Original Article

MLS128 antibody-induced suppression of colon cancer cell growth is mediated by a desmocollin and a 110 kDa glycoprotein

Sarah C. Shuck[1], Teresa Hong[2], Markus Kalkum[2], Ryo Igarashi[1], Kota Kajiya[1], John Termini[1], Kazuo Yamamoto[3], Yoko Fujita-Yamaguchi[4,*]

[1] *Department of Molecular Medicine, Beckman Research Institute, City of Hope, Duarte, CA, USA;*
[2] *Department of Molecular Imaging and Therapy, Beckman Research Institute, City of Hope, Duarte, CA, USA;*
[3] *Department of Integrated Biosciences, Graduate School of Frontier Sciences, The University of Tokyo, Kashiwa, Japan;*
[4] *Department of Diabetes Complications & Metabolism, Beckman Research Institute, City of Hope, Duarte, CA, USA.*

Summary   **Protein glycosylation is a diverse form of post-translational modification. Two to three consecutive O-linked N-acetylgalactosamines (Tn-antigens) are recognized by antibodies such as MLS128. MLS128 mAb inhibited cell growth and bound to a 110 kDa glycoprotein (GP) in LS180 and HT29 colon cancer cells. However, purification and identification of the 110 kDa GP was unsuccessful due to its low abundance. The present study used a highly sophisticated and sensitive mass spectrometry method to identify proteins immunoprecipitated with MLS128 and separated by two-dimensional gel electrophoresis. Three desmosome components were identified. Of these, desmocollin and desmoglein shared many similar characteristics, including molecular mass, pI, and potential Tn-antigen sites. Western blotting analyses of LS180 cell lysates revealed a common 110 kDa band recognized by MLS128 and anti-desmocollin, but not by anti-desmoglein. Immunofluorescence microscopy of LS180 cells revealed that desmocollin is membrane-bound, while desmoglein is primarily localized in the cytosol. Confocal microscopy demonstrated colocalization of the desmocollin-specific antibody with the MLS128 antibody on the cell membrane, suggesting that desmocollin may contain Tn-antigens recognized by MLS128. Treatment of LS180 cells with siRNA to knock down desmocollin expression or a desmocollin-specific antibody decreased cell viability, suggesting a critical role for this protein in cell growth and survival. N-glycosidase F digestion of the 110 kDa GP and desmocollin suggested that although both proteins contain N-glycosylation sites, they are not identical. These findings suggest that desmocollin colocalizes with the 110 kDa GP and that growth inhibition induced by the MLS128 antibody may be mediated through a mechanism that involves desmocollin.**

Keywords: Desmocollin, colon cancer, MLS128, receptor, growth inhibitory mAb, anti-Tn antigen, desmosome

1. Introduction

Mouse immunization with cancer cells, glycoproteins, and mucins has been performed to produce cancer-specific monoclonal antibodies (mAbs); many of which have been isolated and characterized as carbohydrate-specific. Two antibodies identified in this way, MLS128 and 83D4, were found to recognize Tn-antigens, which are two or three consecutively arranged N-acetylgalactosamines (GalNAc) conjugated to serine and/or threonine residues (*1-4*). The Tn-antigen is one of the most common aberrations associated with cancer progression and metastasis, and thus is an excellent target for development of cancer diagnostics and therapeutics (*5-9*). MLS128 (IgG3) was derived from a mouse immunized with mucins secreted by LS180 human colon

carcinoma cells (*10*) whereas 83D4 mAb (IgM) was derived from a mouse immunized with human breast cancer tissue-paraffin sections (*11*).

Previous studies in our laboratory revealed that MLS128 binds a membrane glycoprotein (GP) with a molecular weight of 110 kDa. This 110 kDa GP contains N-glycans in addition to O-glycans consisting of 2 or 3 consecutive Tn-antigens which are recognized by MLS128 (*12*). Tn-antigens are found in over 67% of colon cancer cell lines tested, but not in MCF-7 breast cancer cell lines (*12,13*). MLS128 antibody treatment inhibited growth of LS180 and HT29 colon cancer cells (*13,14*). Efforts to purify and identify the 110 kDa GP bound by MLS128 have been very difficult, mainly due to its low abundance.

In this study, our goal was to identify the 110 kDa GP. To do this, we immunoprecipitated membrane proteins with MLS128, separated them by 2-D gel electrophoresis, digested them with trypsin/Lys-C, and analyzed them by liquid chromatography multistage mass spectrometry (LC-MS/MS). Proteomics analysis identified several putative candidates for the 110 kDa GP, which we further examined in various cell lines using Western blotting, immunofluorescence microscopy, antibody treatments, and siRNA knockdowns.

2. Materials and Methods

2.1. *Materials*

Production and characterization of the MLS128 antibody was previously described (*1,11*). Desmocollin (ab150382) and desmoglein (ab124798) antibodies, horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG3, anti-rabbit IgG (H+L), and Fluoroshield DAPI mounting media were obtained from Abcam (Cambridge, MA). Goat anti-rabbit Alexa Fluor® 488, goat anti-mouse Alexa Fluor® 647, Mem-PER Membrane protein isolation kit, and protease inhibitor (87886) were from Thermo Fisher Scientific (Waltham, MA). Cell culture media (Dulbecco's Modified Eagles Medium (DMEM) and McCoy's 5A) were purchased from Gibco (Grand Island, NY). N-glycosidase F was purchased from Roche Applied Science (Indianapolis, IN).

2.2. *Cell lines and culture*

Cell lines used in this study, human colon adenocarcinoma LS180 cells, human breast carcinoma MCF-7 cells, and human normal mammary epithelial MCF-10A cells, were obtained from American Tissue Type Culture Collection. LS180 colon cancer cells were cultured in DMEM containing 10% fetal bovine serum (FBS), 4.5 mg/mL D-glucose, and 110 μg/mL pyruvic acid as described (*12*). MCF-7 breast cancer and MCF-10A cells were cultured in DMEM containing 10% FBS.

### 2.3. *Preparation of whole cell lysates and membrane and cytosolic fractions*

LS180 cells were cultured in 75 cm flasks as previously described (*12*). Whole cell extracts were prepared by scraping followed by centrifugation at 200 × g for 5 min and solubilized on ice for 15 min in solubilization buffer (50 mM Tris-HCl buffer, pH 7.4, containing 1% NP40, 2 mM EDTA, 100 mM NaCl, 10 mM sodium orthovanadate, 1 mM PMSF, and protease inhibitors). Supernatants were obtained from solubilized cells by centrifugation at 17,000 × g for 10 min. Protein concentrations were measured using Bradford reagent (Bio-Rad, Hercules, CA). Membrane and cytosolic fractions were prepared using the Mem-PER membrane protein isolation kit (Thermo Fisher Scientific) according to manufacturer's instructions.

2.4. *Western blot analyses*

Solubilized proteins (10 μg) from LS180 cells were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto poly vinylidene difluoride (PVDF) membranes. The membrane was blocked with 3% BSA in 50 mM Tris-HCl buffer, pH 7.4, containing 0.15 M NaCl and 0.1% Tween 20 (TBST) for 1 h at room temperature. Western blotting was carried out with primary antibodies as indicated, and then detected with HRP-conjugated secondary antibodies and color development using Ez West blue (ATTO Co., Tokyo, Japan).

2.5. *Treatment of LS180 cell membrane and cytosolic fractions with N-glycosidase F and Western blotting*

Cytosolic and membrane protein fractions were isolated as described above. Fractions (200 μg) were incubated at 90°C for 3 min in 40 μL of PBS containing 0.5% SDS and 1% β-mercaptoethanol. After cooling to room temperature, 0, 1, or 3 U of N-glycosidase F and 10 μL of 5% NP-40 were added. After overnight incubation at 37°C, the reaction was stopped by addition of SDS-PAGE sample buffer (62.5 mM Tris-HCl, pH 8). Cytosolic and membrane fractions (10 μg) were loaded onto a 4-12% gradient Bis-Tris gel (NuPAGE, Thermo Fisher Scientific) and electrophoresed at 170 V for 1 h. Gels were transferred onto PVDF membranes at 50 V for 2 h followed by blocking with 2% blotting-grade blocker (Bio-Rad) in PBS with 0.05% Tween-20. Membranes were immunoblotted with MLS128 or anti-desmocollin (1:1,000) in blocking buffer for 1 h and then washed 3 times with PBS containing 0.05% Tween (wash buffer). Secondary antibodies, goat anti-mouse anti for MLS128 or goat anti-rabbit for anti-desmocollin were added and incubated for 1 h. Membranes were then washed 3 times with the wash buffer and visualized using Clarity Western ECL
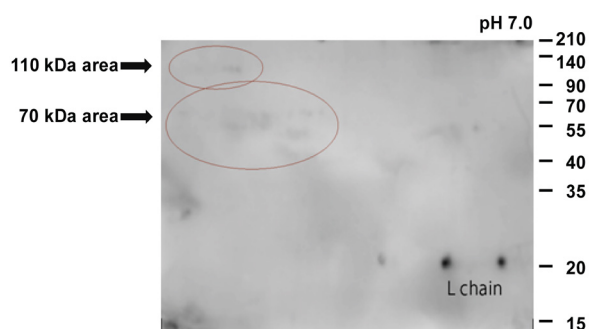
Figure 1. Western blotting with MLS128 of two-dimensional (2-D) electrophoresis of MLS128-IP sample. MLS128 antibody was used to IP proteins from 1.4×10^7 LS180 cells. Resulting IP samples were subjected to 2-D gel electrophoresis as previously described (*13*). 1st dimension was conducted at pH 4-7 linear gradient on the 11 cm strip which was placed on 10% SDS-PAGE gel then electrophoresed under reducing conditions. Shown is the Western blotting of the resulting 2D-gel in which very faint positive staining with MLS128 can be seen. Since IgG light chain (L chain) contains O-glycans, and since the MLS128 was used for IP, those are clearly detected.

Substrate (Bio-Rad) with imaging on a ChemiDoc Imaging Station (Bio-Rad).

2.6. *2-D gel electrophoresis and LC-MS/MS analyses*

To identify peptides derived from 110 kDa proteins, in-gel digestion and mass spectrometric analyses were performed as previously described (*15,16*). Immunoprecipitation (IP) of 110 kDa GP was carried out and resulting IP samples were subjected to 2-D gel electrophoresis as previously described (*14*). The 2-D gels were stained with SimplyBlue SafeStain solution (Life Technologies, Carlsbad, CA) to visualize proteins. Two areas designated in Figure 1 (110 and 70 kDa) were excised and destained in ammonium bicarbonate (100 mM)/acetonitrile (45%) followed by in-gel digestion, which included reduction with Tris(carboxyethyl) phosphine (10 mM), alkylation with iodoacetamide (50 mM) and digestion with Trypsin/Lys-C Mix, Mass Spec Grade (300 ng per band, Promega, Fitchburg, WI), in 100 mM ammonium bicarbonate, pH 7.9. Extracted peptides were acidified with formic acid (1%) and injected into the LC-MS system.

Mass spectrometric analyses of the digested peptides were conducted on an Orbitrap Fusion Tribrid mass spectrometer (Thermo Fisher Scientific) equipped with an Easynano UHPLC, using a 75 μm × 250 mm Pepmap RSLC reverse phase column with a PepMap 1000 trapping column (Thermo Fisher Scientific). Ten μL of digested peptide samples were loaded at 4 μL per min. LC was performed with a gradient mobile phase system containing mobile phase A (0.1% formic acid) and mobile phase B (100% acetonitrile/0.1% formic acid). A 40 min gradient was conducted from 3% to 80% B, followed by 45-60 minutes at 90% B. Flow rate was 300 nL/min. Full mass scans (200-4,000 Da)

were collected by the orbitrap mass analyzer, operated at 120K resolution. MS/MS spectra were generated in data-dependent mode using collision-induced dissociation (CID) and the linear trap mass analyzer. The resulting data sets were analyzed with PEAKS Studio (Bioinformatics Solutions Inc., Waterloo, ON, Canada) and Proteome Discoverer (Thermo Fisher Scientific) and matched against a non-redundant human protein database (Swiss Prot and NR). Database searches were carried out by considering three missed enzymatic cleavages, a precursor ion mass tolerance of 5 ppm, and 0.02 Da mass tolerance for fragment ions. Search parameters also included cysteine carbamidomethylation and methionine oxidation as expected amino acid modifications.

2.7. *Confocal microscopy for detection of desmocollin and MLS128*

Cells were plated at a density of 30,000 cells/well on collagen-coated coverslips in a 24-well plate. Following overnight adherence, media were removed, cells were washed with PBS, and then fixed with 4% paraformaldehyde with 0.2% glutaraldehyde for 30 min at room temperature. Paraformaldehyde was then inactivated by the addition of 25 mM glycine for 10 min at room temperature. Cells were then washed for 5 min three times with PBS containing 0.01% Tween-20 and then blocked for 15 min with 5% BSA in PBS. Blocking buffer was then removed and primary antibodies (anti-desmocollin and MLS128) added at a final dilution of 1:50 with incubation at room temperature for 1 h. It should be noted that while MLS128 recognizes two to three consecutive O-linked GalNAc, the desmocollin antibody (Abcam ab 150382) recognizes an epitope within amino acids 850-950 on the C-terminus of desmocollin. Following incubation, cells were washed for 5 min 3 times with PBS and then secondary antibodies were added at a dilution of 1:100 and incubated overnight at 4°C. Cells were then washed and coverslips mounted onto Rite-On Frosted Slides (Thermo Fisher Scientific) using Fluoroshield DAPI mounting media (Abcam). Immunofluorescence was detected using a Zeiss LSM 880 confocal microscope with an LCI Plan-Neofluar 63×/1.3 NA Multi-Imm Corr objective (Zeiss). Laser scanning modes used include diode 405 nm, argon Laser 458, 488, and 514 nm, and helium/neon Lasers at 594 and 633 nm. Optical slices were obtained using 0.7 μm intervals. Images were processed using Zen Blue software (Zeiss version 2.3). Colocalization between MLS128 and desmocollin antibodies was determined using Zen Blue. The Pearson correlation coefficient was determined for MLS128 and desmocollin antibodies within each cell. There was a minimum of 100 cells analyzed in three independent experiments. Data is presented as the average Pearson correlation coefficient and standard deviation for all experiments.

2.8. *siRNA knockdown of desmocollin*

LS180 cells were plated at a density of 50,000 cells/ well in 24-well plates and allowed to adhere overnight. Desmocollin-targeting siRNAs SASI_Hs01_00172856 (siRNA 1, approximate start site nucleotide 388) and SASI_Hs02_00338620 (siRNA 2, approximate start site nucleotide 732), as well as non-coding siRNAs were obtained from Sigma-Aldrich (St. Louis, MI). siRNAs were transfected using RNAi max transfection reagent (Thermo Fisher Scientific) according to manufacturer's instructions and incubated for 48 h. Following incubation, RNA was isolated using Trizol (Zymo Research, Irvine, CA) and concentrations determined using NanoDropTM with absorbance readings at 260 nm (Thermo Fisher Scientific).

The extent of desmocollin knockdown was determined by synthesizing cDNA from isolated RNA (500 ng), dNTPs (40 mM), RNase inhibitor (1U, Promega), random primers (3 µg, Invitrogen, Walthum, MA), reverse transcriptase (200U, BioChain, Newark, CA), and reaction buffer (5× reaction buffer is composed of 250 mM Tris-HCl, pH 8.3, 375 mM KCl, 15 mM MgC12, and 50 mM DTT, BioChain). The cDNA synthesis reaction was performed at 25°C for 10 min, 45°C for 30 min, and 85°C for 5 min. qRT-PCR was performed with primers specific for desmocollin. The forward primer was 5'-TTTGAGTGAGGAGTGTTTGGCA-3' and the reverse primer sequence was 5'-AAGTTACCGAACTGTTGTGTGT-3'. Tubulin forward (5'-TTCAATCTCCCTCCAAGCTC-3') and reverse (5'-GGGAAGGATTCCACTTGACA-3') primers were used for normalization. Reactions included cDNA, primers, and Applied Biosystems™ Power SYBR Green PCR Master Mix (Thermo Fisher Scientific) and were performed with initial denaturation at 95°C for 10 min followed by 44 cycles of denaturation (95°C for 15s) and annealing and extension (at 60°C for 30s). Relative gene expression was calculated using cycle threshold values for each gene.

2.9. *Effects of antibody and siRNA treatment on cell viability*

LS180 cells were plated onto 96-well plates at a density of 104 cells/well in DMEM supplemented with 1% FBS and 4.5 g/L D-glucose. After adhering overnight, cells were treated with increasing amounts of anti-desmocollin, anti-desmoglein, MLS128, or anti-IgG antibodies for 48 h. Following treatment, wells were washed with PBS and then incubated with crystal violet solution (0.5% crystal violet in 25% MeOH) for 10 min at room temperature. Wells were then washed twice with PBS and crystal violet solution, then cells were solubilized by the addition of 1% SDS. Absorbance was measured at 570 nm on a BioTek Synergy 4 spectrophotometer using Gen5 2.09 software (BioTek, Winooski, VT). Cell viability was normalized to IgG control antibody.

To determine viability following siRNA treatment, cells were plated as described above and then treated with increasing concentrations of control, SASI_ Hs01_00172856 (siRNA1), or SASI_Hs02_00338620 (siRNA2) for 48 hr. Viability was then determined using crystal violet analysis as described above.

3. Results

3.1. *2-D gel electrophoresis and identification of candidate proteins by LC-MS/MS*

MLS128-IP samples were prepared from 1.4×10^7 LS180 cells and proteins separated using 2-D gel electrophoresis. The gel was transferred and probed with MLS128 antibody. Seven 2-D gels were prepared for further analysis. A typical result of 2-D gel analysis is shown in Figure 1. The areas marked on the Western blot were cut out from seven slightly-stained 2-D gels for proteomic analyses. Fifty µL of peptide digests from A (110 kDa area) and B (70 kDa area) of Gel #1 and #2 were prepared. For the 1st experiment, 10 µL of each sample and for the 2nd and 3rd experiment, twice-concentrated samples were analyzed by LC-MS/MS. The results are summarized in Table 1 and identified protein amino acid sequences are presented in Table 2.

Identified 110 kDa GP candidates include "desmocollin 1", consisting of 894 amino acids with molecular weight (MW) of 99.8 kDa and isoelectric point (pI) of 5.25, "desmoglein 1" consisting of 1049 amino acids, MW of 113.7 kDa, and pI of 4.90, and "junction plakoglobin" containing 745 amino acids,

Table 1. List of candidates for 110kDa GP detected by an Orbitrap Fusion hybrid mass spectrometer

Items	Candidate proteins	110 kD area (probability*)	70 kD area
Experiment 1	Desmoglein-1 preproprotein	2 (80-94%)	2 (over 95%)
Experiment 2	Desmoglein-1 preproprotein	2 (over 95%)	7 (over 95%)
	Desmocollin-1	2 (80-94%)	2 (over 95%)
	Junction plakoglobin	1 (over 95%)	2 (over 95%)
Experiment 3	Junction plakoglobin	1 (80-94%)	3 (over 95%)
	Desmoglein-1 preproprotein	4 (over 95%)	2 (over 95%)
	Desmocollin-1		3 (over 95%)

*protein probability values as calculated by Scaffold proteome software at 1% protein FDR.

Table 2. Examples of peptide sequences derived from the candidate proteins

Protein	Peptide	Amino acids
Desmocollin	IEDDNDNAPYFEHR	232-245
Desmocollin	VTATDLDEPDTLHTR	264-278
Desmocollin	VQDQDLPNTPHSK	377-389
Desmoglein	IHSDCAANQQVTYR	73-86
Desmoglein	ISGVGIDQPPYGIFVINQK	87-105
Desmoglein	IIRQEPSDSPMFIINR	198-213
Desmoglein	TMNNFLDREQYGQYALAVR	220-238
Desmoglein	TYVVTGNMGSNDK	391-303
Desmoglein	DGGADGMSAECECNIK	243-258
Desmoglein	YVMGNNPADLLAVDSR	423-438
Desmoglein	ESSNVVVTER	916-925
Junction plakoglobin	SAIVHLINYQDDAELATR	125-142
Junction plakoglobin	LLNDEDPVVVTK	150-161
Junction plakoglobin	TMQNTSDLDTAR	192-293
Junction plakoglobin	ISEDKNPDYR	652-661

Figure 2. MLS128 and desmocollin antibodies recognized a 110 kDa protein. LS180 cell lysates were prepared and analyzed by Western blotting with MLS128 antibody **(A)** anti-desmocollin **(B)**, and anti-desmoglein **(C)**.
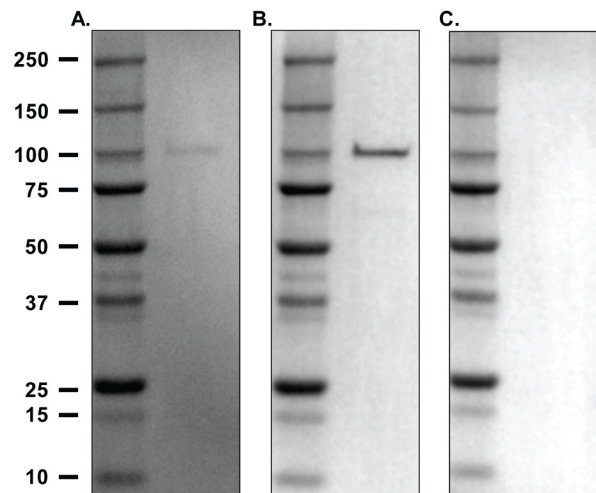
MW of 81.74 kDa, and pI of 5.75. In addition to similar MW and pI expected for the 110 kDa GP, all three candidates contain potential O-glycosylation sites for MLS128 recognition. In addition, two candidates, desmocollin and desmoglein, contain potential N-glycosylation sites which are known to be present in the 110 kDa GP [(*12*), Figure 6 in this article]. Desmocollin contains 12 potential MLS128 binding sites along with two N-glycosylation sites. Desmoglein contains 22 potential MLS128 binding sites and 3 N-glycosylation sites. Junction plakoglobin has 13 potential MLS128 binding sites but no N-glycosylation sites. Thus, we focused on desmocollin and desmoglein for the following studies.

3.2. *MLS128 and desmocollin antibodies bind a 110 kDa protein*

Western blotting analyses of LS180 cell lysates showed both MLS128 and desmocollin antibodies recognized a 110 kDa band (Figure 2A and 2B), whereas the desmoglein antibody did not (Figure 2C). These results suggested that desmocollin was the best candidate for the 110 kDa GP.

3.3. *MLS128 colocalizes with desmocollin in LS180 cells*

To determine if MLS128 and desmocollin antibodies colocalize, immunofluorescence microscopy was performed using LS180 colon cancer cells as well as breast cancer (MCF7) and normal mammary epithelial (MCF10A) cell lines. In LS180 cells, desmocollin was localized to the membrane and showed significant colocalization with the MLS128 antibody (Figure 3A upper panel and 3B). Desmoglein, however, showed more diffuse staining throughout the cytoplasm and had lower correlation for colocalization (Figure 3A lower panel and B). The breast cancer cell line MCF7 showed significantly lower levels of staining with anti-

desmocollin and anti-desmoglein compared to LS180 cells (Figure 3C). Interestingly, the normal breast epithelial line MCF10A showed no staining by anti-desmocollin, anti-desmoglein, or MLS128 antibodies (Figure 3D). Since desmosomes, together with adherens junctions, represent the major adhesive cell-junctions of epithelial cells, it was expected that human normal mammary epithelial MCF10A cells should be expressing desmosomal proteins. Why we did not detect desmosomal proteins may in fact be due to the cells being sparsely grown instead of tightly associated. It is notable that MCF7 but not MCF10A cells express MLS128 receptors, which is consistent to previous observations by Western blotting (*13*). Above-mentioned experiments showing colocalization of MLS128 and anti-desmocollin suggested that desmocollin may be the 110 kDa protein recognized by the MLS128 antibody or may be associated with this protein.

3.4. *Effect of desmocollin antibody or siRNA treatment on LS180 cell viability*

Previously, we reported LS180 cell growth is inhibited by MLS128 (*13*), therefore, we investigated if treatment with desmocollin or desmoglein antibodies also decreases LS180 cell growth. LS180 cells were treated with increasing concentrations of anti-desmocollin, anti-desmoglein, or MLS128 antibody for 48 hr. Crystal violet staining was used to determine cell viability. While all antibodies were cytotoxic, the desmocollin antibody was the most cytotoxic, displaying a 20-times lower IC_{50} compared to MLS128 and desmoglein antibodies (Figure 4).

To further investigate the role of desmocollin in LS180 cell viability, we transfected cells with two siRNAs targeting desmocollin (see Methods for sequence). Transfection was performed for 48 h and
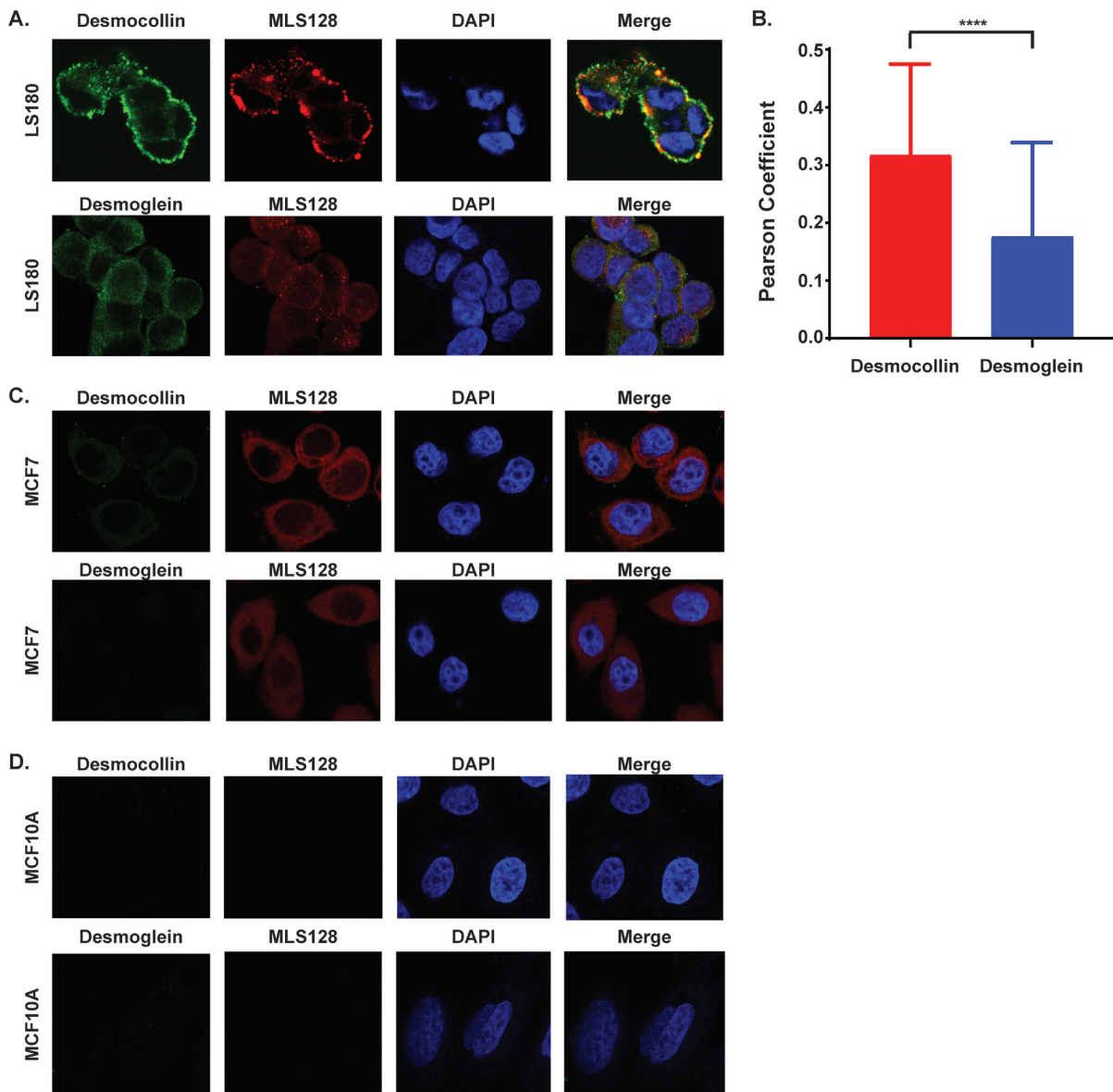
Figure 3. Immunofluorescence staining of LS180, MCF-7, and MCF-10A cells suggests colocalization of anti-desmocollin and MLS128 antibodies. Cells were stained with anti-desmocollin or anti-desmoglein (green) along with MLS128 (red) antibodies **(A, C, D)**. **(A)** shows the immunostaining of LS180 cells; **(B)** Colocalization was determined using Zen Blue software with calculation of Pearson correlation coefficients. Average coefficients and standard deviations were calculated from a minimum of 100 cells in three independent experiments **(C)** and **(D)** represent the immunostaining of MCF-7 and MCF-10A, respectively.
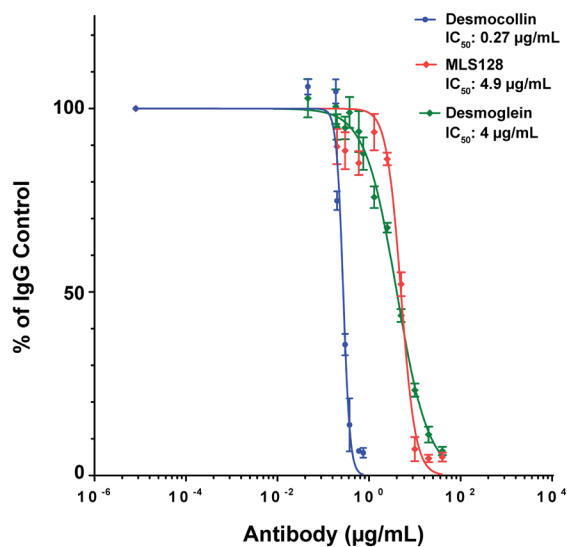
Figure 4. Treatment of anti-desmocollin, anti-desmoglein, and MLS128 antibodies on LS180 cell decreased viability. LS180 cells were treated with increasing amounts of each antibody and viability measured using crystal violet. Viability was normalized to treatment with an IgG control antibody.
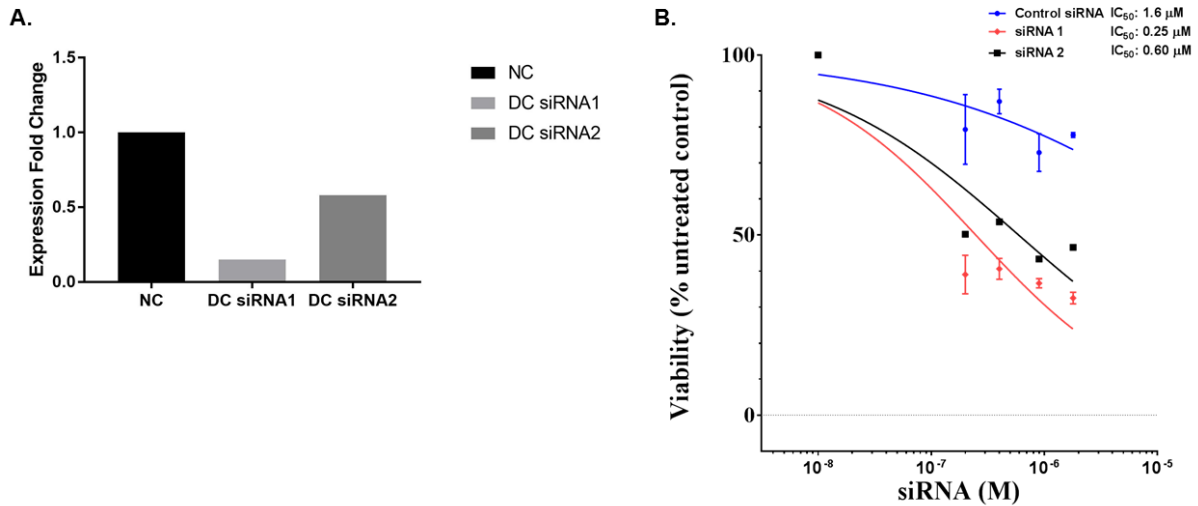
Figure 5. Desmocollin siRNA decreased LS180 cell viability. (A), LS180 cells were treated with control scramble or two different desmocollin siRNAs for 48 hr. A. siRNA1 and siRNA2 decreased desmocollin expression by ~90% and ~50%, respectively. **(B)**, LS180 cells were treated with increasing amounts of control or desmocollin siRNAs for 48 hr. Viability was determined using crystal violet and was normalized to untreated control.
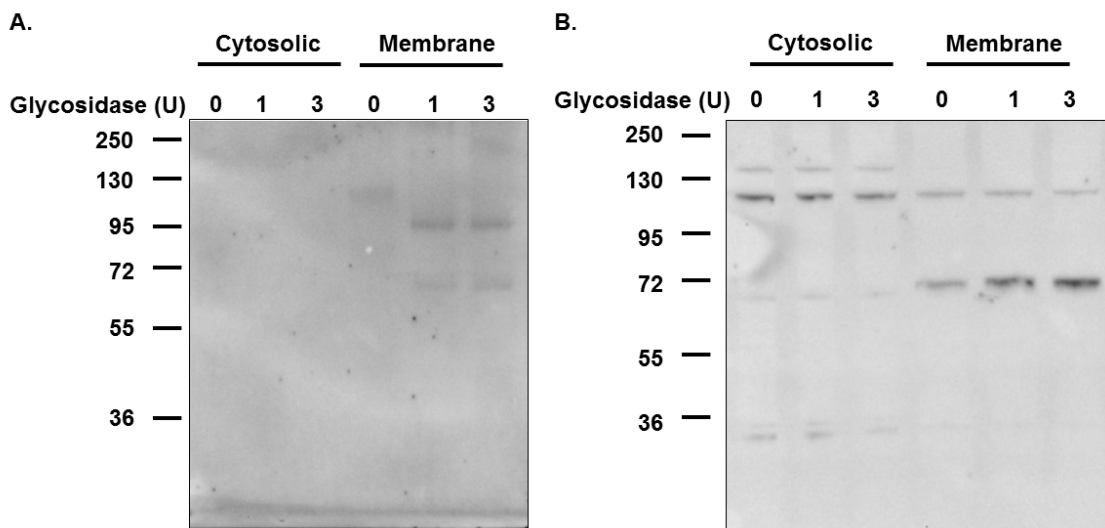

Figure 6. N-glycosidase F digestion and Western blotting analyses of cytosolic and membrane fractions of LS180 colon cancer cells suggest the presence of N-glycosylation in both the 110 kDa GP and desmocollin. Both fractions were treated with N-glycosidase F (0. 1, or 3 unit) and then analyzed by Western blotting with MLS 128 **(A)** or anti-desmocollin **(B)**.

then qPCR was used to quantify mRNA transcript levels. siRNA1 targeting desmocollin decreased mRNA levels by ~90% while siRNA2 only decreased levels by ~50% (Figure 5A). We next analyzed how the treatment with siRNAs affected cell viability. We found that cells transfected for 48 h with siRNA targeting desmocollin were less viable than those treated with scrambled siRNA. In accordance with the PCR data, siRNA1 had an IC_{50} value three times lower than siRNA2 (Figure 5B).

3.5. *The 110 kDa GP and desmocollin contain N-glycosylation modifications*

LS180 membrane and cytosolic fractions were digested with N-Glycosidase F followed by Western blotting with MLS128 or anti-desmocollin. A 110 kDa protein was recognized in the membrane fraction by both the MLS128 and desmocollin antibodies. Following glycosidase treatment, bands recognized by MLS128 decreased in size to ~95 and 70 kDa (Figure 6A); while the desmocollin antibody recognized two bands at 110 and 70 kDa (Figure 6B). Following N-glycosidase treatment, the 110 kDa band intensity decreased slightly while the 70 kDa band intensity increased (Figure 6B). This result suggested that the 110 kDa band recognized by both desmocollin and MLS128 antibodies may both have N-glycosylation sites, but whether the 110 kDa recognized by anti-desmocollin represents the same GP bound by MLS128 requires further study.
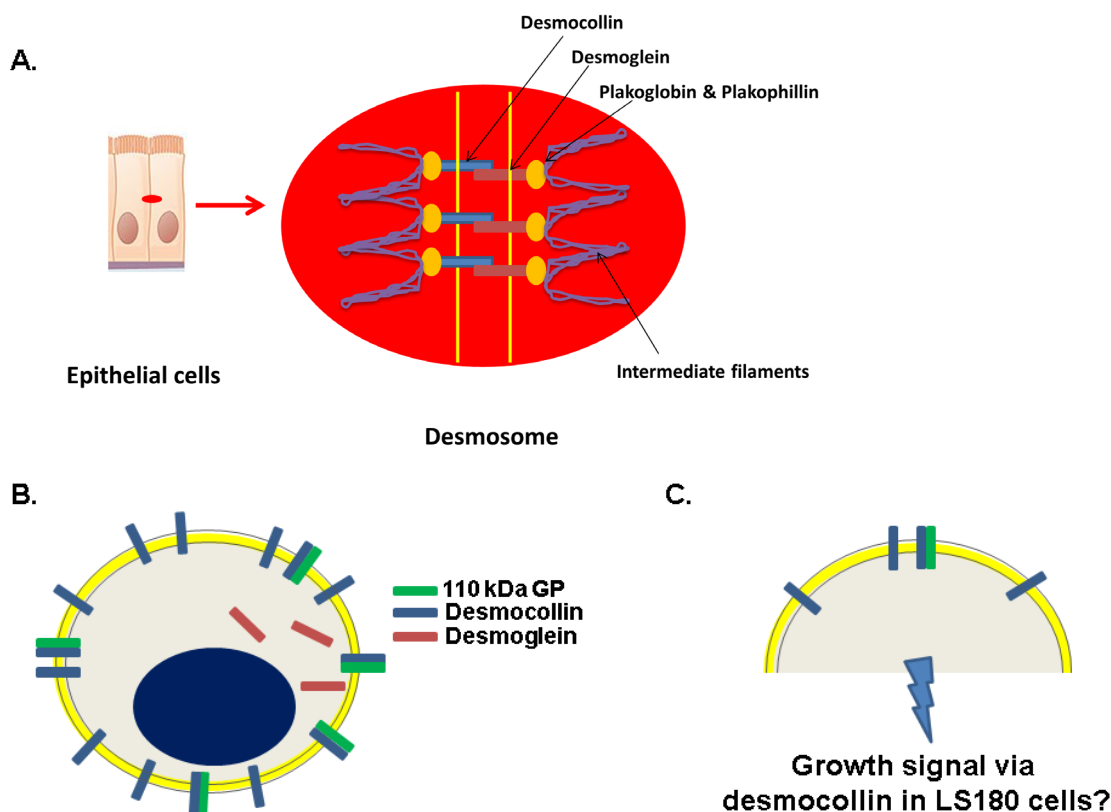
Figure 7. Potential roles of desmocollin and 110 kDa GP in LS180 colon cancer cell growth. (A), a desmosome structure in non-cancerous epithelial cells is illustrated. **(B)**, desmocollin (light blue), 110 kDa GP (green), and desmoglein (pale red) in LS180 cells are highlighted to reflect the results of this study. **(C)** illustrates our hypothesis that LS180 cell growth is mediated by desmocollin and 110 kDa GP. Other illustrations include desmosome (red), plakoglobin (orange), intermediate filaments (purple), nuclei (blue) and cell membranes (yellow).

4. Discussion

Western blotting of LS180 and HT29 colon cancer cells with MLS128 antibody clearly showed the detection of a 110 kDa protein (*12,14*). Purification of the 110 kDa GP from these cells, however, has been unsuccessful although our laboratory has extensive experience in affinity chromatography (*17*).

This study thus used a very sophisticated and sensitive mass spectrometry method that can detect extremely low peptide levels. Three desmosome proteins as illustrated in Figure 7A were identified using this method, and among these, desmocollin had characteristics similar to the 110 kDa GP. It has a molecular weight of ~110 kDa, contains putative Tn-antigen sites that can be recognized by MLS128, and contains N-glycosylation sites. Desmoglein was another candidate protein that also shared these characteristics, but the desmoglein antibody did not recognize a 110 kDa band in Western blot analysis (Figure 2C). Immunofluorescence microscopy of LS180 cells revealed that desmocollin was membrane-bound while desmoglein was localized to the cytosol (Figure 3A). This indicated random dispersion of desmosome components on the cell membrane and in the cytosol in LS180 colon cancer cells, whereas in normal cells

desmocollin and desmoglein hold epithelial cells in tight association (Figure 7B). Many of the membrane-bound desmocollin proteins were colocalized with the MLS128 binding molecules as clearly seen in the merged panel of Figure 3A. Statistical analysis of Pearson correlation coefficients revealed significant colocalization between desmocollin and MLS128 antibodies (Figure 3B).

We demonstrated that blocking desmocollin function by antibody or siRNA decreased cell viability, suggesting a critical role for desmocollin in LS180 cell growth and survival. Colocalization of MLS128 and desmocollin antibodies suggested either that some but not all desmocollin molecules contained Tn-antigens recognized by MLS128, or that the 110 kDa GP was associated with desmocollin. These results provide new insight into how MLS128 antibody inhibits colon cancer cell growth.

We previously reported that MLS128 binding to its receptor on LS180 cells caused downregulation of the IGF-I receptor, involved in cell growth and survival, and that both 110 kDa GP and IGF-I receptor were colocalized in microdomains (*14*). The previous study thus suggested that MLS128 treatment resulted in modulation of the signaling molecules localized in the microdomains. New findings from this study suggest that growth inhibition by MLS128 antibody treatment

is likely to be mediated by desmocollin (Figure 7C). It is plausible to state that although desmocollin functions in keeping epithelial cells adhered, in colon cancer cells it plays another important role in promoting cell growth and survival. Consistent with our hypothesis, previous studies by other groups supported that loss of desmosome adhesion is a prerequisite for the epithelial-mesenchymal transition, implicating epithelial cell invasion and metastasis (*18*), and that modulation in expression levels of the three desmocollin subtypes could play an important role in colorectal cancer (*19*).

In summary, our goal to identify the 110 kDa GP has not been completed. It is possible that we may have missed 110 kDa GP-derived peptides for the following reason. Since heavy O- and N-glycosylations are anticipated, the enzyme-specific cleavage sites of 110 kDa GP may not be accessible to digestion by Trypsin/Lys-C, resulting in no elution of its peptides from the 2-D gels. Alternatively, glycosylated peptides may not produce sufficient sequence information in the CID MS/MS experiments conducted. This study, however, provides an attractive working hypothesis that growth inhibition induced by MLS128 antibody is mediated via the 110 kDa GP/desmocollin dimer or association of the two molecules in microdomains.

Acknowledgements

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