity, sections were incubated with primary antibodies overnight at 4°C, followed by processing with a polymer horseradish peroxidase detection system (PV-9000). The primary antibodies used were the same as in Western blotting, with the addition of Ki67 (#ab16667, Abcam). The expression levels were evaluated by H-score method. Further, slides of vital organs were processed and stained with H&E. IHC score and histopathology of H&E staining were reviewed independently by two experienced pathologists.
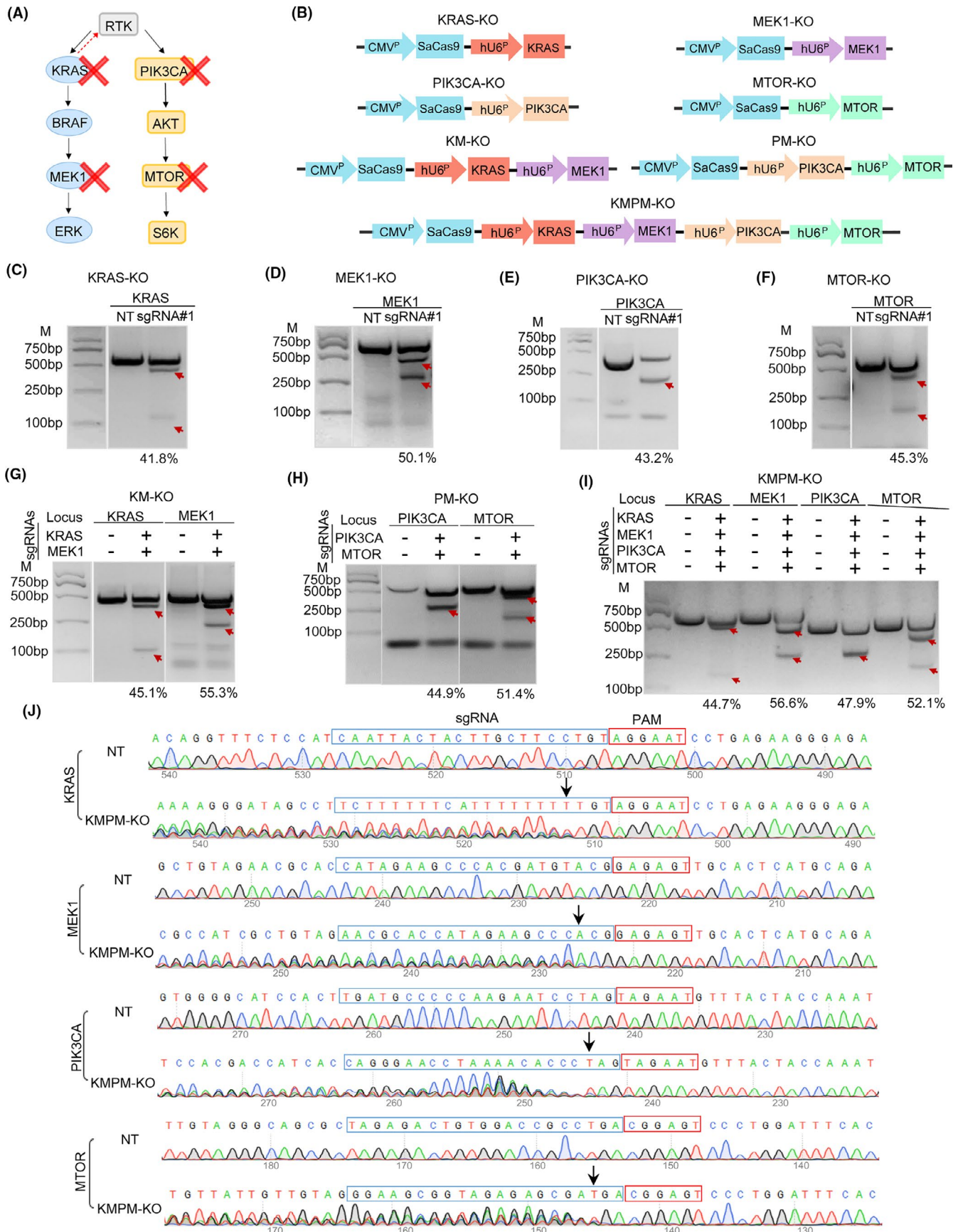
FIGURE 1 Constructing single- and multiple-editing vectors targeting the MAPK and PI3K pathways. A, A diagram of components in the MAPK and PI3K pathways and indicated editing targets. B, A diagram of single- and multiple-editing vectors of KRAS, MEK1, PIK3CA, and MTOR. P indicates promoter, and sgRNA cassettes are symbolized by corresponding gene names. C-I, Editing effects of single- and multiple-editing vectors in 293T cells were respectively detected by T7E1 assays. The red arrows denote the cleavage products of the T7E1 assay. J, Sanger sequencing of target genomic regions of quadruple-editing in 293T cells. The arrow indicates the start site of the on-target mutation
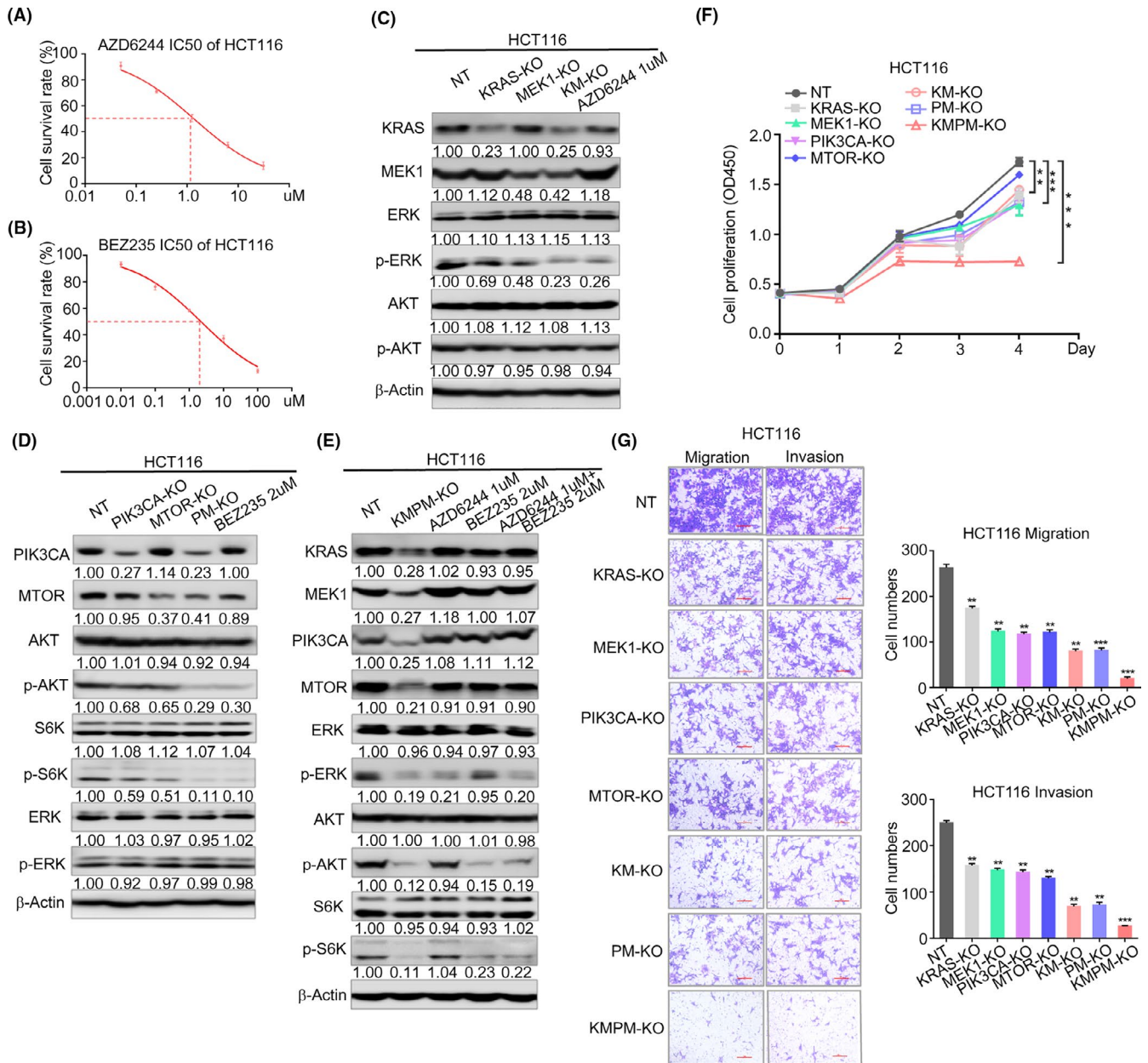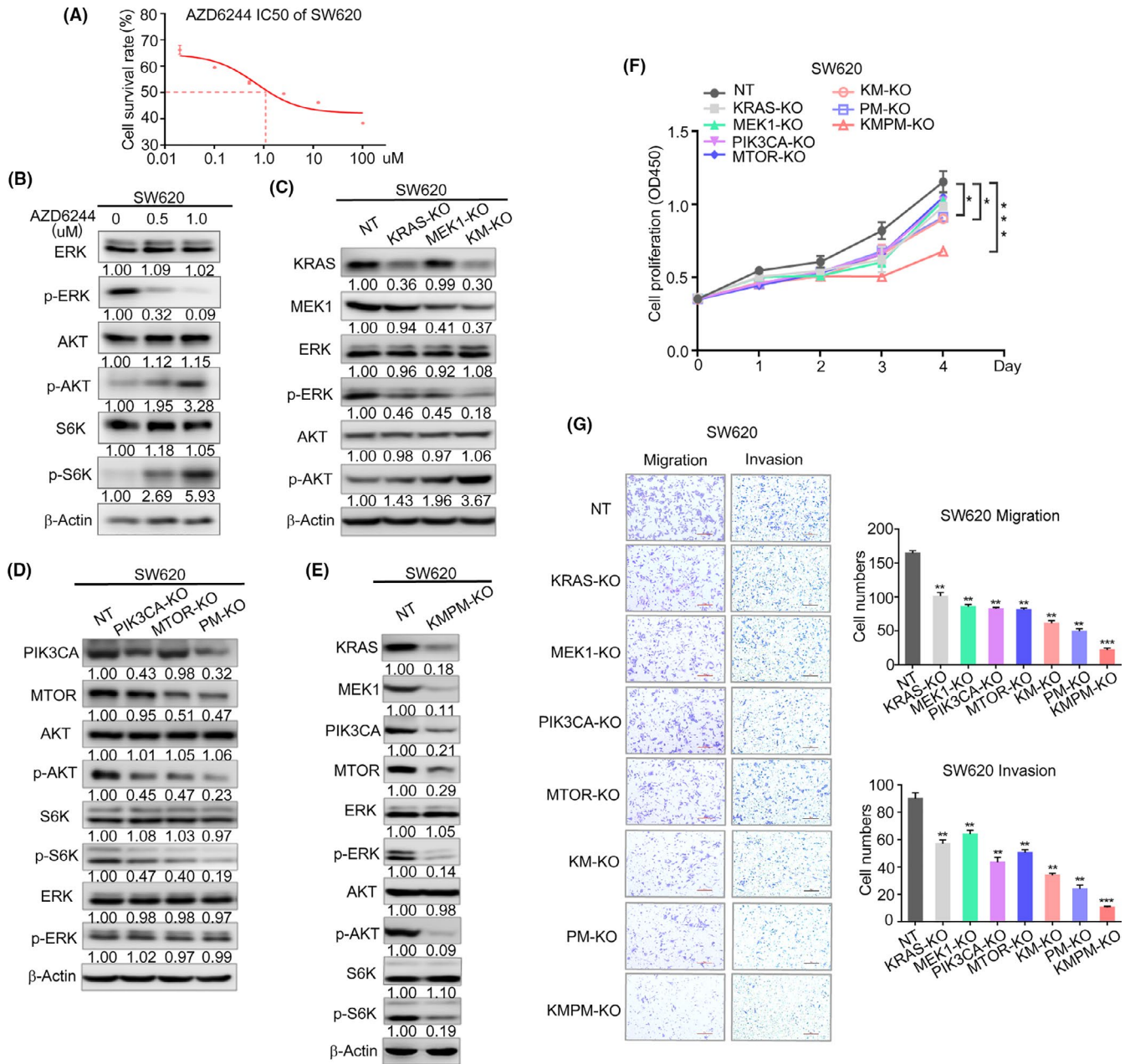
FIGURE 2 Quadruple-editing inhibited the MAPK and PI3K pathways in colorectal cancer (CRC) cells with double mutations of KRAS and PIK3CA. A, B, The IC_{50} of HCT116 treated by AZD6244 or BEZ235 was respectively examined and is indicated by dotted line. C-E, MAPK and PI3K signaling examined by Western blotting in HCT116 cells treated with single- or multiple-editing system transfection, or AZD6244 and BEZ235. The quantification of densitometry normalized to β-actin is presented below the blotting results. F, G, Proliferation, migration, and invasion of HCT116 cells with single- and multiple-editing of the four targets. The results were the average of three replicates

2.11 | RT-qPCR

Total RNA was extracted using TRIzol reagent (Invitrogen) and transcribed with TransScript First-Strand cDNA Synthesis SuperMix (Transgene Biotech). RT-qPCR was performed with GoTaq qPCR Master Mix (Promega). The relative expression levels of genes were normalized to β-actin (qPCR primers in Table S2).

2.12 | Protein expression and purification

The EpCAM adaptor, MUC1 adaptor, truncated adaptor losing scFv fragment, and the protector protein with His tags were respectively expressed in HEK293T cells. The proteins were extracted using RIPA buffer, purified with Ni-NTA beads (Byeotime), and rinsed with PBS and Amicon Centrifugal Filters (Millipore).

FIGURE 3 Quadruple-editing inhibited the MAPK and PI3K pathways in KRAS-mutated colorectal cancer (CRC) cells with compensated activation of PI3K. A, The IC_{50} of SW620 treated by AZD6244 was examined and is indicated by dotted line. B-E, Western blotting of MAPK and PI3K signaling in SW620 cells treated with different concentrations of AZD6244 (B), or with single- and multiple-editing system (C-E). The blotting densitometry of editing targets and signaling proteins were normalized to that of β-actin and are presented below the blotting results. F, G, Proliferation, migration, and invasion of SW620 cells with single- and multiple-editing of the four targets. The results were the average of three replicates

2.13 | Statistical analysis

Statistical analysis was carried out respectively by two-tailed Student's t-test and two-way analysis of variance (ANOVA) using SPSS 20.0. The Kolmogorov-Smirnov test was employed to indicate whether samples were normally distributed. The statistical significance was presented as * $0.01 \leq P < .05$, ** $0.001 \leq P < .01$, *** $P < .001$.

3 | RESULTS

3.1 | Construction of single-, double-, and quadruple-editing vectors targeting the MAPK and PI3K pathways

To effectively inhibit the MAPK and PI3K pathways, KRAS, MEK1, PIK3CA, and MTOR were selected as depletion candidates (Figure 1A).
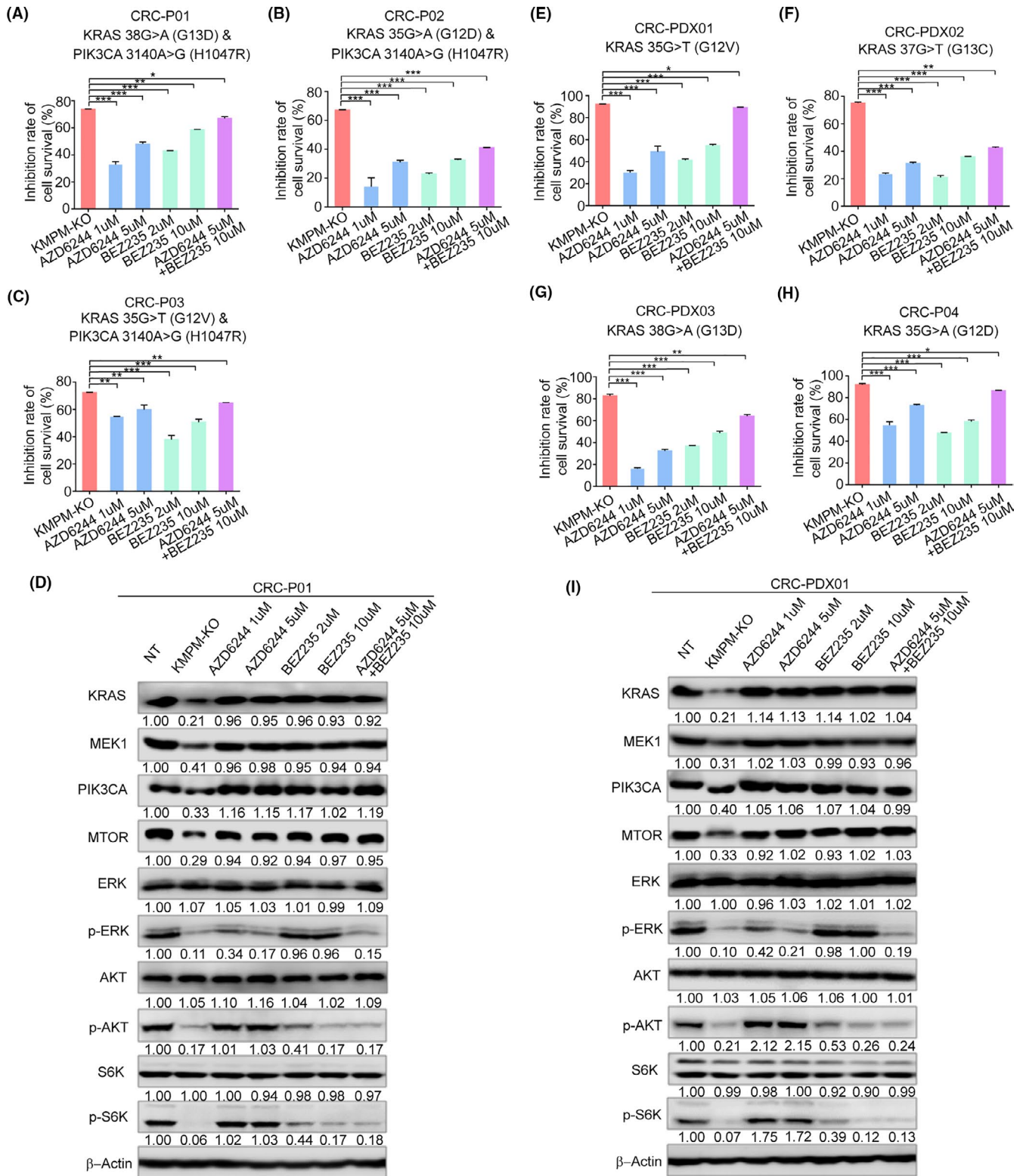
FIGURE 4 Quadruple-editing inhibited the survival of primary colorectal cancer (CRC) cells with diverse mutations of KRAS and PIK3CA. A-C, E-H, Survival inhibition rates of cells treated with quadruple-editing, or increased amounts of AZD6244 and BEZ235 alone or in combination were calculated according to the formula, (1-viability of treated cells/viability of untreated control cells) × 100. Histograms showed averages of replicated experiments. D, I, MAPK and PI3K signaling was examined by Western blotting after primary tumor cells of CRC-P01 (D) and CRC-PDX01 (I) were treated as above and harvested. The quantification of densitometry normalized to β-actin is presented below the blotting results

In addition to single-editing vectors, multiple-editing vectors including double-depletion of KRAS and MEK1 (KM-KO) and PIK3CA and MTOR (PM-KO), as well as quadruple-depletion of the four genes (KMPM-KO) were constructed (Figure 1B). The sequences of two top-ranked sgRNAs for each target were selected through Benchling tool (Table S1). After constructing each of them into lentiviral vectors expressing CRISPR/SaCas9 system (Figure S1A), depletion efficiencies of every sgRNA were examined by T7E1 assays and Sanger sequencing (Figure 1C-F, Figure S2). SgRNAs with more potent depleting efficiencies were selected for construction of multiple-editing vectors. The editing efficiencies of double- and quadruple-depletions are shown in Figure 1G-I. All gRNA expressions in KMPM-KO were comparable with each other as tested by RT-qPCR (Figure S3). Additionally, on-target mutations of corresponding genes by quadruple-editing were further confirmed by Sanger sequencing (Figure 1J).

3.2 | Quadruple-editing efficiently inhibited the MAPK and PI3K pathways in CRC cells with oncogenic mutations of KRAS and PIK3CA

Editing efficiencies of constructed single- and multiple-depleting systems were first examined in HCT116 cells with oncogenic mutations of KRAS (G13D) and PIK3CA (H1047R), compared with the effects of MAPK and PI3K inhibitors (AZD6244 and BEZ235). Western blotting was performed after obtaining IC_{50} of AZD6244 and BEZ235 in HCT116 cells (Figure 2A,B). As shown in Figure 2C, dual inhibition of KRAS and MEK1 could suppress the MAPK pathway more potently than single-editing, similar to AZD6244 treatment. Downregulation of PI3K signaling by PM-KO showed comparable effects with BEZ235, which were superior to single-depletion (Figure 2D). Besides, quadruple-depletion significantly suppressed both pathways, no less than the combined effects of AZD6244 and BEZ235 (Figure 2E). Moreover, KMPM-KO significantly inhibited the proliferation, migration, and invasion of HCT116 cells, with a greater inhibitory effect than single- and double-depletion (Figure 2F,G). These results revealed that quadruple-editing could efficiently and specifically inactivate both the MAPK and PI3K pathways and effectively inhibited the malignant phenotypes of CRC cells with oncogenic mutations of KRAS and PIK3CA.

3.3 | Quadruple-editing effectively blocked the compensated PI3K activation in KRAS-mutated CRC cells with MAPK suppression
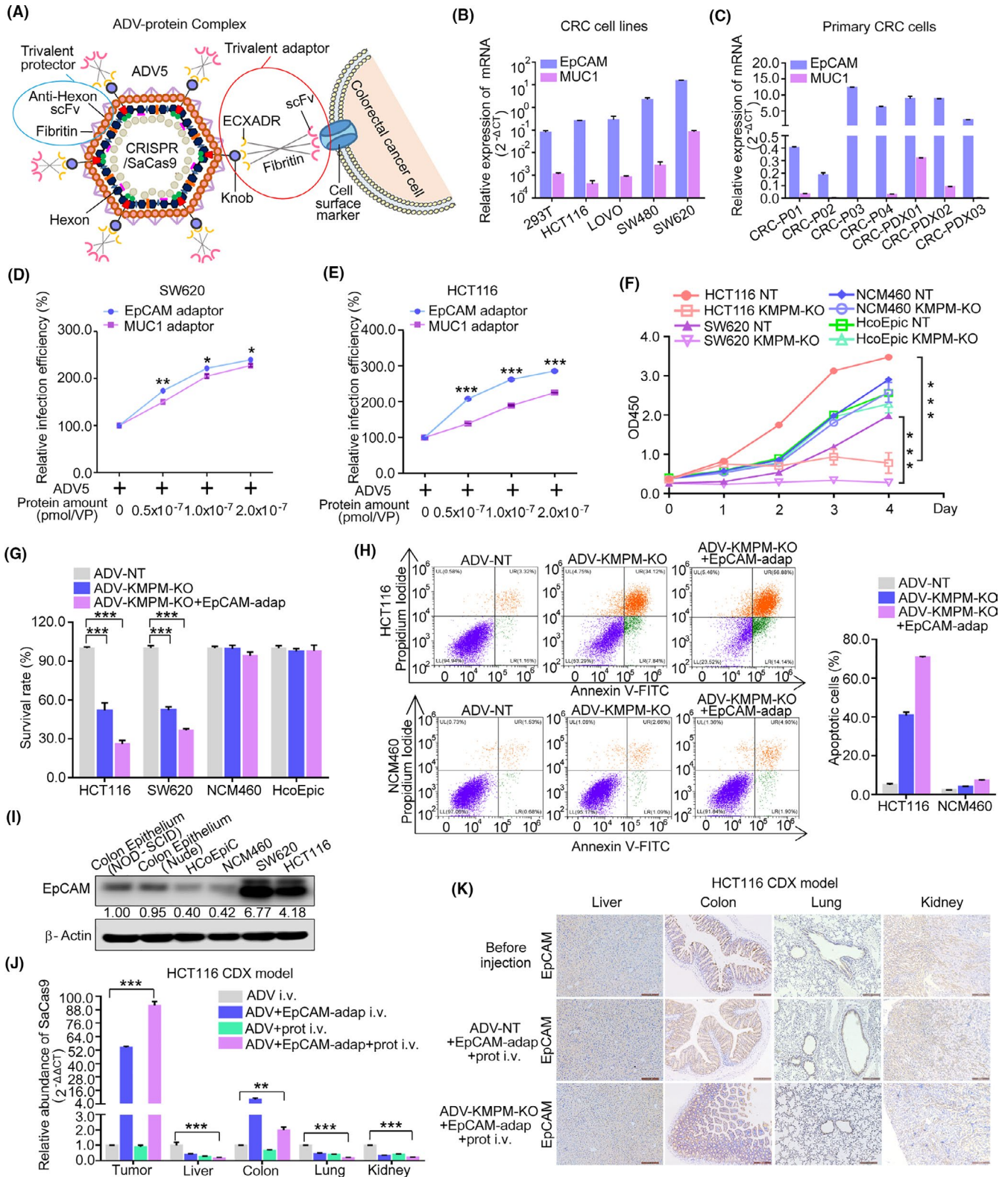
The effects of single- and multiple-editing systems targeting the MAPK and PI3K pathways were further examined in SW620 cells with mutant KRAS (G12V) and wild-type PIK3CA. Just like the effects of AZD6244, a compensatory activation of PI3K signaling was elicited when depleting single- or dual-target genes in the MAPK pathway, the effect of KM-KO was more robust than KRAS-KO or MEK1-KO (Figure 3A-C), which suggested that dual-editing of the MAPK pathway was insufficient to suppress tumor growth of KRAS-mutated CRC with wild-type PIK3CA. Targeting genes in the PI3K pathway showed no influence on MAPK signaling, irrespective of single- or dual-depletion (Figure 3D). However, simultaneous depletion of the four target genes significantly suppressed both pathways, suggesting quadruple-editing could efficiently block the compensated activation of the PI3K pathway under MAPK suppression (Figure 3E). Correspondingly, KMPM-KO could markedly inhibit the proliferation, migration, and invasion of SW620 cells, more effectively than single- and double-depletion (Figure 3F,G). Therefore, quadruple-editing of both the MAPK and PI3K pathways could effectively block tumor progression of KRAS-mutated CRC cells, no matter whether compensatory activation of PI3K signaling took place or not.
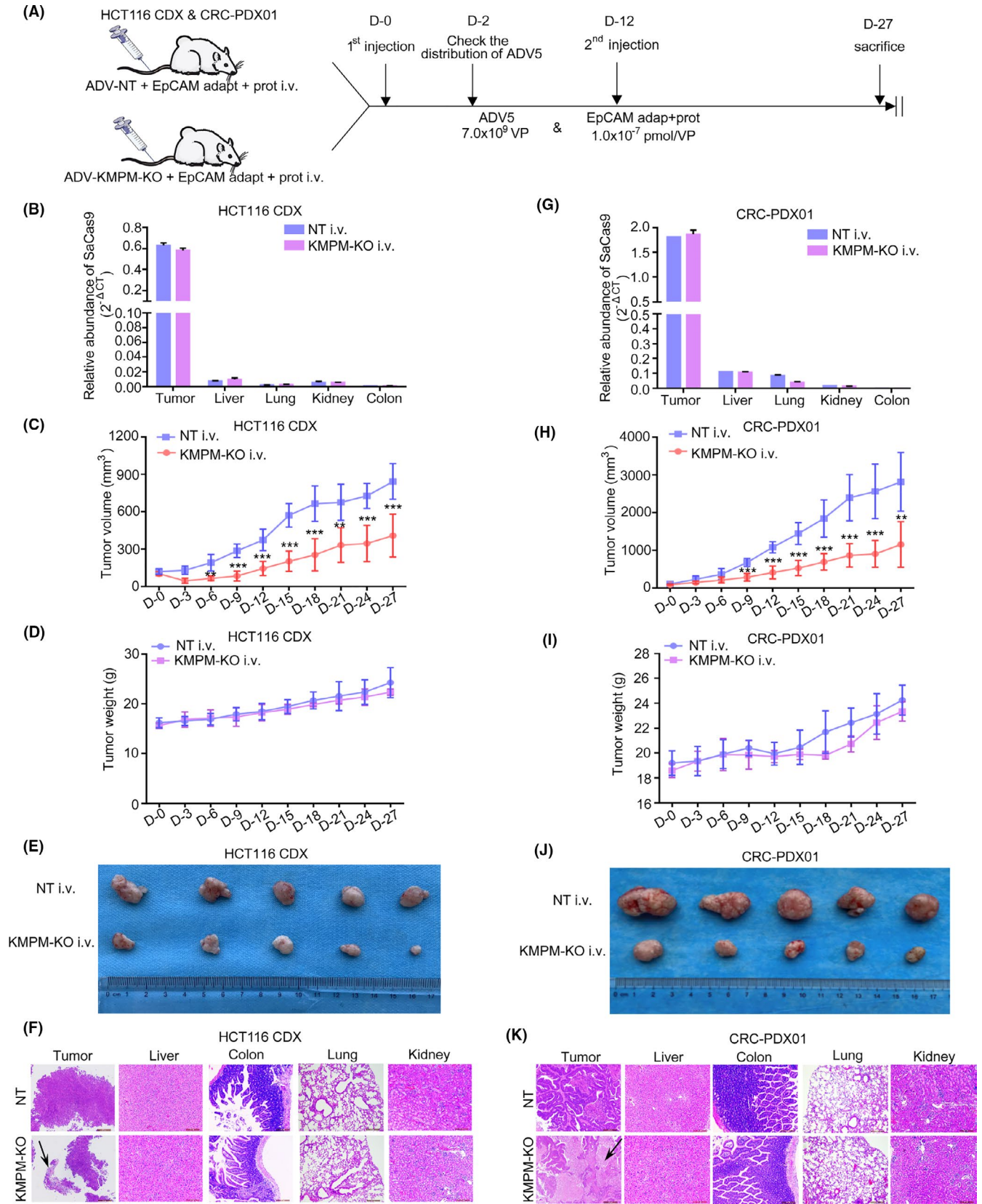
3.4 | Quadruple-editing inhibited the survival of primary CRC cells with diverse mutations of KRAS and PIK3CA

To further confirm the antitumor effects of quadruple-editing on CRC cells, four primary tumor cells and three PDX tumor cells with different types of KRAS and PIK3CA mutations were studied (Table S3). These tumor cells were infected with the lentivirus

FIGURE 5 Intravenously delivered quadruple-editing ADV-protein complex (APC) to colorectal cancer (CRC) cells showed high efficiency and low toxicity. A, The design of APC retargeting the CRISPR/SaCas9 system to CRC. B, C, Expression levels of EpCAM and MUC1 in CRC cell lines (B) and primary tumor cells (C). D, E, Infection efficiencies of GFP-expressing ADV5 to SW620 (D) and HCT116 (E) cells integrated with various amounts of EpCAM and MUC1 adaptor detected by flow cytometry. The relative infection efficiencies were calculated through comparing the GFP-positive ratios of corresponding cells infected with adaptor-coated ADV5 with those infected with ADV5 only. The results were the average of two replicates. F, Growth curve of CRC and human normal colonic epithelial cell lines infected by KMPM-KO ADV and its control. G, H, Growth inhibition (G) and apoptotic rates (H) of cells treated with KMPM-KO with or without EpCAM adaptor were detected by CCK8 and flow cytometry. I, EpCAM expression in normal mice, human colonic epithelial, and CRC cells were detected by Western blotting, with β-actin as an internal control. J, Virus distribution in HCT116 cell-line-derived xenograft model administered with ADV5 or ADV5 precoated by adaptors, protectors, or both of them intravenously (iv). The tumor tissues and vital organs were collected 48 h after delivery. The distribution of ADV5 was indicated by the relative expression of SaCas9. The results were the average of two replicates. K, EpCAM expression was examined by immunohistochemistry in vital organs before and after systemic delivery of quadruple-editing ADV-protein complex (APC). The brown scale bar indicates 200 μmol/L

(A) ADV-protein Complex

(B) CRC cell lines

(C) Primary CRC cells

(D) SW620

(E) HCT116

(F)

(G)

(H)

(I)

(J) HCT116 CDX model

(K) HCT116 CDX model

(A)

HCT116 CDX & CRC-PDX01

ADV-NT + EpCAM adapt + prot i.v.

ADV-KMPM-KO + EpCAM adapt + prot i.v.

D-0
1st injection

D-2
Check the distribution of ADV5

D-12
2nd injection

D-27
sacrifice

ADV5
7.0x10⁹ VP

&

EpCAM adap+prot
1.0x10⁻⁷ pmol/VP

(B) HCT116 CDX

Relative abundance of SaCas9 (2^−ΔCT)

NT i.v.
KMPM-KO i.v.

Tumor Liver Lung Kidney Colon

(G) CRC-PDX01

Relative abundance of SaCas9 (2^−ΔCT)

NT i.v.
KMPM-KO i.v.

Tumor Liver Lung Kidney Colon

(C) HCT116 CDX

Tumor volume (mm³)

NT i.v.
KMPM-KO i.v.

D-0 D-3 D-6 D-9 D-12 D-15 D-18 D-21 D-24 D-27

(H) CRC-PDX01

Tumor volume (mm³)

NT i.v.
KMPM-KO i.v.

D-0 D-3 D-6 D-9 D-12 D-15 D-18 D-21 D-24 D-27

(D) HCT116 CDX

Tumor weight (g)

NT i.v.
KMPM-KO i.v.

D-0 D-3 D-6 D-9 D-12 D-15 D-18 D-21 D-24 D-27

(I) CRC-PDX01

Tumor weight (g)

NT i.v.
KMPM-KO i.v.

D-0 D-3 D-6 D-9 D-12 D-15 D-18 D-21 D-24 D-27

(E) HCT116 CDX

NT i.v.

KMPM-KO i.v.

(J) CRC-PDX01

NT i.v.

KMPM-KO i.v.

(F) HCT116 CDX

Tumor Liver Colon Lung Kidney

NT

KMPM-KO

(K) CRC-PDX01

Tumor Liver Colon Lung Kidney

NT

KMPM-KO

FIGURE 6 Quadruple-editing of KRAS, MEK1, PIK3CA, and MTOR blocked the progression of KRAS-mutated colorectal cancer (CRC) in vivo. A, A diagram of intravenous administration of quadruple-editing ADV-protein complex (APC) in HCT116 cell-line-derived xenograft (CDX) and CRC-PDX01 PDX models. B, G, ADV5 distributions in CDX (B) and PDX (G) mice receiving KMPM-KO APC and its control were detected 48 h after systemic delivery. ADV5 abundance was indicated by the relative expression of SaCas9, revealed by RT-qPCR. The results were the average of two replicates. C, D, H, I, Tumor growth curves and body weights of CDX (C, D) and PDX (H, I) mice with quadruple-depletion. The number of mice was respectively five for both models. E, J, Tumor tissues collected from CDX (E) and PDX (J) mice with quadruple-depletion at the final observation point (day 27). F, K, Morphological status of vital organs in CDX (F) and PDX (K) models were detected by H&E staining at the final observation point (day 27). The arrow indicated cell necrosis in tumor tissue. The brown scale bar indicates 200 μmol/L

of quadruple-editing or treated with different concentrations of AZD6244 and BEZ235 alone or in combination, respectively. The results indicated that, in all the seven cases, quadruple-editing led to more remarkable inhibition of cell survival than a single treatment with AZD6244 or BEZ235 and showed similar or stronger inhibitory effects to those of combined therapies (Figure 4A-C,E-H). Furthermore, Western blotting results suggested that the inhibitory effects of quadruple-editing were more noticeable than those of single treatment with AZD6244 or BEZ235 and were similar or a little superior to those of combined therapies in primary CRC cells carrying KRAS/PIK3CA double mutations (CRC-P01) or KRAS single mutation (CRC-PDX01) (Figure 4D,I). The abovementioned results suggested that quadruple-editing had prevalent antitumor effects on KRAS-mutated CRC cells.

3.5 | Intravenous delivery of the quadruple-editing system in APC to CRC cells showed high efficiency and low toxicity

To increase infection efficiency and avoid off-target tissue tropism of ADV5 under systemic delivery, an adaptor recognizing CRC-specific cell surface protein and a hexon-shielding protector were engineered and integrated with ADV5 (Figure 5A). A phage T4 fibritin polypeptide formed the linker inside the adaptor or protector, which could induce the trimerization of adaptor and protector proteins, upregulating their affinities with ADV5.[19,20] After literature review, EpCAM and MUC1 were selected as potential recognition targets on CRC for adaptor synthesis.[29,30] EpCAM expression was much higher than MUC1 in human CRC cell lines and cells isolated from primary and PDX tumors (Figure 5B,C).

GFP-expressing ADV5 was integrated with an EpCAM adaptor, a MUC1 adaptor, and a truncated adaptor without scFv, respectively, and infected HCT116 and SW620 cells to test viral transduction efficiency. Compared with the truncated adaptor, which blocked the interaction between ADV5 and SW620 cells, the EpCAM adaptor retargeted the virus to CRC cells by anti-EpCAM scFv with high efficiency (Figure S4). The EpCAM adaptor was chosen to form the APC because it mediated stronger ADV infection than the MUC1 adaptor for both CRC cells (Figure 5D,E, Figure S5). Moreover, the EpCAM adaptor could also recognize mouse EpCAM, increasing ADV transduction efficiency in EpCAM-positive LLC cells but reducing it in EpCAM-negative CT26 cells (Figure S6).

The colon shows the highest EpCAM expression among normal human tissues;[31,32] thus, it is more vulnerable to side effects caused by the quadruple-editing system. Human normal colonic epithelial cells were employed to examine toxicity. We found nearly unchanged survival rate of NCM460 and HCoEpiC cells under the treatment of KMPM-KO ADV, even with the addition of an EpCAM adaptor, compared with the dramatically inhibited proliferation of CRC cells, with more obvious suppression after adding an EpCAM adaptor (Figure 5F,G). The apoptotic rates of treated cells further confirmed these results (Figure 5H). Despite its abundant expression in normal colon rather than other tissues, EpCAM was expressed remarkably higher in CRC cells than in normal mice colon and human colonic epithelial cells (Figure 5I). Systemic delivery of SaCas9-expressing ADV5 with EpCAM adaptor significantly enhanced ADV5 infection efficiency and reduced tropism to vital organs, except the colon. Anti-hexon scFv generally protected tissues from ADV5-unspecific infection, and using both of them further reduced the viral abundance in liver, lung, and kidney, except a nearly twofold increase in the colon (Figure 5J). Nevertheless, the detection of EpCAM by IHC in these organs before and after intravenous injection of quadruple-editing APC showed no injury of EpCAM-positive epithelial cells by such treatment (Figure 5K).

3.6 | Quadruple-editing blocked the progression of KRAS-mutated CRC cells through systemic delivery

To evaluate the therapeutic potential of the quadruple-editing system, KMPM-KO and NT packaged by APC were intravenously injected into HCT116 CDX and CRC-PDX01 PDX models, respectively (Figure 6A). Both KMPM-KO and NT were mainly distributed in CRC tumor tissues with similar expression levels on day 2 post administration (Figure 6B,G). Suppressed tumor growth and substantially reduced tumor volume strongly suggested that quadruple-editing of the MAPK and PI3K pathways blocked the progression of KRAS-mutated CRC cells, although without visible body weight changes (Figure 6C-E,H-J). Furthermore, H&E staining of different organs in CDX and PDX mice showed that no injuries were observed. Obvious necrosis could only be found in tumors treated with KMPM-KO (Figure 6F,K).

The results of T7E1 assay showed that the genomic regions of the four targets were all efficiently edited in both animal models (Figure 7A,D). According to the results of IHC and Western blotting,

(A)

(B)

(C)

(D)

(E)

(F)
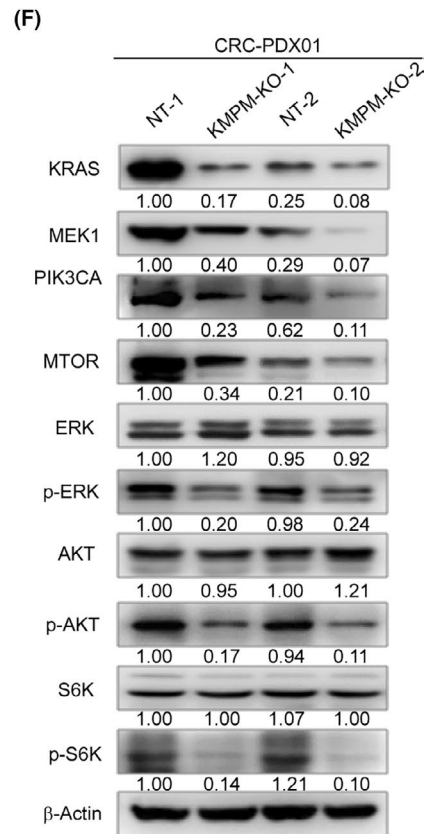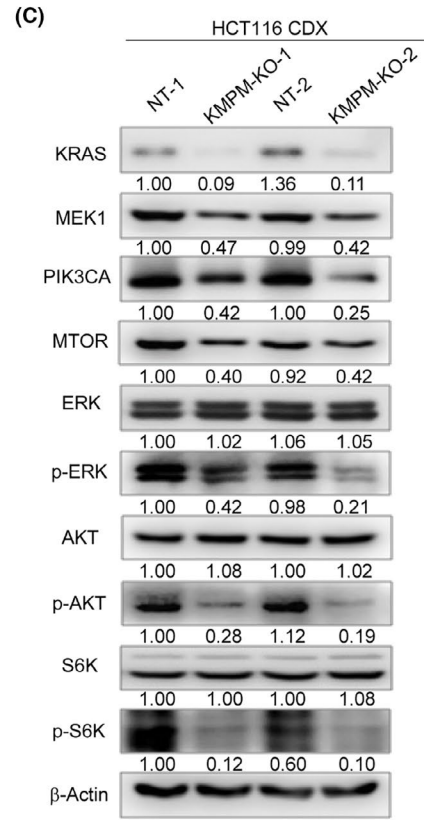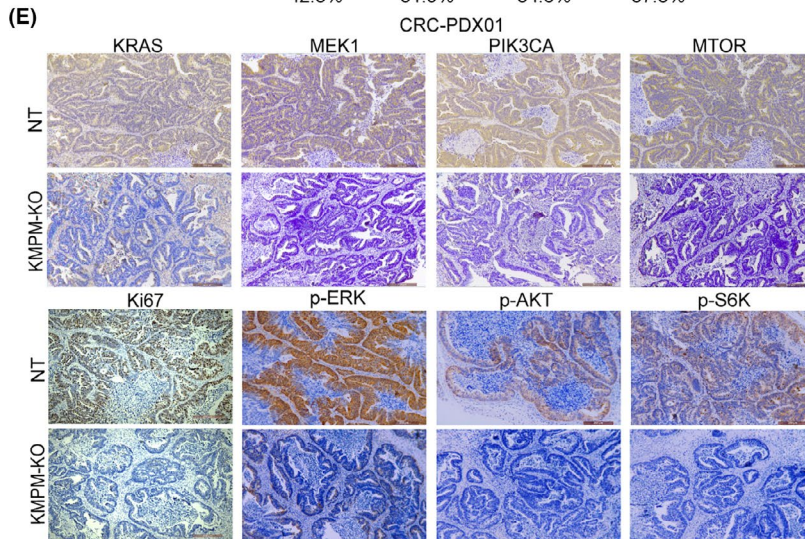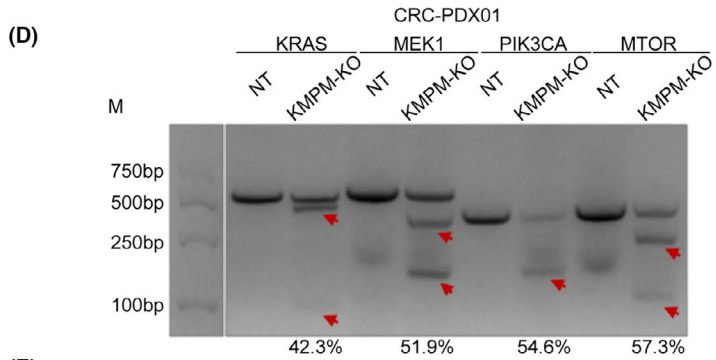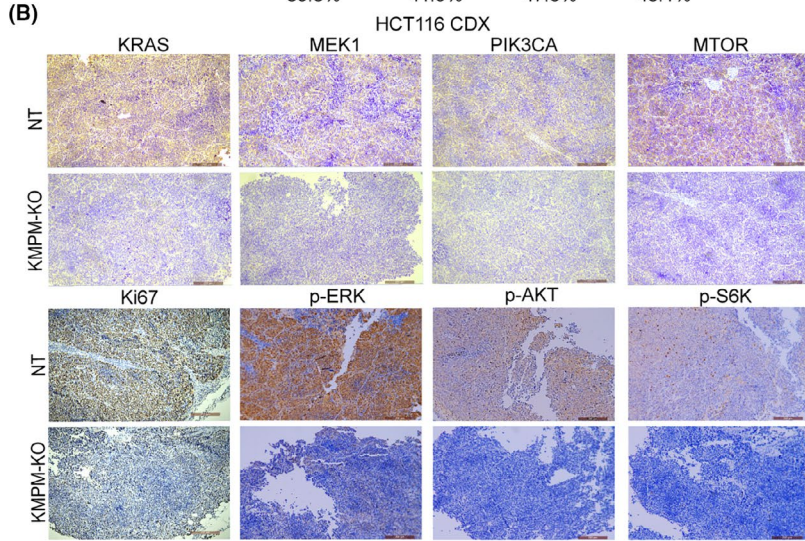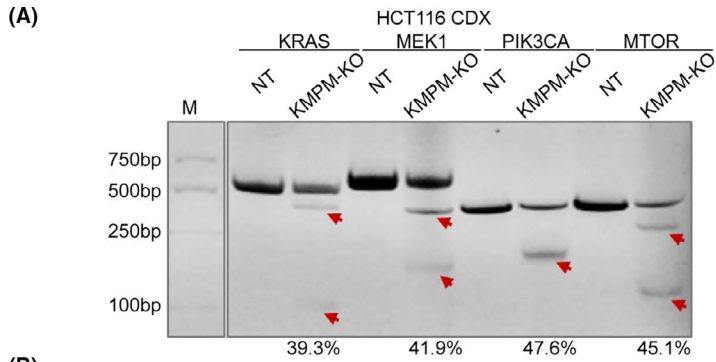
FIGURE 7 Quadruple-editing blocked MAPK and PI3K signaling in KRAS-mutated colorectal cancer (CRC) in vivo. A, D, T7E1 assays detected the editing efficiencies of the four target genes in tumor tissues of HCT116 cell-line-derived xenograft (CDX) (A) and CRC-PDX01 (D) mice at day 27. The red arrows denote the cleavage products of the T7E1 assay. B, E, immunohistochemistry examined the depleting effects of the four target proteins, as well as Ki67, p-ERK, p-AKT, and pS6K levels in tumor tissues of CDX (B) and PDX (E) mice. The brown scale bar indicates 200 μm. C, F, Western blotting examined the inhibiting effects of the MAPK and PI3K pathways by ADV-protein complex (APC) in tumor tissues of CDX (C) and PDX (F) mice. The blotting densitometry of targets and phosphorylated proteins were normalized to that of β-actin

the expression levels of the four target genes and the phosphorylation status of components in the MAPK and PI3K pathways were significantly downregulated in KMPM-KO samples (Figure 7B,C,E,F). Ki67 staining indicated a remarkably suppressed proliferation in tumor cells after quadruple-depletion (Figure 7B,E). Quadruple-editing APC targeting dual pathway could efficiently block the progression of KRAS-mutated CRC cells in vivo.

3.7 | The status of on-target and off-target mutations in tumor tissues treated by quadruple-editing

The mutation status of HCT116 CDX tumor tissues after quadruple-editing was examined by WES. The sequencing depth was over 100×. Totally, seven types of on-target deletions were found at genomic regions of the four target genes (Figure 8A). Among 167 potential off-target loci of these genes predicted by Benchling tool, only one locus of MTOR was detected with a real off-target single-nucleotide variant (SNV) (Chr16, 28603692 C > T), whose mutation frequency was 1.68% (Figure 8B). Furthermore, the total number of variants detected in NT and KMPM-KO samples was almost equal (24420 vs 24539). The number of insertion/deletions (INDELs) and SNVs detected at different genomic regions was also similar in NT and KMPM-KO samples (Figure 8C,D). Additionally, the majority of variants detected at different chromosomes were common ones shared by NT and KMPM-KO samples (Figure 8E). These results strongly suggested minimal effect of quadruple-editing on the mutation status of CRC.

4 | DISCUSSION

Although recent development of inhibitors against KRAS G12C and MEK has shed light on CRC treatment, the diversity of KRAS variants and aberrant activation of the PI3K pathway are still critical bottlenecks for the therapy of KRAS-mutated CRC cases. There are more than ten types of oncogenic mutations in codon 12/13 of KRAS, and most of them have no promising inhibitors.[33] The PI3K pathway could be activated by RTKs, compromising the antitumor effect of MEK inhibitor. The overlapping toxicities limit the clinical significance of combined therapies with inhibitors of the MAPK and PI3K pathways.[7-9] The proposed quadruple-editing system could simultaneously deplete four key components of the MAPK and PI3K pathways and inhibit the progression of CRC with diverse KRAS

mutations and compensated PI3K activation under MAPK suppression (Figure S7). According to the results of WES analysis of tumor tissues with gene editing, quadruple-depletion rarely induced off-target mutation and had minor influence on the mutation status of CRC. No visible injury could be observed in the vital organs of mice with gene editing, especially colon, which may be due to the inactivated status of the RAS/MAPK and PI3K/AKT pathways in well-differentiated human colonic epithelial cells, the remarkably lower expression of pathway proteins in normal colon than in CRC, and the less recognizability of EpCAM expressed on normal epithelia by its corresponding antibodies than this molecule expressed on cancer.[34-38] These results inferred high specificity and low toxicity of the quadruple-editing system under clinical settings. However, it is highly essential to investigate the individual and combined effects of available inhibitors and quadruple-editing vector in future studies to explore the clinical application of the CRISPR system in CRC treatment.

KRAS, MEK, PIK3CA, and MTOR are the most common therapeutic targets for CRC treatment at present. KRAS and PIK3CA are essential editing targets in KRAS-mutated CRC due to their high mutation frequencies.[5,39] MEK and MTOR were chosen for further depletion because of the crosstalk between the MAPK and PI3K pathways.[40] Many studies have demonstrated the potent suppression effect of dual inhibition. Combination of BRAF and MEK inhibitors was more efficient than BRAF inhibitor alone in BRAF-mutant melanoma and non–small cell lung cancer; it even showed a prolonged overall survival in a CRC clinical trial.[41-45] Moreover, dual inhibitors of PI3K/MTOR displayed a stronger effect than MTOR inhibitor alone in not only hematological tumors but also solid tumors.[46-48] Furthermore, based on our data, depleting the signaling intermediates together with KRAS or PIK3CA provided not only better antitumor effects but also stronger signaling inhibition than single-depletion. Therefore, targeting two genes in one pathway is a better strategy than single targeting.

ADV has received much attention as an effective gene delivery vector, while a major drawback of ADV-based gene therapy is the hepatotoxicity due to a massive uptake of ADV5 in the liver.[18] The EpCAM adaptor reduced liver tropism tremendously not only due to the redirection of ADV to EpCAM, which was expressed very little in liver, but also because of the occlusion of the receptor-binding site on the ADV fiber knob by the coverage of the EpCAM adaptor.[20-22,49,50] The slightly increased viral abundance in the colon by the application of APC is yet unsatisfying, although no impairment could be observed. Better tumor-specific adaptors still need to be identified for CRC and different types of cancer. For example,
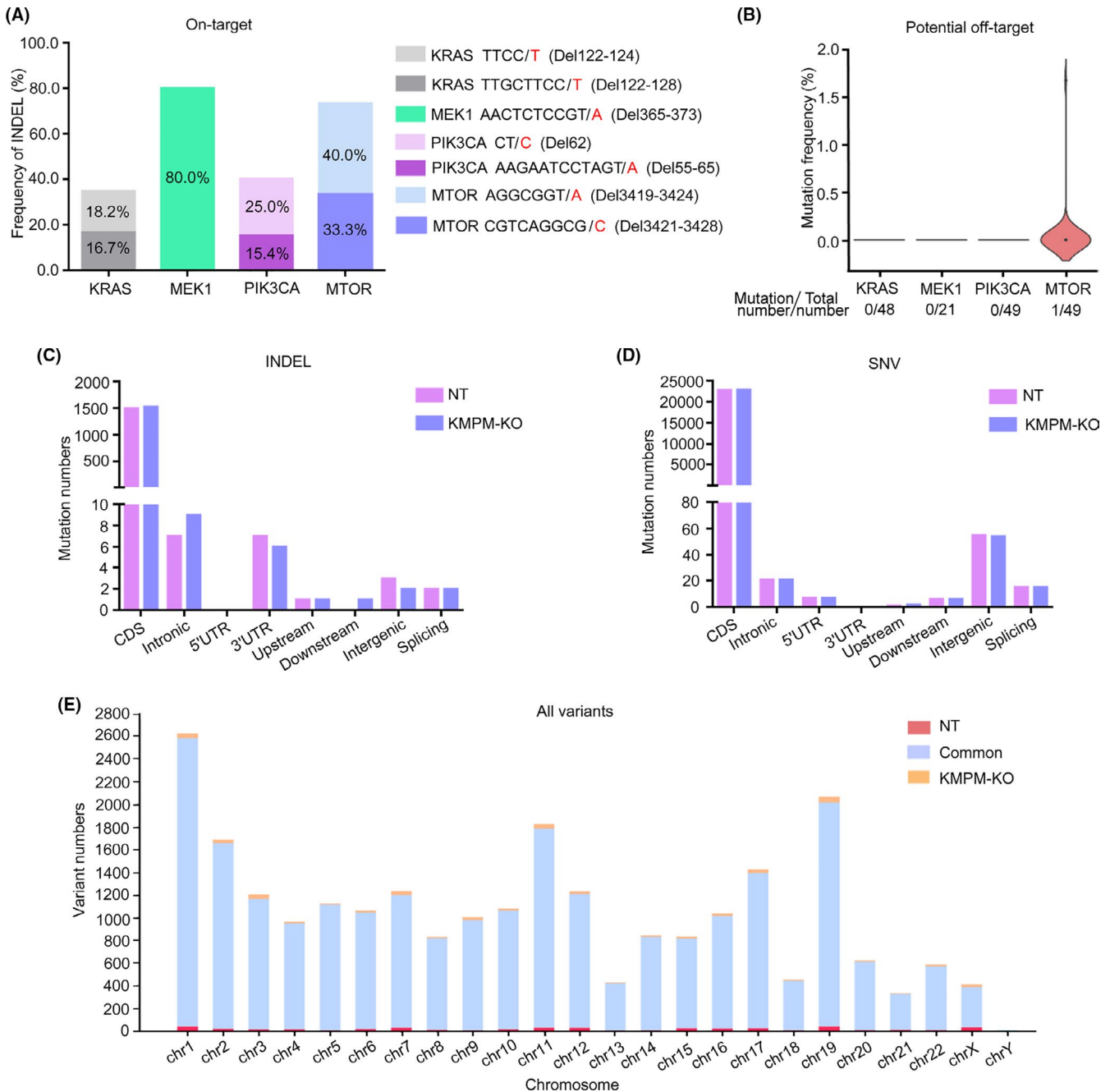
FIGURE 8 On-target and off-target mutations in tumor tissues with quadruple-editing. A, On-target mutations detected by whole-exome sequencing (WES) in tumor tissues of HCT116 cell-line-derived xenograft (CDX) model with quadruple-editing. B, Frequencies of real off-target mutations detected at potential mutation sites were presented in violin plots. The total numbers of real mutations detected by WES vs. potential off-target sites predicted by Benchling tool are listed below the graphs. C, D, The numbers of insertion/deletions (INDELs) (C) and single-nucleotide variants (SNVs) (D) were detected at different genomic regions in CDX tumor tissues treated with quadruple-editing and control. E, The variation numbers detected at different chromosomes of KMPM-KO and NT groups are presented in bar plots

proteins of the MUCIN family could be an exchangeable choice of adaptor for gastric and pancreatic cancer instead of EpCAM.[51,52]

In summary, CRISPR-mediated quadruple-depletion toward the MAPK and PI3K pathways intravenously delivered by APC could be a promising therapeutic option for KRAS-mutated CRC cells. In the future, adaptor and sgRNA libraries will be established, and the genome-editing therapy will have extensive applications in different types of cancer.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.