Supplementary Information

Antibody-secreting macrophages generated using CpG-free plasmid eliminate tumor cells through antibody-dependent cellular phagocytosis

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Construction of plasmids

The Lucia gene in the pCpGfree-Lucia purchased from InvivoGen (InvivoGen, San Diego, CA, USA) was replaced by the green fluorescent protein (GFP) gene amplified from pCFP4-CXCR4-IRES-EGFP by PCR using the GFP-Fw and GFP-Rv primers (Table S1), which generated pCGf-GFP. For the construction of pCGfd-GFP with the reduced size, the S/MAR and β-globin MAR sequences were removed from pCGf-GFP. Briefly, the sequences necessary for pCGfd-GFP were amplified by the PCR using two pairs of the dMAR-1 and dMAR-2 primers (Table S1). The amplified DNA fragments were joined by using the Gibson assembly method (New England Biolabs, Beverly, MA, USA), which generated pCGfd-GFP. All of the plasmid sequences were confirmed by sequencing (Cosmo Genetech, Seoul, Korea).

For the construction of pCGfd-aEGFR-scFv-Fc plasmid, the sequences coding anti-EGFR scFv (derived from Cetuximab) and human IgG-Fc-C223P were synthesized by Bioneer (Daejeon, Korea). The leader sequence of murine immunoglobulin kappa light chain was created in the front of the sequencing coding anti-EGFR scFv by the successive extension PCR using three forward primers (EGFR-1st-Fw, SP-2nd-Fw, SP-3rd-Fw), and one reverse EGFR-scFv-Rv primer (Table S1). Human IgG-Fc-C223P sequences were added at the end of anti-EGFR-scFv sequences by the fusion PCR using EGFR and Fc primers (Table S1). The resulting DNA fragment (leader sequence-anti-EGFR scFv-Fc) was subcloned into pCGfd by using the Gibson assembly method, which generated pCGfd-aEGFR-scFv-Fc.

The one vector CRISPR/cas9 system based on the pCGfd vector was constructed for efficient gene editing. The U6 promoter gRNA scaffold from pX330S-2 (58778; Addgene, Watertown, MA, USA) and the 1BPNLS-Cas9-1BPNLS-2AGFP gene from pCAG-1BPNLS-Cas9-1BPNLS-2AGFP (87109; Addgene) was amplified using U6-gRSca and NLS-GFP primers (Table S1). The resulting DNA fragments were subcloned into pCGfd vector, which generated pCGfd-gR-Cas9-2A-GFP. gRNA targeting EGFR gene was designed by using CCTop (https://crispr.cos.uni-heidelberg.de/) and subcloned into the pCGfd-gR-Cas9-2A-GFP, which produced pCGfd-gR-EGFR-Cas9-2A-GFP.

Detection of EGFR using flow cytometry and immunoblotting

Cells were harvested and fixed with 2% paraformaldehyde (Bio-solution, Ansan, Korea) for 10 min. After fixing, the cells were permeabilized with permeabilization buffer (eBioscience, San Diego, CA, USA). The permeabilzed cells were incubated with normal rabbit IgG (NI01; Sigma-Aldrich) or anti-EGFR antibody (Merck-Millipore) for 30 min at 4 ℃. After incubation, the cells were incubated with Alexa Fluor 647-tagged anti-rabbit IgG secondary antibody (Thermo Fisher Scientific) for 30 min at 4 ℃, and then analyzed by flow cytometry (FACS verse).

To detect the EGFR expressions in the wild-type and EGFR-knockout ($EGFR^{KO}$) A431 cells, the harvested cells were lysed with sample buffer and boiled for 10 min. The samples were then separated by SDS-PAGE using an 8% gel, and then transferred to a polyvinylidene fluoride (PVDF) membrane. The membrane was blocked by incubating with 5% skim milk for 1 h, followed by incubation with anti-EGFR (Merck-Millipore) and β-actin (Sigma Aldrich) antibodies for overnight at 4 ℃. After incubation, the membrane was washed three times with TBST. The membrane was then incubated with HRP-conjugated anti-rabbit (Enzo Life Science, Farmingdale, NY, USA) or mouse IgG (Thermo Fisher Scientific) secondary antibodies for 1 h. The target protein bands were visualized using a chemiluminescence ECL solution.

Table S1. Primer sequences

Primer name	Primer sequences	Product
GFP-Fw	5’-CATGCCATGGTGAGCAAGGGCGAGG-3’	GFP
GFP-Rv	5’-CTAGCTAGC TTACTTGTACAGCTCGTCCATG-3’	GFP
dMAR-1-Fw	5’-CCTCTACAAATGTGGTATGGTAAAATCAGCAGTTCAACCTG-3’	pCGfd-GFP
dMAR-1-Rv	5’-ATTGACTCCTGCAGGAATTCTAATTTTAATTAAAACAGGTAGTTG-3’	pCGfd-GFP
dMAR-2-Fw	5’-GAATTCCTGCAGGAGTCAAT-3’	pCGfd-GFP
dMAR-2-Rv	5’-CCATACCACATTTGTAGAGG-3’	pCGfd-GFP
EGFR-1st-Fw	5’-TCTGGGTGTCAGGCACCTGTGGAGACATCTTGCTGACTCAGTCTCC-3’	Anti-EGFR-scFv
SP-2nd-Fw	5’-GGCCCAAGTGCTGATGCTGTTGCTGCTCTGGGTGTCAGGCACCTGT-3’	Anti-EGFR-scFv
SP-3rd-Fw	5’-GGTGTACAGTAGCTTCCACCATGGACTCCCAGGCCCAAGTGCTGATGCTGT-3’	Anti-EGFR-scFv
EGFR-scFv-Rv	5’- TCTGGAGATTTGGGCTCAACTGCAGAGACAGTGACCAGAG-3’	Anti-EGFR-scFv
Fc-Fw	5’-GTTGAGCCCAAATCTCCAGAC-3’	Anti-EGFR-scFv-Fc
Fc-Rv	5’- CTTATCATGTCTGGCCAGCTAGCTCATTTACCCGGAGACAG-3’	Anti-EGFR-scFv-Fc
U6-gRSca-Fw	5’-CGTTAATTAAGGCATGTGAGGGCCTATTTC-3’	U6 promoter gRNA
U6-gRSca-Rv	5’-CGGAATTC TAGAGCCATTTGTCTGCAG-3’	U6 promoter gRNA
NLS-GFP-Fw	5’-GGTGTACAGTAGCTTCCACCATGAAGCGGACTGCTGATGG-3’	1BPNLS-Cas9-1BPNLS-2AGFP
NLS-GFP-Rv	5'-CTTATCATGTCTGGCCAGCTAGCTCACTTGTACAGCTCGTCCATGC-3'	1BPNLS-Cas9-1BPNLS-2AGFP
gRhEGFR-Fw	5’-CACCGGAAAACCTGCAGATCATCAG	guide RNA EGFR
gRhEGFR-Rv	5’-AAACCTGATGATCTGCAGGTTTTCC-3’	guide RNA EGFR

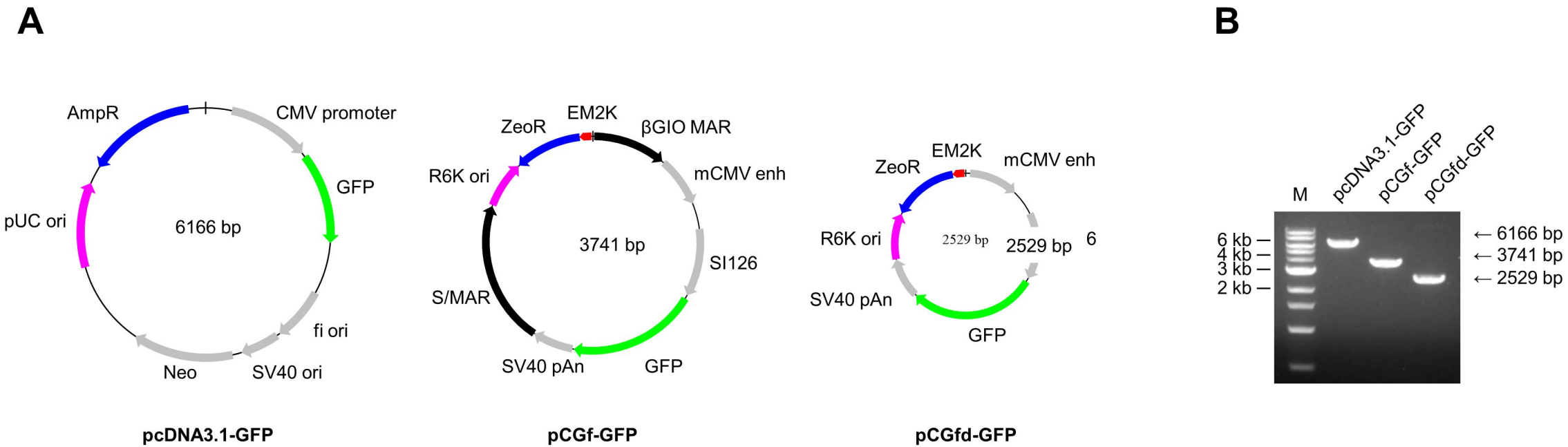

Fig. S1. Establishment of pcDNA3.1-GFP, pCGf-GFP, and pCGfd-GFP vectors. (A) Plasmid maps for pcDNA3.1-GFP, pCGf-GFP, and pCGfd-GFP. (B) Plasmids were incubated with the restriction enzyme, NheI, and then separated in 1% agarose gel. M: DNA size marker

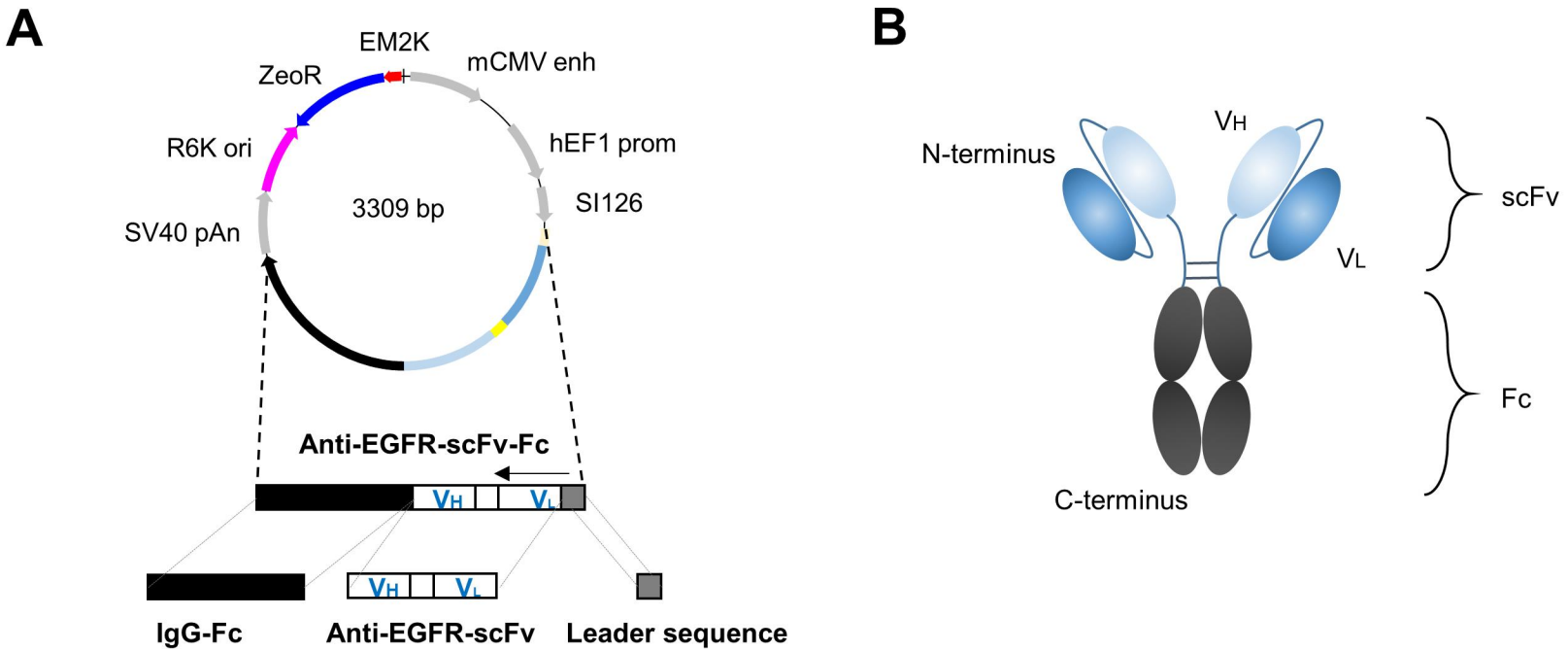

Fig. S2. The vector for secretory expression of anti-EGFR scFv-Fc. (A) Plasmid map of pCGfd-aEGFR-scFv-Fc. The sequences coding the leader sequence, anti-EGFR-scFv, and IgG-Fc are represented. (B) Domain structure of anti-EGFR scFv-Fc antibody.

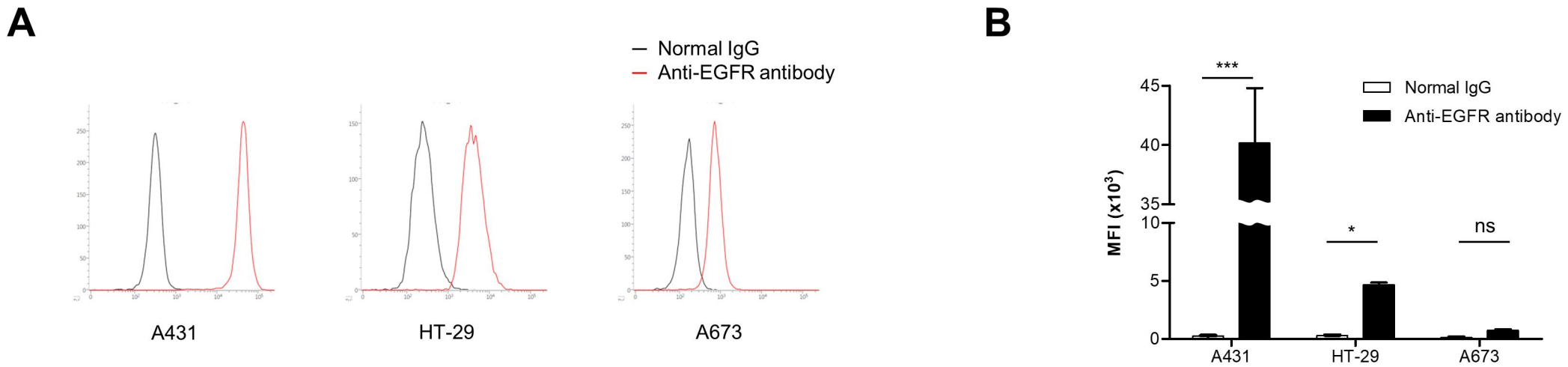

Fig. S3. The EGFR expression levels of A431, HT-29, and A673 cells. (A) Representative histograms of EGFR expressions. The EGFR expressions of A431, HT-29, and A673 cells were analyzed by the flow cytometry using an anti-EGFR antibody. (B) Mean fluorescence intensities (MFIs) of EGFR expressions were compared. Data are the means ± standard deviation, n = 3. Statistical significance was determined by Two-way ANOVA, ns = non-significance, *$p<0.05$, and ***$p<0.001$.

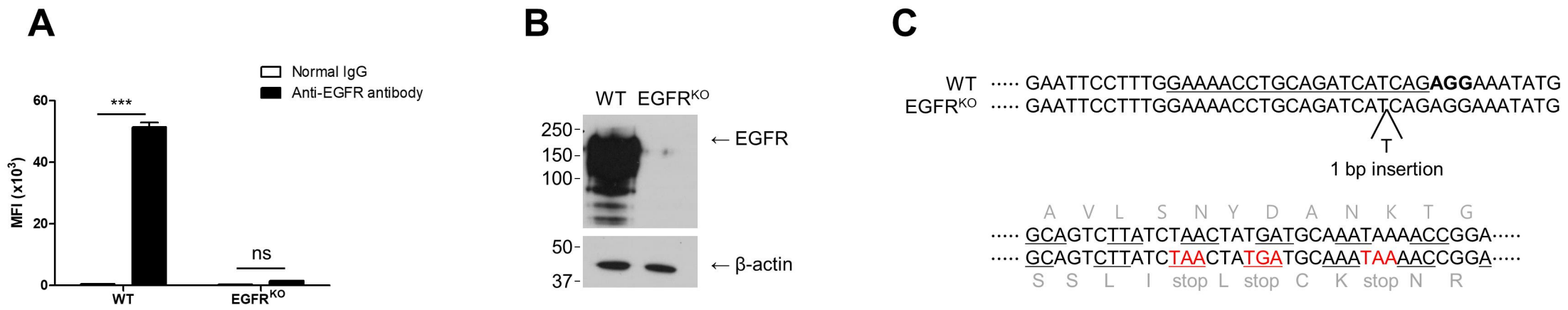

Fig. S4. Establishment of EGFR^{KO} A431 cells. The EGFR^{KO} A431 cells were generated using CRISPR/Cas9. The EGFR expression levels of wild-type (WT) and EGFR^{KO} cells were analyzed by flow cytometry (A) and immunoblotting (B) using an anti-EGFR antibody. Data are presented as the mean ± standard deviation, n = 3. Statistical significance was determined by two-way ANOVA, ns = non-significance, and ***p<0.001. (C) The alignment of the EGFR sequences between the wild type and EGFR^{KO} clone. Red letters indicate the stop codons induced by one bp insertion in the EGFR^{KO} clone.