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Article

Expression and *in vitro* function of anti-cancer mAbs in transgenic *Arabidopsis thaliana*

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ABSTRACT

The anti-colorectal cancer monoclonal antibody CO17-1A (mAb CO), which recognizes the tumor-associated antigen EpCAM, was expressed in transgenic *Arabidopsis* plants. PCR and western blot analyses showed the insertion and expression of heavy chain (HC)/HC fused to the KDEL ER retention modif (HCK) and light chain (LC) of mAb CO and mAb CO with HCK (mAb COK) in *Arabidopsis* transformants. Both plant-derived mAb^{P} CO and mAb^{P} COK were purified from a biomass of approximately 1,000 seedlings grown in a greenhouse. In sandwich ELISA, both mAb^{P} CO showed a slightly higher binding affinity for the target, EpCAM, compared to mAb^{M} CO. In cell ELISA, both mAbs^{P} COs showed binding affinity to the human colorectal cancer cell line SW480. Furthermore, mAb^{M} CO, mAb^{P} CO, and mAb^{P} COK exhibited dose and time-dependent regression effects on SW480 cells *in vitro*. In summation, both mAb^{P} CO and mAb^{P} COK, expressed in *Arabidopsis*, recognized the target antigen EpCAM and showed anti-

proliferative activity against human colorectal cancer cells.

Keywords: *Arabidopsis*, Colorectal cancer, Monoclonal antibody, Recombinant protein, Transgenic plant

Running Title: Plant-derived mAb to inhibit cancer cell growth

INTRODUCTION

Colorectal cancer, presenting as an abnormal growth of malignant cells in the inner layer of the colon and rectum, is a very common, lethal disease, comprising 9 percent of all cancers worldwide (1). The demand for anti-colorectal cancer antibody reagents is steadily increasing. Immunotherapeutic recombinant proteins such as antibodies have been produced via fermentation systems using yeast, bacteria, and mammalian cells (2-5). However, these systems are known to have certain drawbacks related to bulk production, quality, and safety (6-9). Plants are considered as a promising alternative bioreactor source materials for the production of recombinant biopharmaceutical proteins via *in vivo* whole plant or *in vitro* plant cell platform techniques (6, 10-13). Production of anti-colorectal cancer mAbs in transgenic plants offers a promising avenue for providing their large quantities with comparatively free of human and animal contaminants at a low cost (9, 14). Therefore, the plant-derived recombinant products have been tested in early phase clinical trials to monitor safety and efficacy in use (15, 16).

Among diverse plant platforms, *Arabidopsis thaliana* plant has several strengths such as a relatively short life span, high total soluble protein (TSP) yields, and cost-effective transformation methods (17-19). The endoplasmic reticulum (ER) retrieval motif has been fused to the C-terminus of the heavy chain (HC) of mAb thereby accumulation in ER retention signal peptide for high yields of anti-colorectal cancer mAb(4, 13, 20).

In this study, anti-colorectal cancer mAb^{P}s (mAb^{P}CO and mAb^{P}COK) were expressed in *Arabidopsis*. The expression level and *in vitro* anti-cancer activities of the antibodies were compared between mAb^{P}CO and mAb^{P}COK in *Arabidopsis* and mammalian-derived mAb CO17-1A (mAb^{M}CO) as a parental antibody. This is the first report that discussed the expression of functional anti-colorectal cancer antibodies mAbCO, and mAbCOK in *Arabidopsis* plants.

RESULTS

Generation of T_1 transgenic *Arabidopsis* plants to express mAb^{P}CO and mAb^{P}COK.

To investigate the effect of the ER retention motif (ERRM) on the expression and *in vitro* function of anti-colorectal cancer mAbs, both plant binary vectors, pBI CO17-1A (21) and pBI CO17-1AK (22), were delivered via *Agrobacterium tumefaciens* GV3101 to *Arabidopsis* to express the anti-colorectal cancer mAb^{P}CO and mAb^{P}COK, respectively (Fig. 1A). The ERRM was added to the C-terminus of HC in pBI CO17-1AK in order to retain mAb CO in ER, thereby enhancing its accumulation in the plant cells. The expression levels of transgenic plants expressing mAb^{P}CO (CO) and mAb^{P}COK (COK) were compared.

For *Arabidopsis* transformation, *Agrobacterium* was introduced to flowering plants using the floral-dip method (23), resulting eventually in mature seeds. Transgenic seedlings with green true leaves (20~30) were then selected from approximately 1,000 seeds germinated on *in vitro* germination media containing kanamycin. Most seeds sown in kanamycin-containing media germinated, but failed to produce true leaves and roots that were not transformants (Data not shown). In Agrobacterium-floral dip transformations with both pBI CO17-1A and pBI CO17-1AK expression vectors, the transformation rates were 1.8 and 2.1%, respectively. All putative, surviving seedlings with true leaves of CO (21) and COK (24) were grown in soil pots (Fig. 1B, upper). PCR detected HC and LC bands of the expected size in all tested CO and COK transgenic plants (Fig. 1B, middle). T_2 plants obtained from T_1 plants with high protein expression levels were used for bulk production of anti-colorectal cancer mAb from transgenic plants.

Expression and purification of mAb^{P}CO and mAb^{P}COK in *Arabidopsis*.

HC and LC expression levels of both mAb CO and mAb COK in CO and COK *Arabidopsis* transgenic plants, respectively, were compared (Fig. 1B bottom). All seedlings with true leaves and PCR bands did not exhibit HC and LC expression in both CO and COK transgenic plants (data not shown). COK expression was significantly higher than that of CO (Fig. 1B bottom). HC and LC bands were not detected in non-transgenic *Arabidopsis* plant (-) (Fig. 1B bottom). Quantitative western blotting and nanodrop protein analyses indicated that the relative expression levels of CO and COK were 0.52 and 2.42, respectively (Data not shown). T_2 seeds of both transgenic *Arabidopsis* plants highly expressing anti-colorectal cancer mAb CO and mAb COK were sown, and T_2 seedlings were produced (Fig. 1C upper). Purified $mAb^P CO$ and $mAb^P COK$ were obtained from 300 g of fresh biomass containing both transgenic CO and COK plants. The amounts of purified $mAb^P CO$ and $mAb^P COK$ were 750 µg and 3,400 µg, respectively. HC and LC bands of purified $mAb^P CO$ and $mAb^P COK$ detected via SDS-PAGE were at the expected sizes of 50 and 25 kDa, respectively. The LC band sizes of both $mAb^P CO$ and $mAb^P COK$ were slightly higher than those of the LC of counterpart $mAb^M CO$ (Fig. 1C bottom).

Binding activity of $mAb^P CO$ and $mAb^P COK$ to recombinant EpCAM molecules.

Sandwich ELISA was conducted to reconfirm binding affinity to EpCAM as detailed in the schematic diagram (Fig 2 right). Sandwich ELISA results indicated that both $mAb^P CO$ and $mAb^P COK$ had higher absorbance values compared to that of $mAb^M CO$, the positive control. Both $mAb^P CO$ and $mAb^P COK$ showed a higher absorbance value than the positive control, $mAb^M CO$. Absorbance values of both $mAb^P CO$ and $mAb^P COK$ were similar to EpCAM-associated ELISA results (Fig. 2).

Binding activity of purified mAb^{P}CO and mAb^{P}COK to SW480.

Cell ELISA was performed to assess the binding affinities of mAb^{P}CO and mAb^{P}COK to SW480 (Fig. 3A). Among the 4 mAbs (mAb^{M}CO as a positive control, mAb^{P}SO as a negative control) mAb^{M}CO showed the highest absorbance values. Compared to mAb^{P}CO, mAb^{P}COK showed slightly higher binding affinity to SW480 cells. The absence of an absorbance value for mAb^{P}SO indicated the absence of binding affinity. To investigate the region of SW480 to which mAb^{P}CO, mAb^{P}COK, and mAb^{M}CO bind, immunocytochemical analysis (ICC) was performed (Fig. 3B). ICC results showed that mAb^{M}CO was mainly bound to the surface membrane of SW480 cells, whereas both mAb^{P}CO and mAb^{P}COK were bound throughout SW480 cells (Fig. 3B). Both mAb^{P}CO and mAb^{P}COK exhibited similarities in the pattern of binding to SW480 cancer cells. The negative control groups (1×PBS) did not bind to SW480 cells.

Dose- and time-dependent effects of mAb^{P}CO and mAb^{P}COK on cell growth inhibition of colorectal cancer cell line SW480.

A dose-dependent tumor cell regression assay indicated that the number of intact live cancer cells was not significantly decreased by mAb^{M}CO, mAb^{P}CO, or mAb^{P}COK below the concentration of 250 ng (Fig. 4A, C). However, at concentrations above 500 ng per well, both mAb^{P}CO and mAb^{P}COK decreased the number of intact, live SW480 cells, to a value similar to that of parental mAb^{M}CO (Fig. 4A, C). A time-dependent tumor cell regression assay (Fig. 4B, D) indicated that inhibition of cell growth appeared approximately 4 hr after the anti-cancer antibody treatment (mAb^{M}CO, mAb^{P}CO, and mAb^{P}COK) (Fig. 4B, C). The lowest number of cells was observed at 6 hr after inoculation with the three anti-cancer mAbs. After 6 hr, the cancer cells appeared to proliferate again. 1× PBS treatment, as a negative control did not reduce the number of cancer cells.

DISCUSSION

The current study explored the expression and *in vitro* function of anti-colorectal cancer mAbs produced in transgenic *Arabidopsis* plants. The *HC/HCK* and *LC* genes of the recombinant therapeutic protein mAb CO, which recognizes the target antigen, EpCAM, highly expressed in human colorectal cancer cells (SW480) were expressed in *Arabidopsis* plants, which have high total soluble protein levels (19) Transgenic *Arabidopsis* expressing anti-colorectal cancer mAb^{P}CO and mAb^{P}COK (CO and COK, respectively) were obtained via *Agrobacterium*-mediated transformation. The expression and amount of purified mAb^{P}COK were approximately two times higher than that of mAb^{P}CO (4, 24). We hypothesized that the ER retrieval motif enabled retention of proteins in the intracellular organelle of plant cells, resulting in higher expression levels. These results were consistent with previous reports, indicating that production levels of recombinant mAbs and vaccines tagged with the KDEL ER retention signal were significantly higher than those tagged with KDEL and the high mannose *N*-glycan structure (4, 5, 12, 20, 25, 26).

The relative binding affinity of each mAb to target intact SW480 cells from the EpCAM positive cell line was quantitatively analyzed via Cell ELISA. Compared to mAb^{M}CO, mAb^{P}CO showed a slightly weaker binding affinity to the target cancer cell. Whereas mAb^{M} bound to entire cells, mAb^{P} mainly bound to the surface membrane of cells. These results were anticipated since the regions of epitopes that mAb^{M} and mAb^{P} bind to may be different. The origin sequences of mAb^{P}CO and mAb^{P}COK were specifically selected to recognize the extra cellular region of EpCAM proteins. The mAb^{M}CO (Anti-EpCAM antibody) recognized the epitopes of both extracellular and intracellular EpCAM proteins.

Cell regression assay indicated that the intact SW480 tumor cell population was efficiently decreased in a dose- and time-dependent manner in the mAb^{P}CO treatment groups, as well as in the mAb^{M}CO treatment groups. These observations suggest that interaction between the target antigen, EpCAM, and antibodies may induce apoptotic signaling to cancer cells without the addition of complement and serum (29, 30). Therefore, these assays may help determine optimal dosages and administration frequencies required for efficient anti-cancer therapy.

In summation, our research indicates that *Arabidopsis* may be an alternative platform for producing therapeutic proteins such as antibodies and vaccines because these proteins demonstrate a level of biological efficacy that is very similar to that of mammalian-derived monoclonal antibodies.

MATERIALS AND METHODS

See Supplementary information.

ACKNOWLEDGMENTS

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CONFLICTS OF INTEREST

The authors have no conflicting interests.

FIGURE LEGENDS

Figure 1. Generation of transgenic *Arabidopsis* plant expressing anti-colorectal mAbs CO and COK, and purification of plant-derived mAb (mAb^{P}). (A) Schematic diagram of the mAb^{P}CO17-1A (mAb^{P}CO) and mAb^{P}CO17-1AK (mAb^{P}COK) gene expression cassette construction in a plant expression vector pBI121 used for the *Agrobacterium* floral dip transformation. The promoters Pin2p and Ca2p regulate the light and heavy chains, respectively. KDEL is the 3′ endoplasmic reticulum (ER) retention motif. Pin2p, promoter of *Pin2* from potato; Ca2p, cauliflower mosaic virus 35S promoter; A, an alfalfa mosaic virus untranslated leader sequence of RNA4; Pin2T, terminator of *Pin2* from potato; NOST, terminator of *nopaline synthase* (NOS). (B) Generation and identification of T_1 transformants expressing mAb^{P}CO and mAb^{P}CO using antibiotic selection, soil growth, PCR, and western blotting. Soil growth of transformants after T_1 seedlings was selected on MS media containing kanamycin (upper). Surviving seedlings were transferred to a pot and placed in a growth chamber with 16 hr of light and 8 hr of darkness at 23°C. Rosette leaves were sampled from T_1 seedlings to confirm target gene insertion using PCR (middle) and protein expression level using western blotting (bottom). (C) SDS-PAGE gel (bottom) to confirm purity of mAb^{P}CO and mAb^{P}COK, purified from transgenic *Arabidopsis* plant biomass (upper).

Figure 2. ELISA analysis to confirm binding process of mAb^{M}CO, mAb^{P}CO, and mAb^{P}COK against the antigenic protein EpCAM-Fc. Sandwich ELISA to determine protein binding activity of anti-colorectal cancer mAbs to the EpCAM-Fc antigen molecule. Antibodies (mAb^{M}CO, mAb^{P}CO, and mAb^{P}COK) (15, 30, 60, and 120 ng gradient) were coated on Maxisorp 96-well microplates (Nunc, Roskilde, Denmark), and the epithelial cell adhesion molecule (EpCAM-Fc) antigen was placed in each well. HRP-conjugated goat anti-human IgG Fc fragment-specific antibody and TMB solution (KPL) were used to detect absorbance values. Absorbance at 450 nm was read using a UV-Vis microplate spectrophotometer (Biotek).

Figure 3. Cell ELISA and immunocytochemistry to determine the binding affinity of mAb^{M}CO, mAb^{P}CO, and mAb^{P}COK for SW480 cells. (A) mAb^{M}CO, mAb^{P}CO, mAb^{P}COK, and the plant-derived anti-rabies mAbSO57 (mAb^{P}SO), serially diluted to a range from 5,000 $ng \cdot \mu L^{-1}$ to 39 $ng \cdot \mu L^{-1}$ were applied to ELISA plates coated with human SW480 cells showing high EpCAM (epithelial cell adhesion molecule) expression. The negative control was used 1×PBS. Absorbance at 450 nm was measured using the Epoch Microplate Reader (Biotek). (B) Immunocytochemistry was used to determine the affinity of mAb^{P}s (mAb^{P}CO and mAb^{P}COK for binding to SW480. Sw480 cells were fixed in 10% formalin for 2 hr and processed for paraffin embedding. mAb^{M}CO, mAb^{P}CO, and mAb^{P}COK were used as primary antibodies. Samples were visualized with an HRP-conjugated goat anti-mouse/rabbit antibody and NovaRED (Dako) and counterstained with Mayer′s hematoxylin (Muto Pure Chemicals CO., Tokyo, Japan). Positive control was mAb^{M}CO, respectively. [magnification, X 400; BX53F (Olympus)]. **mAbPCO and mAbPCOK are shown where asterisks indicate significant differences ($p < 0.05$).**

Figure 4. Dose and Time-dependent effects of $mAb^{M}CO$, $mAb^{P}CO$, and $mAb^{P}COK$ on human colorectal cancer SW480 cell growth. SW480 cells (1×10^{5} cells per well) were seeded on the coverslips of 24 well cell culture plates. To investigate (A) the dose-dependent and (B) time-dependent effects of antibodies, $mAb^{M}CO$, $mAb^{P}CO$, and $mAb^{P}COK$ (1,000, 500, 250, 125, and 62.5 ng) were applied to the SW480 cells. (A) Antibody of $mAb^{M}CO$, $mAb^{P}CO$, and $mAb^{P}COK$ (1,000, 500, 250, 125, and 62.5 ng) were applied to the SW480 cells at concentration of 1,000, 500, 250, 125, and 62.5 ng. (B) $mAb^{M}CO$, $mAb^{P}CO$, and $mAb^{P}COK$ were added to each well, where SW480 cells were seeded (1×10^{5} cells per well) and incubated for 2, 4, 6, and 8 hr. Positive control was used $mAb^{M}CO$. The cells were incubated under conditions of 37°C and 5% $CO_{2,}$ for 2 hr. Positive control used was ^{M}CO, respectively. Two randomly selected areas of each plate were photographed, and the cell numbers were counted. The slides were observed under a microscope [magnification, X 200; BX53F (Olympus)]. (C) The relative SW480 cell number was counted after adding serially diluted antibodies to each well at 2 hr. These experiments were performed in duplicate. (B) Relative cell numbers were determined after incubating for 2, 4, 6, and 8 hr with the antibodies mentioned above. These experiments were performed in duplicate, and error bars are shown on the graph.

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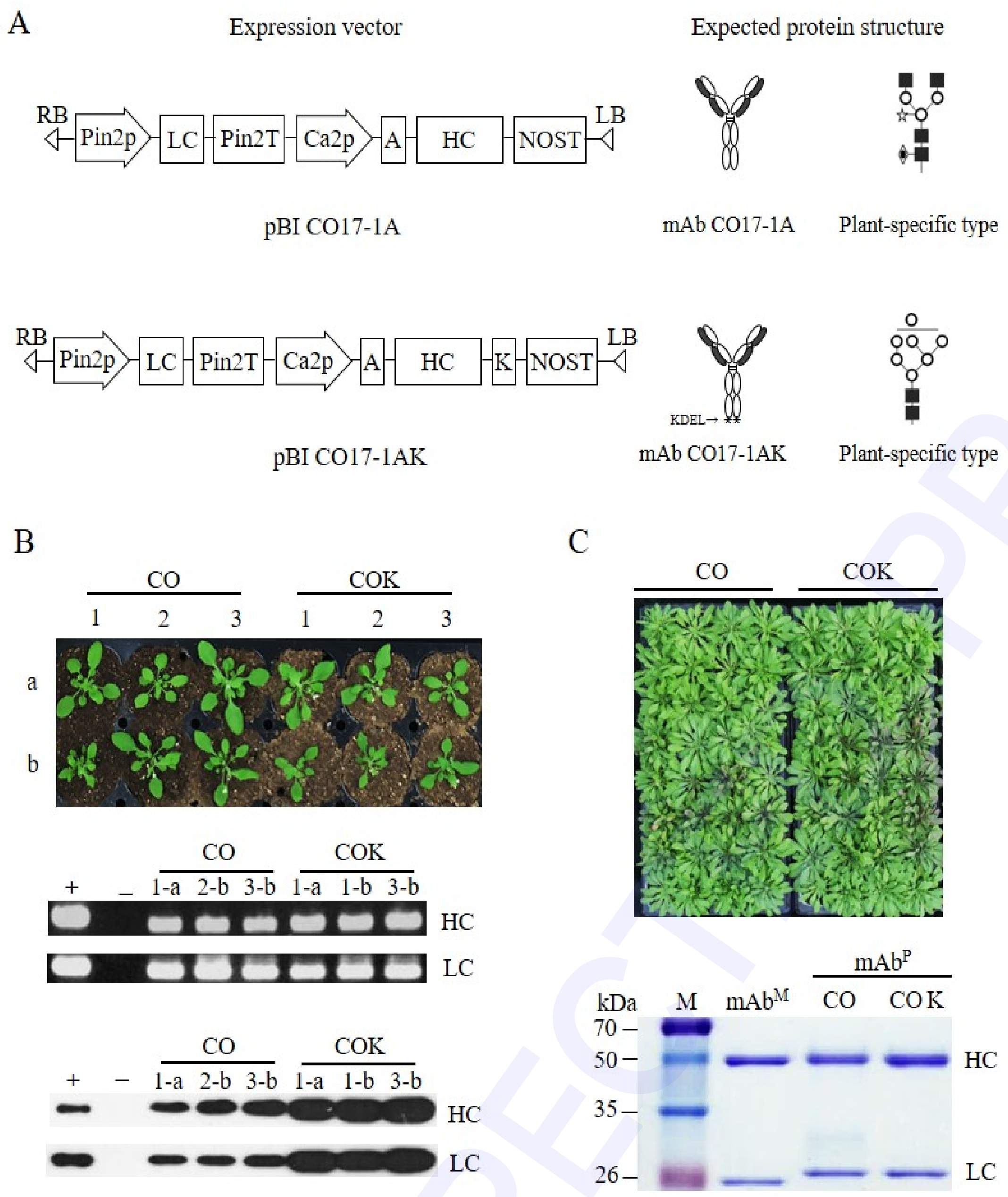

Fig. 1. Figure 1. Generation of transgenic Arabidopsis plant expressing anti-colorectal mAbs CO and COK, and purification of plant-derived mAb (mAbp).

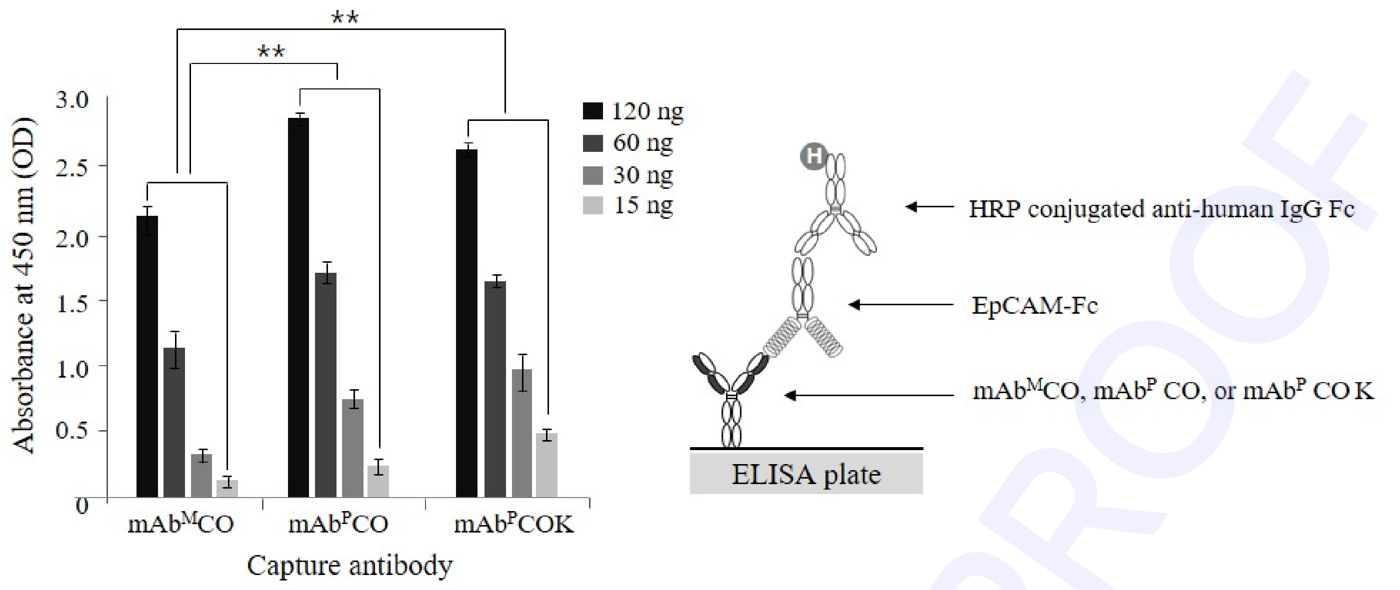

Fig. 2. Figure 2. ELISA analysis to confirm binding process of mAbMCO, mAbPCO, and mAbPCOK against the antigenic protein EpCAM-Fc.

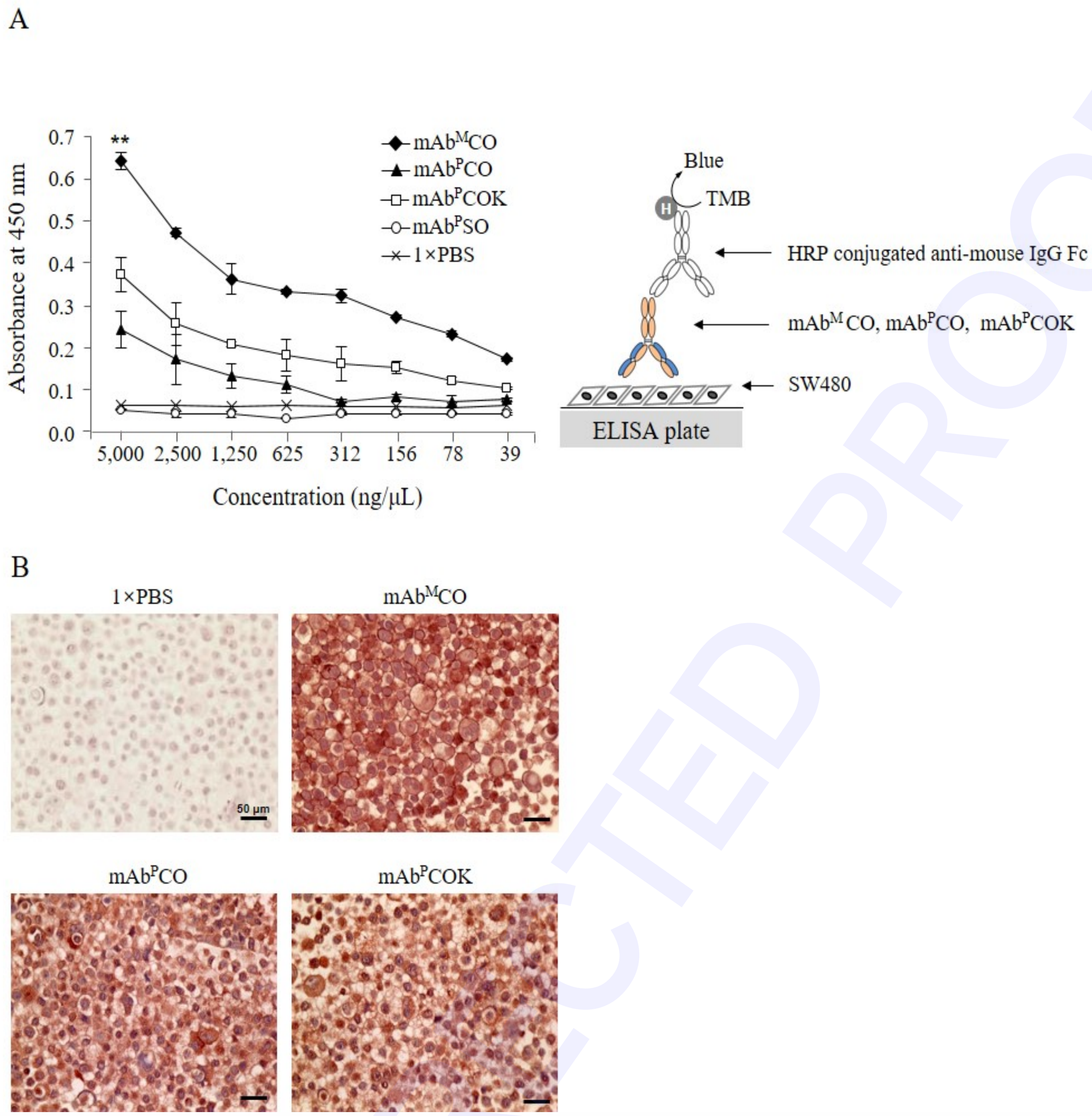

Fig. 3. Figure 3. Cell ELISA and immunocytochemistry to determine the binding affinity of mAbMCO, mAbPCO, and mAbPCOK for SW480 cells.

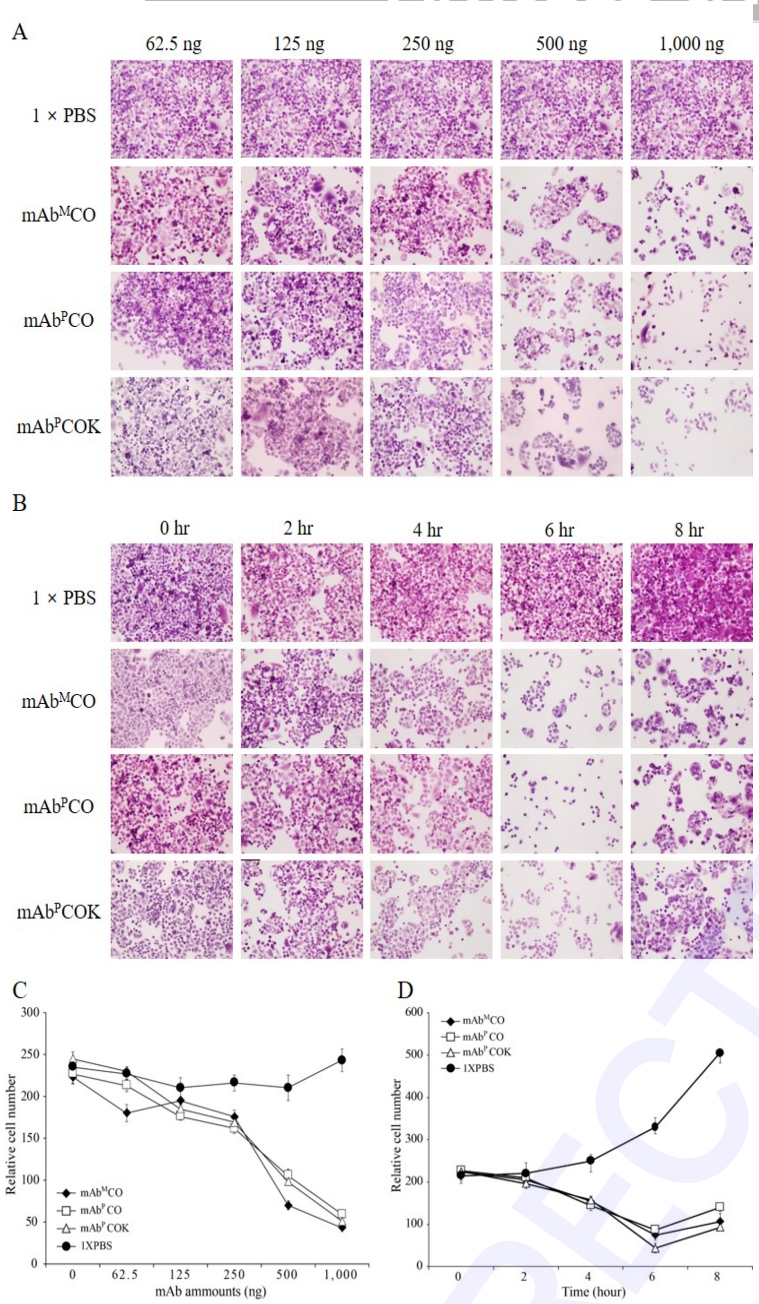

Fig. 4. Figure 4. Dose and Time-dependent effects of mAbMCO, mAbPCO, and mAbPCOK on human colorectal cancer SW480 cell growth.

Supplementary Materials and methods

Generation of transgenic Arabidopsis expressing mAb^{P}CO and mAb^{P}COK

Plant expression vectors (pBI CO17-1A and pBI CO17-1AK) were transformed into *Agrobacterium tumefaciens* strain GV3101::pMP90 by electroporation (Fig. 1A). Non-transgenic *Arabidopsis* plants were transformed using the floral dip method (23). T_1 seeds were selected on Murashige and Skoog (MS) medium agar plates [10 g·L^{-1} of sucrose, 8 g·L^{-1} of plant agar, and 4.3 g·L^{-1} of MS B5 vitamin (Duchefa Biochemie, Haarlem, Netherlands)] containing 50 mg·L^{-1} kanamycin and 25 mg·L^{-1} cefotaxime. True leaf-generating shoots were sorted, transferred to a pot, and maintained at 23°C under a 16 hr light/8 hr dark cycle.

Polymerase chain reaction (PCR)

Approximately 100 mg of rosette leaves from 4-week-old, wild-type (WT) (Col-0) *A. thaliana* and transgenic plants expressing mAb^{P}CO and mAb^{P}COK (CO and COK, respectively) were used for PCR analysis. A DNA extraction kit (RBC Bioscience, Seoul, Korea) was used to extract genomic DNA from these leaves, following the manufacturer's protocols. PCR was conducted to confirm the presence of genes for mAb^{P} CO HC (1,404 bp), HCK (1,416 bp), and LC (764 bp) in order to screen the transformants from T_1 plants. Primer design was as follows: HC forward primer, 5′-GCG AAT TCA TGG AAT GGA GCA GAG TCT TTA TC-3′; HC reverse primer, 5′-GAT TAA TCG ATT TTA CCC GGA GTC CG-3′; LC forward primer, 5′-GCC TCG AGA TGG GCA TCA AGA TGG AAT CAC AG-3′; and LC reverse primer, 5′-GAG GTA CCC TAA CAC TCA TTC CTG TTG AAG CTC-3′. Leaves from the Col-0 plant were used as a negative control, and pBI CO17-1A vector was used as a positive control. PCR was replicated thrice.

Purification of anti-cancer mAb

To purify anti-colorectal cancer mAbs (mAb^{P}CO and mAb^{P}COK) from transgenic *Arabidopsis* plants, 300 g of freshly harvested leaves from each transgenic plant (CO and COK) were homogenized in 1.8 L extraction buffer (37.5 mM Tris-HCl pH 7.5, 50 mM NaCl, 15 mM EDTA, 75 mM sodium citrate, and 0.2% sodium thiosulfate) using a grinder (HR2094, Philips, Seoul, Korea). After centrifugation at 8,800 × *g* for 30 min at 4°C, the supernatant was filtered through Miracloth (Biosciences, LaJolla, CA), and its pH was adjusted to 5.1 using acetic acid. The solution was further centrifuged at 10,200 × *g* for 30 min. The supernatant was filtered through Miracloth, and the pH was adjusted to 7.0 by adding 3 M Tris-HCl. Next, ammonium sulfate was added to 8% concentration. Following centrifugation at 8,800 × *g* for 30 min at 4°C, ammonium sulfate was added to 24% concentration to the supernatant and incubated overnight at 4°C. The resulting solution was centrifuged at 4°C for 30 min, and the pellet thus obtained was resuspended in 180 mL extraction buffer. The resultant solution was centrifuged at 10,200 × *g* for 30 min at 4°C. Both mAb^{P}CO and mAb^{P}COK proteins were purified using protein A Sepharose 4 Fast Flow (GE Healthcare, Piscataway, NJ), according to the manufacturer's recommendations. Both mAb^{P}s were dialyzed in 1×PBS (pH 7.4). Protein concentration was determined using a Nano-drop (Biotek, Highland, VT), and the purified protein was visualized via SDS-PAGE. Purified proteins were stored at -70°C for further studies.

Sandwich ELISA analysis

To investigate the protein binding affinity for the target antigen, EpCAM-Fc, serially diluted solutions of mAb^{M}CO [anti-EpCAM mAb (R&D Systems, Minneapolis, MN)], mAb^{P}CO, and mAb^{P}COK were coated on Maxisorp 96-well micro plates (Nunc, Roskilde, Denmark) with

100 μL of coating buffer [50 mM sodium carbonate (Sigma-Aldrich) and 50 mM sodium bicarbonate (Sigma-Aldrich), adjusted to pH 9.6], and incubated at 4°C for 12 hr **(31)**. Following incubation, plates were washed thrice with 200 μL of PBS-T, treated with 200 μL of blocking buffer [3% BSA (Bio World, Dublin, OH) in 1 x PBS-T] and incubated for another 1 hr at RT. The 96 well plates were then incubated for 2 hr at 37°C with 100 μL of blocking buffer containing EpCAM-Fc. After washing the plates thrice, HRP-conjugated anti-human IgG Fc (Jackson, West Grove, PA) was added to each plate, following which, the plates were incubated for 90 min and treated with 3,3′,5,5′-tetramethylbenzidine (TMB) substrate (KPL, Gaithersburg, MA) for 3 min, followed by 100 μL of TMB stop solution (KPL) to stop the reaction. Absorbance at 450 nm was measured using an ELISA reader Epoch (Biotek).

Statistical analysis

To confirm the statistical comparison between plant-derived monoclonal antibodies (mAb CO and mAb COK), optical density (OD) values obtained by ELISA analyses were applied using Minitab statistical program (Minitab Inc., State college, PA).

Cell ELISA analysis

Cell ELISA was conducted to determine the binding affinity of mAb^{M}CO, mAb^{P}CO, and mAb^{P}COK to SW480 cells (ATCC, Manassas, VA). SW480 was cultured in RPMI-1640 medium supplemented with 10% fetal bovine serum (FBS) under conditions of 37°C and 5% CO_2. Each 96-well culture plate (Nunc) was seeded with 4×10^4 cells of SW480 and cultured until the cells reached 80% confluency in the same atmosphere. The cells were fixed with 10% formalin for 30 min at RT and washed using 1×PBS. Next, 200 μL of blocking buffer (1% BSA in 1×PBS) was added to each well, and the plates were incubated for 1 hr at 37°C. For blocking purposes, 200 μL of blocking buffer was applied for 1 hr at RT. Primary antibodies [100 μL; $mAb^{M}CO$, $mAb^{P}CO$, $mAb^{P}COK$, and plant-derived anti-rabies mAb ($mAb^{P}SO$), respectively] were serially diluted to concentrations ranging from 5,000 $ng \cdot \mu L^{-1}$ to 39 $ng \cdot \mu L^{-1}$ and applied to each well. Here, $mAb^{P}SO$ was used as a negative control. After washing thrice with 1×PBS for 5 min, horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG Fcγ fragment (Jackson ImmunoResearch, West Grove, PA) diluted 1:8,000 in an ELISA blocking buffer was added to the above preparation and incubated for 2 hr at RT. Each plate was treated with 3,3′,5,5′-tetramethylbenzidine (TMB) substrate (KPL) for 15 min. The reaction was stopped using 100 μL of TMB stop solution (KPL). Absorbance at 450 nm was measured using the Epoch Microplate Reader (Biotek).

Immunocytochemistry

SW480 (2×10^{7} cells) cultured in a 5% CO_2 incubator at 37°C were washed with 1×PBS buffer (137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 2 mM KH_2PO_4; pH 7.4) and harvested via trypsinization. Harvested cell pellets were fixed in 10% formalin for 2 hr and embedded in paraffin. Slides were treated with 3% H_2O_2 for 10 min and washed twice with 1× PBS, and subsequent treated with protein block serum-free reagent (Dako, Carpinteria, CA) for 30 min. A composition of 2 μg of $mAb^{M}CO$, $mAb^{P}CO$, and $mAb^{P}COK$ were used as the primary antibody treatment. 1× PBS and $mAb^{P}CO$ were used as negative controls. After washing thrice with 1×PBS, the Dako REAL™ EnVision™ Detection System (Dako) was utilized according to the manufacturer’s instructions, following which counterstaining with Mayer′s hematoxylin (Muto Pure Chemicals CO., Tokyo, Japan) was conducted. After dehydration, a cover glass was mounted with Permount solution. The slides were observed with a microscope [magnification, X 400; BX53F (Olympus, Tokyo, Japan)].

Antibody-mediated tumor cell regression assay

Sterilized coverslips with a 12-mm diameter (SPL Life Sciences, Pocheon, Korea) were placed in the 24-well culture plates (Nunc), and SW480 cells (1×10^5 cells per well) were seeded on the coverslips. To investigate dose-dependent cytotoxicity effects of the antibodies, SW480 cells were treated with antibodies (mAb^{M}CO as a positive control; mAb^{P}SO as a negative control) in the amounts of 1,000, 500, 250, 125, and 62.5 ng. The cells were then incubated under conditions of 37°C and 5% CO_2, for 4 hr. To confirm time-dependent regression effects of the antibodies, SW480 cells (1×10^5 cells per well) were seeded on another cell culture plate. Antibodies in the amount of 0.25 μg were added to each well and incubated for 2, 4, 6, or 8 hr under conditions similar to those stated above. Following antibody treatment, each experimental group was fixed in 10% formalin for 30 min and washed with 1×PBS buffer. These fixed cells were then stained using Harris hematoxylin solution (Muto Pure Chemicals CO.**),** followed by counter staining with eosin Y solution (Sigma-Aldrich). Next, the slides were sequentially dehydrated in 95 and 100% alcohol. One to two drops of aqueous permanent mounting medium (Dako) were applied to the coverslips. The slides were observed under a microscope [magnification, X 200; BX53F (Olympus)].