A Mucin1 C-terminal Subunit-directed Monoclonal Antibody Targets Overexpressed Mucin1 in Breast Cancer

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Supplementary Materials and Methods

Native-PAGE and western blotting

Cells lysates (MDA-MB-231 and T47D) were prepared with cell lysis buffer (20 mM Tris·HCl pH 8.0, 5 mM EDTA, 150 mM NaCl, 100 mM NaF, 2 mM Na_3VO_4, 1% NP-40). The cell lysates were centrifuged at 14,000 rpm at 4°C for 20 min, and the supernatants were mixed with 4X sample buffer (0.25M Tri-Cl pH 6.8, 40% glycerol, 0.4% bromophenol blue). The proteins from cell lysates were separated in SDS-free 7% PAGE at 4°C. After electrophoresis, the separated proteins were transferred onto nitrocellulose membranes and blocked with 3% BSA in PBST for 1 h at 4°C. The nitrocellulose membranes were incubated with anti-hMUC1 monoclonal antibody, anti-hMUC1-CT2 antibody, anti-MUC1-NT antibody (Cell Signaling, Danvers, MA, USA, mouse monoclonal Ab to MUC1-N tandem repeats), or anti-β-actin antibody overnight at 4°C. The membranes were treated with horseradish peroxidase-conjugated secondary antibody (Jackson ImmunoResearch), and the immune-reactive bands were detected by an enhanced chemiluminescence reagent (Thermo Fisher Scientific).

Purification of recombinant mouse MUC1 C-terminal protein, and immunoprecipitation and western blotting analyses

The cDNA encoding a peptide sequence of 187 amino acids of mouse MUC1 (from proline 342 to serine 528; GenBank Accession No. NP_038633.1) was obtained from BNL 1ME A.7R.1 cDNA (ATCC® TIB-75™) and amplified by RT-PCR using the following primer sets: sense 5′-CAT ATG CCT ACC AGT TCT GTG TTG G-3′ and anti-sense 5′-CTC GAG GGA CTG GGC AGA GGG AG-3′. Using the *Nco* I and *Xho* I restriction enzyme sites, the amplified cDNA fragments were cloned into the expression vector pET-22b (Novagen) containing a C-terminal His-tag. The mouse MUC1 C-terminal protein was expressed and purified from *Escherichia coli* Rosetta™ (Invitrogen) competent cells as described in "Materials and Methods". The resultant recombinant mouse (rm) protein including extracellular region of MUC1-C was named rmMUC1-EC187.

To investigate whether the anti-hMUC1 monoclonal antibody recognizes mouse MUC1 C-terminal protein, immunoprecipitation and western blotting using purified mouse and human MUC1 C-terminal proteins were carried out. Equal amount of mouse and human MUC1 C-terminal proteins were incubated with mouse anti-hMUC1 monoclonal antibody for

1 h at 4℃ and then Protein A bead was added to the mixture and incubated at 4℃ for 1 h. The immunocomplexes collected by centrifugation were washed and analyzed by western blotting. Membranes were incubated with anti-His tag antibody (Santa Cruz Biotechnology, Dallas, TX).

Antibodies

To analyze signal molecules after anti-hMUC1 monoclonal antibody treatment by western blotting, antibodies to ERK, phosphor-ERK, AKT, phosphor-AKT, p38, phosphor-p38 were purchased from Cell Signaling Technology. To estimate whether apoptosis is induced by anti-hMUC1 monoclonal antibody treatment, the anti-Caspase-3 antibody and anti-PARP antibody were purchased from Cell Signaling Technology.

Annexin V and propidium iodide staining

MDA-MB-231 cells and T47D cells were treated with anti-hMUC1 monoclonal antibody for 72 h. After collection of the cells using trypsin-EDTA, FITC-conjugated Annexin V (eBioscience, San Diego, CA, USA) and propidium iodide (PI, eBioscience) were added to the cells. After staining for 15 min, the cells were analyzed by FACSCalibur (BD Biosciences, San Jose, CA, USA) and Flowing software (Turku Centre for Biotechnology, Finland).

Cell cycle analyses by flow cytometry

MDA-MB-231 cells and T47D cells were treated with anti-hMUC1 monoclonal antibody or normal IgG for 3 days and 5 days. After collection of the cells using trypsin-EDTA, the cells were fixed with 70% ethanol at 4℃ overnight. The fixed cells were washed with PBS containing 1% FBS and treated with 500 μg/ml RNase (Sigma) at 37℃ for 30 min. After washing with PBS containing 1% FBS, the cells were incubated with PI at room temperature for 15 min in the dark. DNA contents were analyzed using FACSCalibur (BD Biosciences) and Flowing software (Turku Centre for Biotechnology).

Supplementary Figure 1

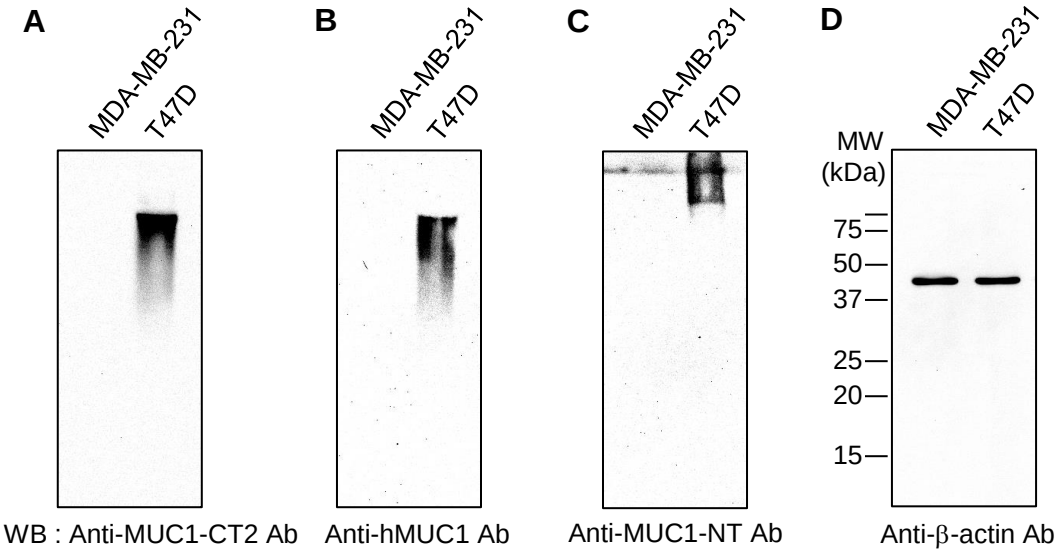


Figure S1. Recognition of native MUC1 by anti-hMUC1 monoclonal antibody. Lysates from MDA-MB-231 and T47D breast cancer cell lines were resolved by SDS-free native-PAGE and western blotting was performed with anti-MUC1-CT2 (A), anti-hMUC1 monoclonal antibody (anti-hMUC1 Ab) (B), and anti-MUC1-NT antibody (mouse monoclonal antibody to MUC1-N) (C). The expression level of β-actin was analyzed by SDS-PAGE and western blotting to confirm equal loading of protein (D).

Supplementary Figure 2

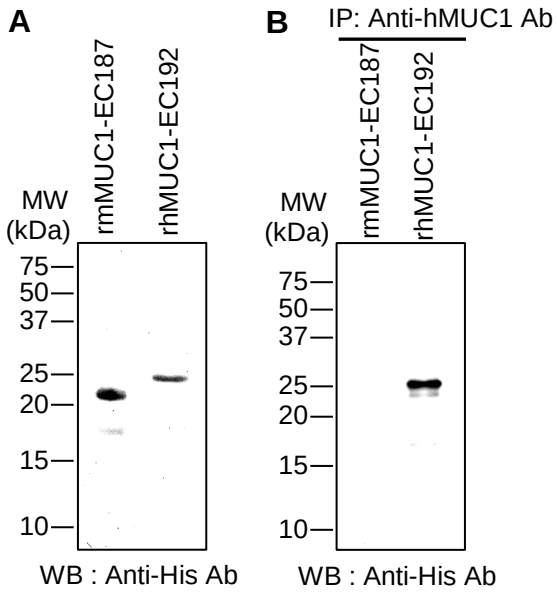


Figure S2. Recognition of human MUC1 by anti-hMUC1 monoclonal antibody. (A) Purified mouse MUC1 C-terminal protein (rmMUC1-EC192) and human MUC1 C-terminal protein (rhMUC1-EC187) were resolved by SDS-PAGE, and western blotting was performed with anti-His tag antibody. (B) Purified mouse and human MUC1 C-terminal proteins were immunoprecipitated with the anti-hMUC1 monoclonal antibody (Anti-hMUC1 Ab) and then immunoblotted with anti-His tag antibody.

Supplementary Figure 3

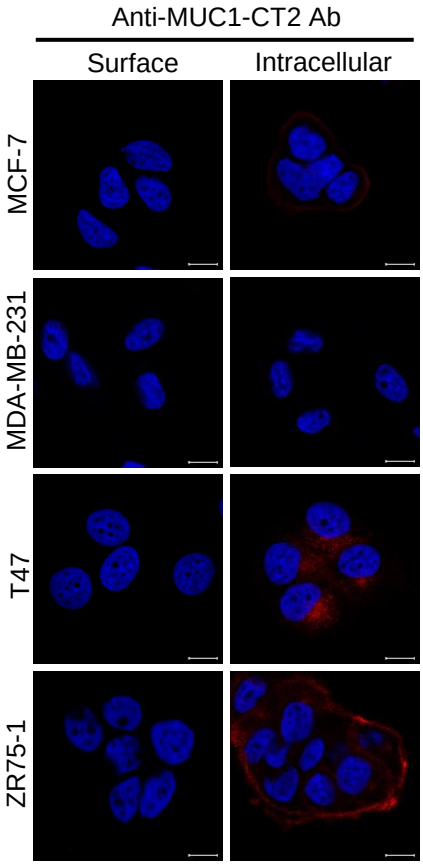


Figure S3. Binding of anti-MUC1-CT2 antibody to MUC-1 protein localized in the intracellular regions. MCF-7, MDA-MB-231, T47D, and ZR75-1 cells were surface-stained or stained in the intracellular region with the anti-MUC1-CT2 antibody, followed by staining using Alexa 488-conjugated secondary antibody. The nuclei were stained with Hoechst 33258. Images were visualized by confocal microscopy. Scale bar, 10 μm.

Supplementary Figure 4

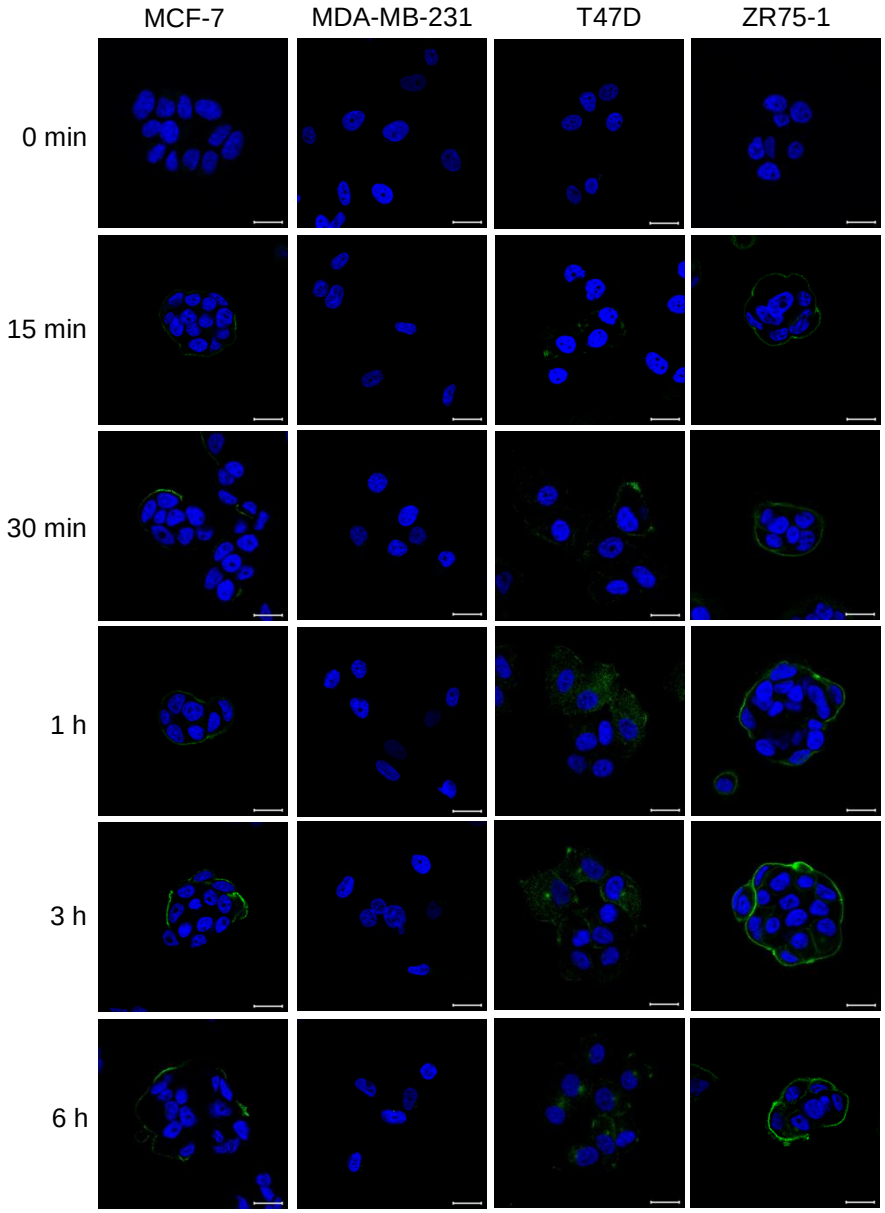


Figure S4. Internalization of anti-hMUC1 antibody in a time-dependent manner. MCF-7, MDA-MB-231, T47D, and ZR75-1 cells were treated with the DyLight 488-labeled anti-hMUC1 antibody and incubated at 37℃ for various time intervals. The nuclei were stained with Hoechst 33258. Confocal microscopy images of MCF-7, MDA-MB-231, T47D, and ZR75-1 cells were captured. Scale bars, 10 μm. The images are representative of data from 3 independent trials.

Supplementary Figure 5

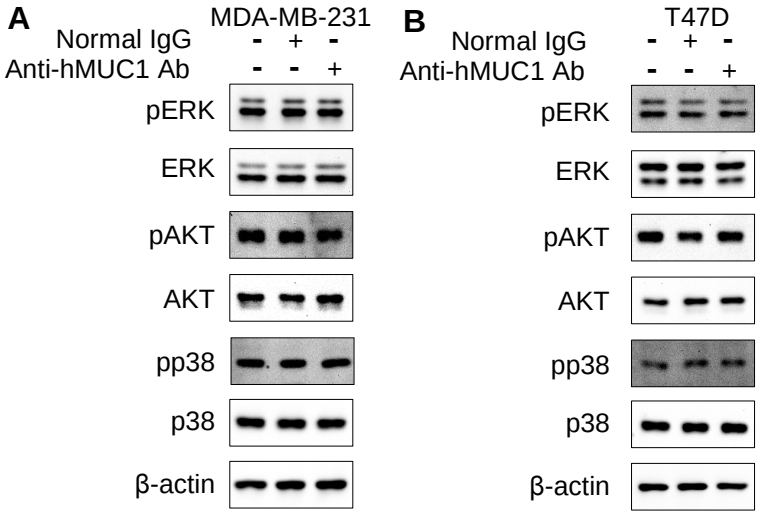
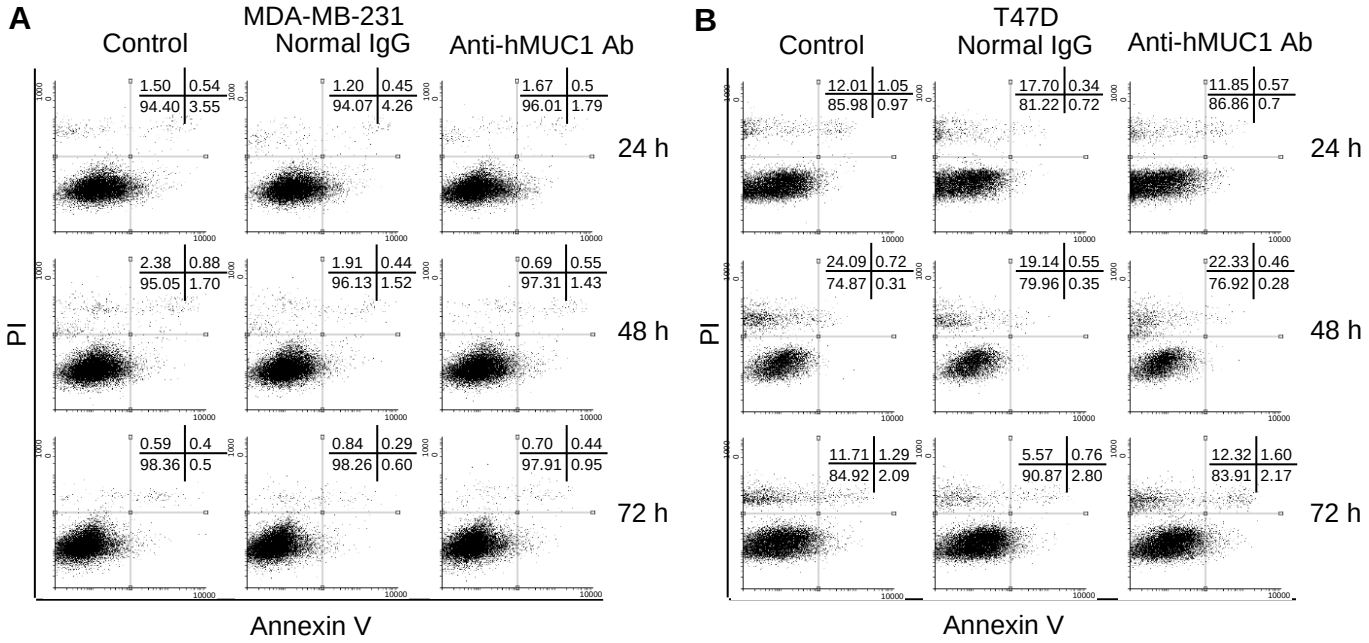


Figure S5. Western blot analysis of signal molecules in anti-hMUC1 monoclonal antibody-treated breast cancer cell lines. MDA-MB-231 cells (A) and T47D cells (B) were treated with anti-hMUC1 monoclonal antibody or normal IgG for 6 h, and western blotting was performed using phosphor-ERK, ERK, phosphor-AKT, AKT, phosphor-p38, p38 and β-actin.

Supplementary Figure 6



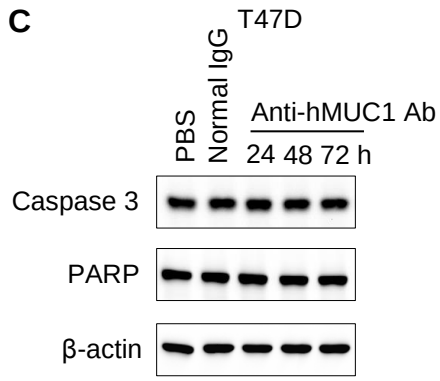


Figure S6. Analysis of apoptosis in anti-hMUC1 monoclonal antibody-treated breast cancer cell lines. MDA-MB-231 cells (A) and T47D cells (B) were treated with anti-hMUC1 monoclonal antibody or normal IgG for 24, 48 and 72 h. The cells were stained with Annexin V and PI, and analyzed with FACSCalibur and Flowing software. (C) T47D cells were treated with anti-hMUC1 monoclonal antibody or normal IgG for 24, 48 and 72 h, and western blotting was done using antibodies to Caspase 3, PARP, and β-actin.

Supplementary Figure 7

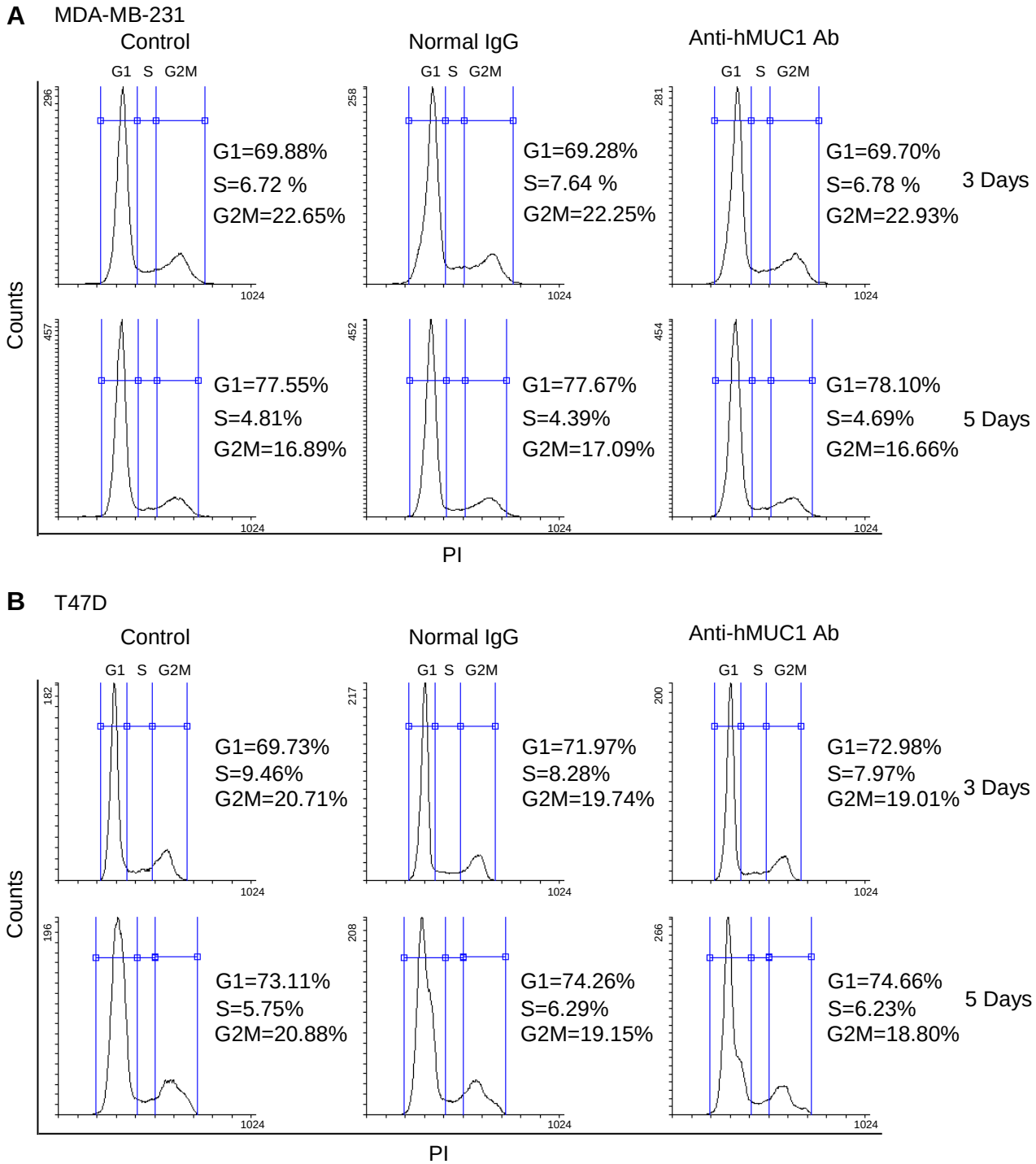


Figure S7. Effect of anti-hMUC1 monoclonal antibody on cell cycle in breast cancer cell lines. MDA-MB-231 cells (A) and T47D cells (B) were treated with anti-hMUC1 monoclonal antibody or normal IgG for 3 and 5 days. The cells were fixed, and stained with propidium iodide. Cell cycle profiles were analyzed with FACSCalibur and Flowing software.

Supplementary Figure 8

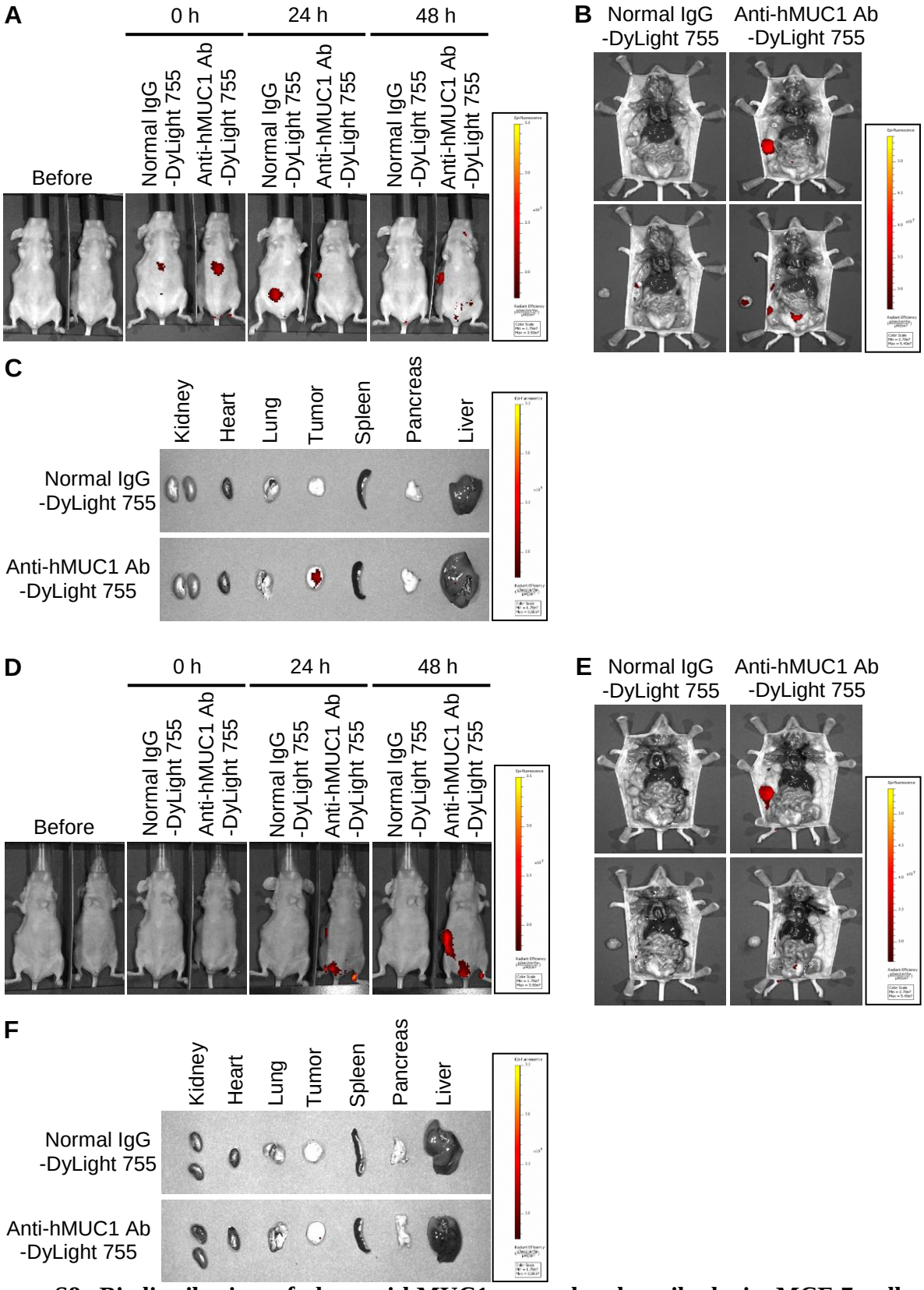


Figure S8. Biodistribution of the anti-hMUC1 monoclonal antibody in MCF-7 cell-derived breast tumor tissues. BALB/C nu/nu mice were subcutaneously injected with MCF-7 cells to induce tumor formation. (A, D) The mice were injected intravenously with

DyLight 755-labeled normal IgG (5 mg/kg) or anti-hMUC1 monoclonal antibody (5 mg/kg), and whole-body fluorescent imaging was performed at 0, 24, and 48 h. (B, E) The localization of the antibody in dissected mice. (C, F) Various organs and tumors were isolated and examined for antibody distribution. The images are data from 2 sets of mice.

Supplementary Figure 9

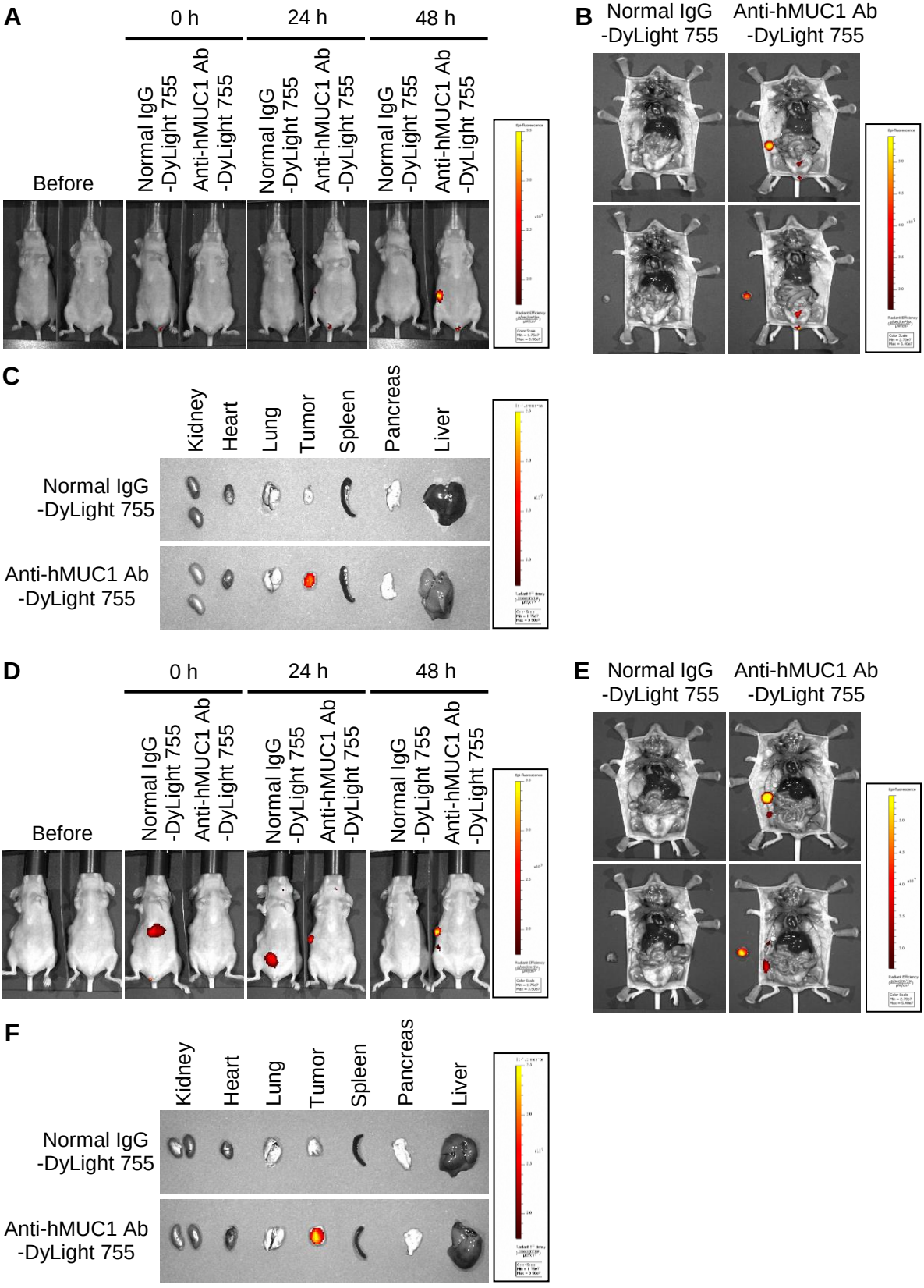


Figure S9. Biodistribution of the anti-hMUC1 monoclonal antibody in T47D cell-derived breast tumor tissues. BALB/C nu/nu mice were subcutaneously injected with T47D cells to induce tumor formation. (A, D) The mice were injected intravenously with DyLight 755-labeled normal IgG (5 mg/kg) or anti-hMUC1 monoclonal antibody (5 mg/kg), and whole-body fluorescent imaging was performed at 0, 24, and 48 h. (B, E) The localization of the antibody in dissected mice. (C, F) The antibody distribution in various organs and tumors. The images are data from 2 sets of mice.

Supplementary Figure 10

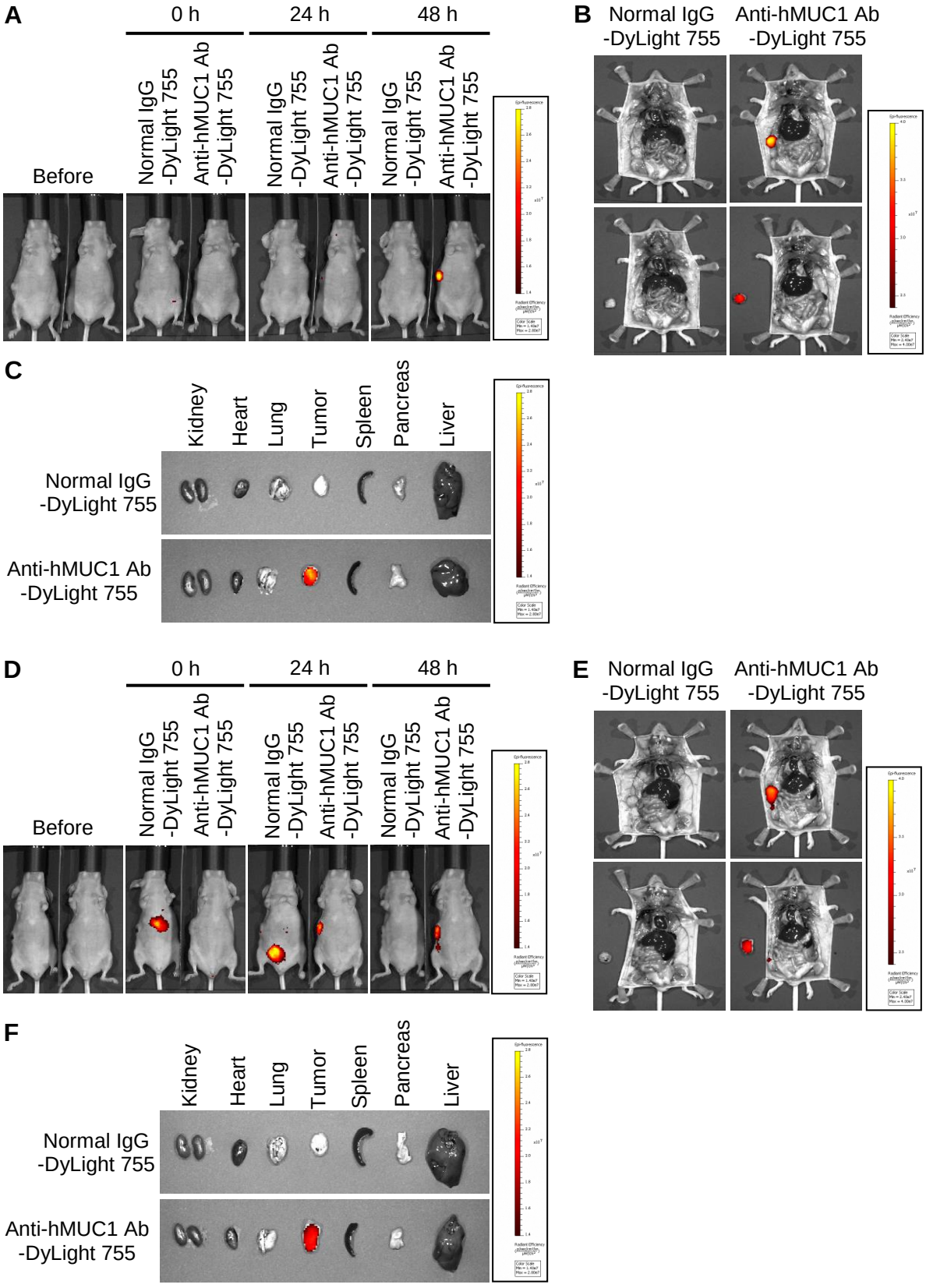


Figure S10. Biodistribution of the anti-hMUC1 monoclonal antibody in ZR75-1 cell-derived breast tumor tissues. BALB/c nu/nu mice were subcutaneously injected with ZR75-

1 cells to induce tumor formation. (A, D) The mice were injected intravenously with DyLight 755-labeled normal IgG (5 mg/kg) or anti-hMUC1 monoclonal antibody (5 mg/kg), and whole-body fluorescent imaging was performed at 0, 24, and 48 hours. (B, E) The localization of the antibody in dissected mice. (C, F) The antibody distribution in various organs and tumors. The images are data from 2 sets of mice.

Table S1: Expression of MUC1 in breast cancer tissues. A312 (II) Breast cancer tissues.

No.	Sex	Age	Black`s nuclear grade	Bloom-Richardson`s histological grarde	Key word	Ab staing
1	F	47	G2	G2	Invasive ductal carcinoma	+
2	F	54	G3	G1	Invasive ductal carcinoma	+
3	F	49	G2	G2	Invasive ductal carcinoma	++
4	F	41	G2	G2	Invasive ductal carcinoma	++++
5	F	52	G2	G2	Invasive ductal carcinoma	+++
6	F	40	G1	G3	Invasive ductal carcinoma	++++
7	F	39	G2	G2	Invasive ductal carcinoma	++
8	F	51	G1	G3	Invasive ductal carcinoma	+++
9	F	57	G2	G3	Invasive ductal carcinoma	++
10	F	53	G2	G2	Invasive ductal carcinoma	+++
11	F	62	G1	G3	Invasive ductal carcinoma	+++
12	F	51	G2	G3	Invasive ductal carcinoma	++++
13	F	41	G1	G3	Invasive ductal carcinoma	+
14	F	31	G1	G3	Invasive ductal carcinoma	++
15	F	52	G1	G3	Invasive ductal carcinoma	+
16	F	54	G2	G2	Invasive ductal carcinoma	-
17	F	45	G1	G3	Invasive ductal carcinoma	++
18	F	54	G2	G1	Invasive ductal carcinoma	+++
19	F	68	G1	G3	Invasive ductal carcinoma	+++
20	F	46	G1	G3	Invasive ductal carcinoma	++++
21	F	41	G2	G2	Invasive ductal carcinoma	++

>75% (4, ++++), 50-74% (3, +++), 11-49% (2, ++), <10% (1, +), Negative (-)