Original Article

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Effects of two monoclonal antibodies, MLS128 against Tn-antigen and 1H7 against insulin-like growth factor-I receptor, on the growth of colon cancer cells

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Summary **MLS128 is an anti-carbohydrate monoclonal antibody (mAb) that binds three or two consecutive Tn-antigens. MLS128 bound 110-210 kDa glycoproteins (GPs) and inhibited the growth of LS180 and HT29 colon and MCF-7 breast cancer cells. One possible mechanism of MLS128's inhibition of growth may be *via* insulin-like growth factor-I receptor (IGF-IR) down-regulation (*Morita et al. BioSci Trends. 2009; 3:32-37*). The current study examined the role of IGF-IR signaling in the growth of colon cancer cells and its possible interaction with MLS128-induced inhibition of cell growth in LS180, LS174T, and HT29 human colon cancer cells treated with MLS128 or anti-IGF-IR 1H7. Both MLS128 and 1H7 treatment significantly inhibited the growth of colon cancer cells. All three colon cancer cell lines expressed IGF-IR. Their growth was in part IGF-I dependent, but inhibition by MLS128 was independent of IGF-IR signaling. All of the colon cancer cell lines expressed an 110 kDa GP for MLS128 binding, but MCF-7 cells expressed MLS128-detectable bands with higher molecular masses. 1H7 treatments caused down-regulation of IGF-IR but did not affect 110 kDa GP levels. MLS128 treatments resulted in partial disappearance of the 110 kDa band but did not affect IGF-IR levels. Western blotting analyses of colon and breast cancer cell lysates revealed that colon and breast cancer cells differed significantly in patterns of expression of growth-related molecules while colon cancer cells were similar but distinctive. In conclusion, MLS128 inhibited the growth of colon cancer cells by binding to the 110 kDa GP receptor. Inhibition of growth by MLS128 did not appear to affect IGF-IR signaling and instead only affected other growth signaling pathways.**

Keywords: Colon cancer, mechanisms of growth inhibition, anti-Tn antigen, anti-IGF-I receptor

1. Introduction

MLS128 is a Tn-antigen specific monoclonal antibody (mAb) (*1*) that binds carbohydrate epitopes consisting of three or two consecutive Tn-antigens (*2-4*). A previous study showed that MLS128 bound 110-210 kDa glycoproteins on cell membranes and inhibited the growth of LS180 and HT29 colon cancer cells as well as MCF-7 breast cancer cells (*5*). One possible mechanism of the inhibition of growth by MLS128 may be *via* insulin-like growth factor-I receptor (IGF-IR) down-regulation (*5*). Thus, the current study focused on the role of IGF-IR signaling in the growth of colon cancer cells and its possible interaction with MLS128-induced inhibition of cancer cell growth.

IGF-IR signaling is known to play an important role in proliferation, anti-apoptosis, and differentiation (*6,7*). IGF-I and -II bind to IGF-IR with a high affinity and thus activate IGF-IR tyrosine-kinase, which in turn stimulates downstream signaling cascades. Increased IGF-IR signaling has been reported to contribute to cancer cell growth and development (*8,9*). IGF-IR is thus an important target for cancer treatment (*10*). In

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order to develop cancer therapeutics, one of the current authors (Y. F.-Y.) previously constructed a single-chain variable fragment (scFv) from the variable domains of 1H7 mAb (*11*), produced it as a chimeric scFv-Fc consisting of anti-IGF-IR mouse scFv and human IgG1 Fc domain, and showed that it inhibited MCF-7 tumor growth (*12*). Inhibition of MCF-7 and T61 breast tumor growth by the scFv-Fc *in vivo* was caused by down-regulation of IGF-IR (*13,14*). Subsequent research suggested that down-regulation due to internalization and degradation is a major mechanism by which anti-IGF-IR antibodies inhibit cell growth (*15-17*).

One cannot reasonably assume, however, that the growth of colon cancer cells depends solely on IGF-IR signaling. In fact, the current study revealed that addition of anti-IGF-IR mAb 1H7 significantly inhibited cell growth of all colon cancer cell lines studied but that this inhibition was only approximately 60%. Individual cancer cells are likely to be equipped with diverse growth signaling pathways, and one or two particular signaling pathways may play a major role in growth of a particular cancer cell line. Expression of several possible growth-related surface molecules has thus been measured in three colon cancer cell lines to ascertain different mechanisms that might play a role in the growth of colon cancer cells. Western blotting analyses of colon and breast cancer cell lysates revealed that colon and breast cancer cells differ significantly with regard to their patterns of expression of growth-related molecules. Although three colon cancer cell lines expressed molecules in similar patterns, all three were distinctive. In summary, this study demonstrated that 1H7 and MLS128 inhibited the growth of colon cancer cells. The 110 kDa GP has been identified as the MLS128 binding receptor in colon cancer cells. Colon cancer cells were in part IGF-IR signaling-dependent. Inhibition of growth by MLS128 does not, however, apparently depend on IGF-IR signaling, suggesting that MLS128 likely inhibits the growth of colon cancer cells by affecting other growth signaling pathways.

2. Materials and Methods

2.1. *Materials*

Production and characterization of MLS128 were previously described (*1-4*). LS180, LS174T, and HT-29 human colon adenocarcinoma cells as well as MCF-7 human breast carcinoma cells were obtained from the American Tissue Type Culture Collection. Rabbit anti-IGF-IRβ, anti-EGFR mAb, rabbit anti-phospho-p44/42 MAPK (Thr202/Tyr204), rabbit anti-phospho-Akt (ser473) (193H12), rabbit anti-p44/42 MAPK, rabbit anti-CD44, anti-phosphotyrosine mAb, and rabbit anti-β actin were purchased from Cell Signaling Technology (Beverly, MA, USA). Rabbit anti-c-Met and anti-E-cadherin antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-rabbit or -mouse secondary antibody labeled with biotin was from Kirkegaard & Perry Lab. (Gaithersburg, MD, USA). IGF-I was obtained from Sigma-Aldrich Biotechnology (St. Louis, MO, USA). Cell culture media (DMEM and McCoy's 5A) were purchased from Invitrogen (Carlsbad, CA, USA).

2.2. *Cell culture*

LS180 and LS174T cells were cultured in DMEM containing 10% fetal bovine serum (FBS) supplemented with 4.5 mg/mL D-glucose and 110 μg/mL pyruvic acid. HT29 cells were cultured in McCoy's 5A containing 10% FBS. MCF-7 cells were cultured in IMEM (GIBCO, Gland Island, NY, USA) containing 5% FBS and 11.25 nM insulin. All culture media included 1% Penicillin-Streptomycin solution (Sigma-Aldrich).

2.3. *Effects of 1H7 or MLS128 on the growth of colon cancer cells*

Cells (1×10^4) were plated in wells of a 96-well plate and cultured in 100 μL of respective media containing 10% FBS for 24 h. Attached cells were then washed twice with PBS and cultured in 100 μL of media containing 1% FBS in the presence or absence of MLS128 (25 μg/mL) or 1H7 (0.36 μg/mL). After culturing for 72 h, cells were collected from each well by treatment with Trypsin-EDTA (Sigma-Aldrich) for 10 min followed by centrifugation at 200 × g for 10 min. Cell pellets were suspended in 0.04% Trypan blue (Sigma-Aldrich). Live cell numbers were counted using a hemacytometer. Quadruple wells were prepared for each data point.

2.4. *Effects of IGF on the growth of colon cancer cells*

To measure the effects of IGF-I on the growth of colon cancer cells, cells (5×10^3) were plated in wells of a 96-well plate and cultured in 100 μL of respective medium containing 10% FBS for 24 h. Attached cells were washed twice with PBS and serum-deprived for 24 h in regular growth media containing 0.1 % BSA instead of FBS (SFM). Media were replaced with SFM containing 0, 0.76, or 76 ng/mL of IGF-I. After culturing for 24, 48, and 72 h, cell growth was determined using a CCK-8 cell counting kit (Dojindo, Kumamoto, Japan) in accordance with the manufacturer's instructions. Absorbance at 450 nm was measured with a plate reader (Bio-Rad, Hercules, CA, USA). Quadruple wells were prepared for each data point.

2.5. *Western blotting analyses of various growth-related proteins in colon and breast cancer cells*

LS180, LS174T, and HT-29 colon cancer cells as well

as MCF-7 breast cancer cells were cultured in their respective media. Cells were collected by scraping, followed by centrifugation at 200 × g for 5 min, and then solubilized in 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 (lysis buffer) on ice for 15 min. Supernatants were obtained from solubilized cells by centrifugation at 17,000 × g for 10 min. Protein concentrations were measured using the Bradford method. The solubilized proteins (5 and 25 μg) from each cell line were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to PVDF membranes. The membrane was blocked with 3% BSA in 50 mM Tris-HCl buffer, pH 7.4, containing 0.15 M NaCl and 1% Tween 20 (TBST) for 1 h at room temperature. Western blotting was carried out with anti-IGF-IR, MLS128, anti-EGFR, anti-c-Met, anti-E-cadherin, and anti-CD44 antibodies as primary antibodies. Bound primary antibodies were then detected with biotin-labeled secondary antibodies using the Vectastain ABC-Amp kit and an alkaline phosphatase kit (Vector Laboratories, Inc. Burlingame, CA, USA). Alternatively, bound primary antibodies were detected with HRP-conjugated secondary antibodies and color development using Ez West blue (ATTO Co., Tokyo, Japan). The blots were analyzed using NIH Image 1.63 Analysis system (Research Service Branch, the National Institute of Mental Health).

2.6. *Effects of 1H7 or MLS128 treatment on IGF-IR and 110 kDa GP levels in colon cancer cell lines according to Western blotting analyses*

LS180, LS174T, and HT29 colon cancer cells (8×10^5) were cultured in respective media containing 10% FBS for 24 h in wells of 6-well plates. Cells were then cultured in the respective media containing 1% FBS in the presence or absence of MLS128 (25 μg/mL) or 1H7 (0.36 μg/mL). After culturing for 24, 48, and 72 h, cells were collected and solubilized in 50 μL of the lysis buffer as described above. The solubilized proteins (2 μg per lane) were separated by SDS-PAGE and transferred to PVDF membranes. The membranes were blocked with 3% BSA in TBST for 1 h at room temperature. After incubation with primary antibodies against 110 kDa GP (MLS128), IGF-IR β subunit, and β-actin for 16 h, bound primary antibodies were detected as described above.

2.7. *Effects of MLS128 on IGF-IR signaling in colon cancer cells*

Cells (8×10^5) were plated in wells of 6-well plate and cultured in respective medium containing 10% FBS for 24 h. Cells were then cultured in SFM containing 0.1% BSA or 25 μg/mL of MLS128. Fifty ng/mL of IGF-I was added to MLS128-treated and non-treated cells. After incubation for 5 or 10 min, cells were collected by centrifugation and subjected to solubilization in 50 μL of the lysis buffer for 15 min, followed by centrifugation at 17,000 × g for 10 min. Recovered supernatants containing solubilized proteins were assayed for protein concentrations. The solubilized proteins (5 μg per lane) were separated by SDS-PAGE and subjected to Western blotting as described above. Primary antibodies against p-Tyr, p-MAPK, and p-Akt were used for detection of IRS-1, MAPK, and Akt phosphorylation, respectively. After incubation at 4°C overnight, bound primary antibodies were detected using biotin-conjugated secondary antibodies and the Vectastain ABC-Amp kit (Vector Laboratories, Inc. Burlingame, CA, USA).

2.8. *Statistical analyses*

Levels of cell growth and Western blotted protein bands were expressed as means ± S.E. from 3 or more experiments. An unpaired Student's *t*-test was used to compare the growth or intensity of the bands in two groups of experiments performed in the absence and presence of mAb.

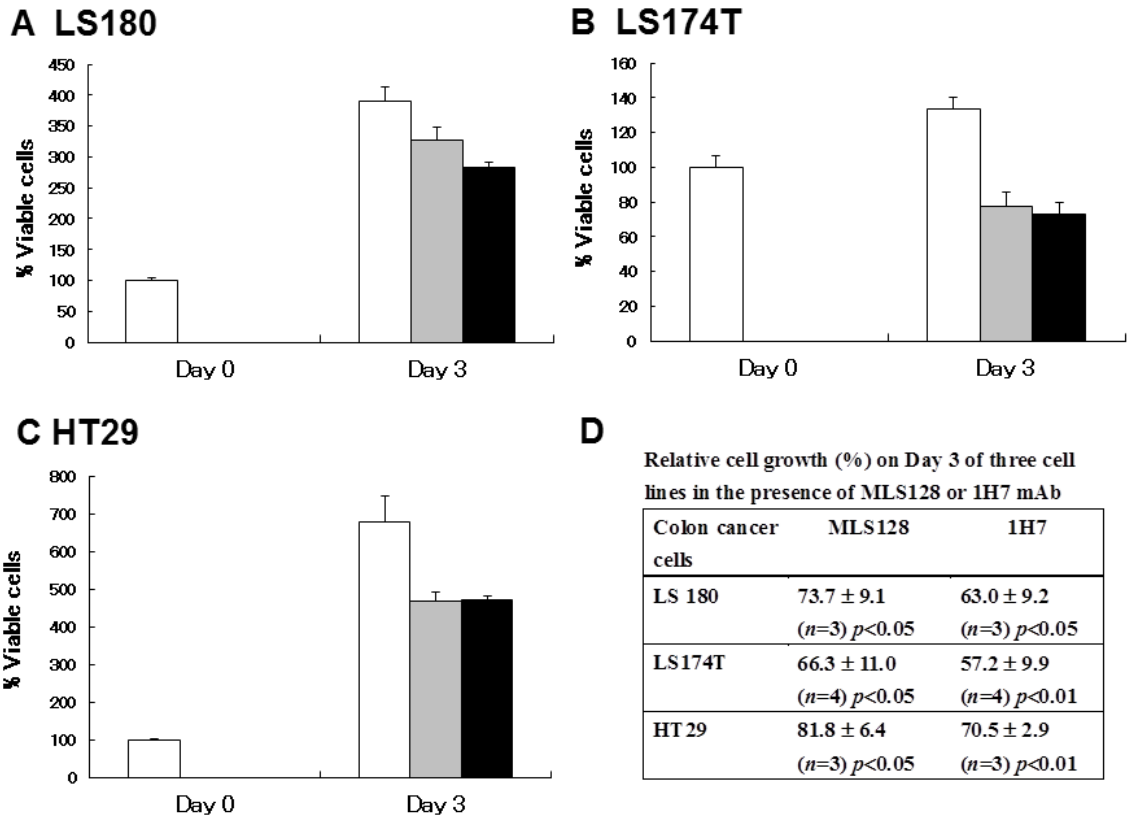
3. Results

3.1. *Addition of MLS128 or 1H7 inhibits growth of three colon cancer cell lines*

A previous study found that MLS128 inhibited the growth of LS180 and HT29 colon cancer cells as well as MCF-7 breast cancer cells (*5*). Thus, the current study compared the inhibition of growth of three colon cancer cell lines, including an additional LS174T colon cancer cell line. Typical results for inhibition of the growth of LS180, LS174T, and HT29 colon cancer cells after 3 days of mAb treatment are shown in Figure 1A, B, and C, respectively. The mean ± S.E. from 3 or 4 experiments with each cancer cell line is summarized in Figure 1D.

MLS128 treatment of LS180, LS174T, and HT29 colon cancer cells for 3 days significantly inhibited cell growth (Figure 1 A, B, and C, respectively). Results confirmed previous findings that MLS128 inhibited on inhibition the growth of LS180 and HT29 cells (*5*). In addition, inhibition of growth was also noted in the LS174T cell line, which is a variant of LS180 that had been maintained with using trypsin in accordance with a subculture protocol (*18*). As shown in Figure 1D, 3 days of MLS128 treatment inhibited the growth of LS180, LS174T, and HT29 colon cancer cells by an average of 26%, 34%, and 18%, respectively.

Anti-IGF-IR 1H7 significantly inhibited the growth of LS180, LS174T, and HT29 colon cancer cell lines by 37%, 43%, and 30%, respectively (Figure 1D). An interesting finding is that monolayer growth of colon cancer cells was significantly inhibited by 1H7. The



D

Relative cell growth (%) on Day 3 of three cell lines in the presence of MLS128 or 1H7 mAb

Colon cancer cells	MLS128	1H7
LS 180	73.7 ± 9.1 (n=3) p<0.05	63.0 ± 9.2 (n=3) p<0.05
LS174T	66.3 ± 11.0 (n=4) p<0.05	57.2 ± 9.9 (n=4) p<0.01
HT29	81.8 ± 6.4 (n=3) p<0.05	70.5 ± 2.9 (n=3) p<0.01

Figure 1. Addition of MLS128 or 1H7 inhibits growth of three colon cancer cell lines. Inhibition of growth of three colon cancer cell lines by MLS128 (grey bars) along with anti-IGF-IR 1H7 (black bars) was compared to the control (white bars). Experiments were performed as described in the Methods. Typical results for growth of LS180, LS174T, and HT29 colon cancer cells after 3 days of mAb treatment are shown in **A**, **B**, and **C**, respectively. The relative growth (% control), average ± S.E. from 3 or 4 experiments for each cancer cell line, is summarized in **D**.

same was not true for monolayer MCF-7 cell growth, which was not significantly inhibited in the presence of 1H7 scFv-Fc (*13*). The situation differs with breast cancer: 1H7 scFv-Fc markedly inhibited anchorage-dependent MCF-7 cell growth *in vitro*, and MSF-7 and T61 breast cancer tumor growth *in vivo via* down-regulation of IGF-IR (*13,14*).

3.2. *Growth of colon cancer cells is IGF-dependent*

LS180 (A), LS174T (B), or HT29 (C) cell growth was observed under serum-free conditions in the absence or presence of IGF-I. The results shown in Figure 2 indicate that the growth of three colon cancer cell lines is in part IGF-I-dependent. These results are consistent with the finding that anti-IGF-IR 1H7 inhibited the growth of all three colon cancer cell lines (Figure 1).

3.3. *Effects of MLS128 on IGF-IR signaling in three colon cancer cell lines*

The effects of MLS128 on IGF-IR signaling were examined in three colon cancer cell lines. MLS128 did not affect the downstream signaling stimulated by IGF-I as determined by immunoblotting of p-Tyr, p-MAPK, and p-Akt (Figure 3). These results suggest that MLS128's inhibition of the growth of colon cancer cells is independent of IGF-IR signaling. While the effects of MLS128 on IGF-IR signaling were not obvious, MLS128 may have different effects on basal phosphorylation of MAPK and Akt, in that MLS128 inhibited MAPK and Akt phosphorylation in LS180 cells, by 0.67 ± 0.04 (n = 3)- and 0.61 ± 0.22 (n = 3)-fold, respectively, but it activated IRS-1 and MAPK phosphorylation in HT29 cells by 1.21 ± 0.08 (n = 3)- and 1.25 ± 0.05 (n = 3)-fold, respectively. These results indicate that MLS128 affects colon cancer cell signaling *via* as yet-unidentified signaling pathways other than the IGF-IR signaling pathway.

3.4. *Effects of 1H7 or MLS128 treatment on IGF-IR levels in colon cancer cell lines*

Since previous studies suggested that MLS128 inhibits LS180 cell growth *via* down-regulation of the IGF-IR (*5*), the amounts of IGF-IR in three colon cell lines after 1H7 or MLS128 treatment were determined by immunoblotting (IB). After treatment with MLS128 or

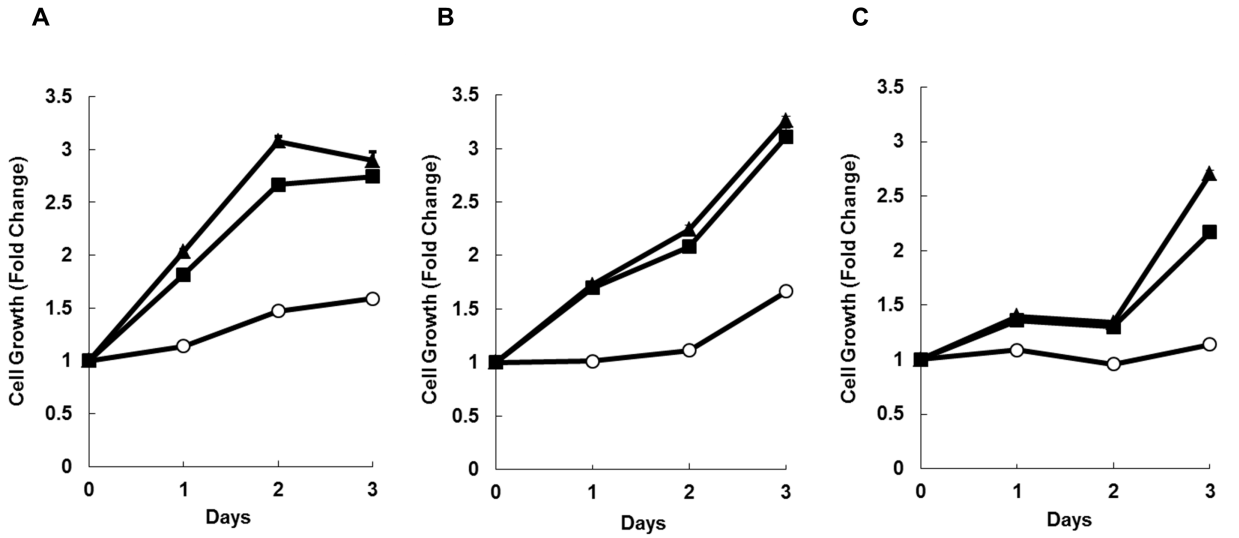


Figure 2. Growth of colon cancer cells is IGF-dependent. LS180, LS174T, or HT29 cell growth was observed under serum-free conditions in the absence (○) or presence of 0.76 (■) or 76 ng/mL (▲) of IGF-I for 3 days as described in the Methods. Typical results from 2-4 experiments are shown.

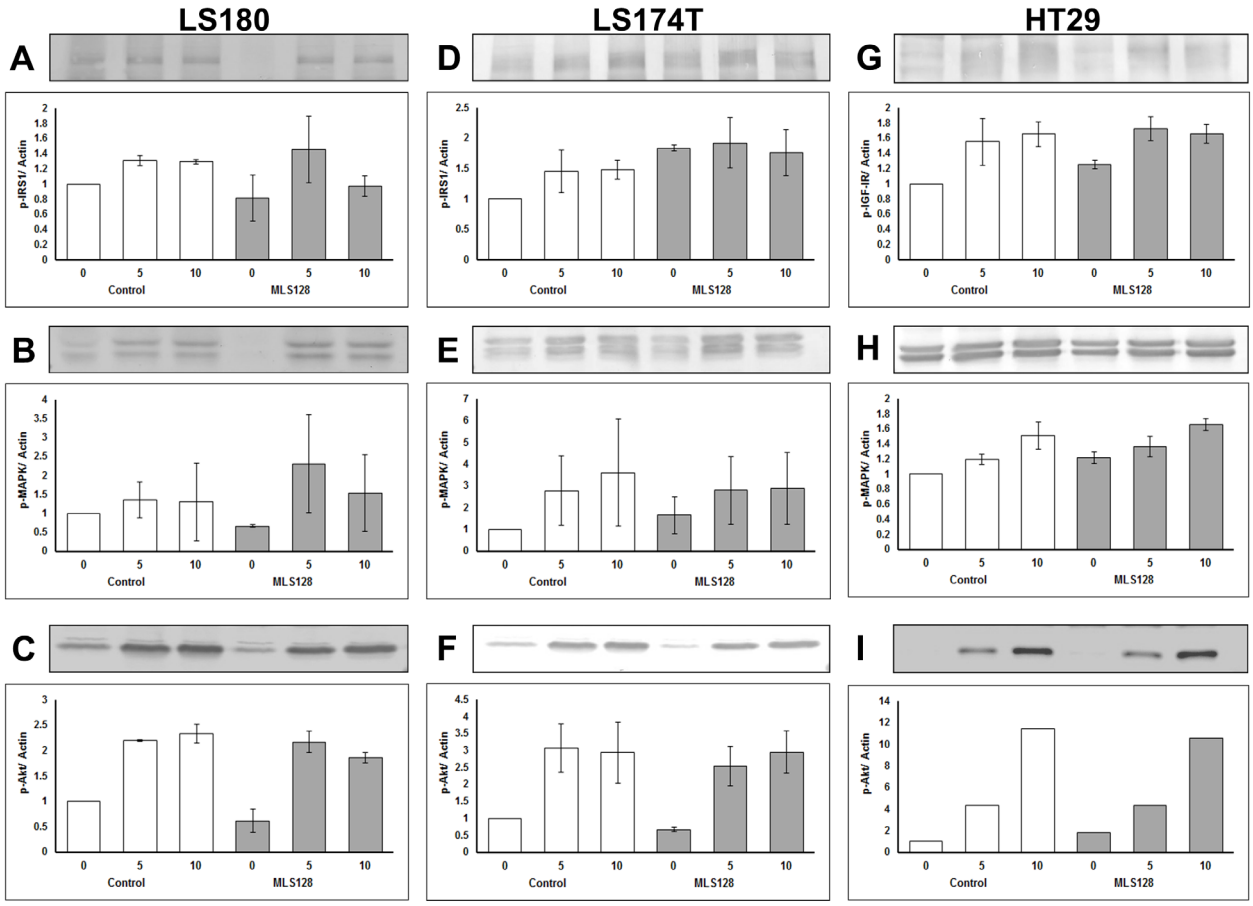


Figure 3. Effects of MLS128 on IGF-IR signaling in three colon cancer cell lines. Effects of MLS128 on IGF-IR signaling in three colon cancer cell lines were examined as described in the Methods. Briefly, 50 ng/mL of IGF-I was added to cells cultured in SFM containing 0.1% BSA (white bars) or 25 μg/mL of MLS128 (grey bars). After incubation for 5 or 10 min, cells were subjected to solubilization. The solubilized proteins (5 μg per lane) were separated by SDS-PAGE and subjected to Western blotting using primary antibodies against p-Tyr (**A**, **D**, and **G**), p-MAPK (**B**, **E**, and **H**), and p-Akt (**C**, **F**, and **I**) for detection of IRS-1, MAPK, and Akt phosphorylation, respectively. Typical immunoblots (upper panel) and average ± S.E. (n = 2-4) (lower panel) of LS180 (**A**-**C**), LS174T (**D**-**F**), and HT29 (**G**-**I**) cells are shown.

1H7 for 1, 2, and 3 days, cells were solubilized. Cell lysates were immunoblotted with anti-IGF-IR β subunit antibody to ascertain whether or not down-regulation of the IGF-IR molecules occurred in the three colon cancer cell lines after each mAb treatment.

Down-regulation of IGF-IR by 1H7 was clearly evident in all three colon cancer cell lines (Figure 4A, C, and E in the left panel). Among the three cell lines, the most significant IGF-IR down-regulation by 1H7 was noted in HT29 cells (Figure 4E, black bars). These results are consistent with the hypothesis that 1H7-induced IGF-IR down-regulation plays a role - at least in part - in inhibiting the growth of colon cancer cells.

As shown in Figure 4A, C, and E, MLS128 treatment of three colon cancer cell lines resulted in only slight down-regulation of IGF-IR in HT29 cells on day 2 and 3 (Figure 4E, grey bars), but the extent of this down-regulation paled incomparison to that induced by 1H7 (Figure 4, left panel). MLS128 treatment of LS180 cells did not cause a reduction in IGF-IR as was previously reported (*5*). Thus, the results of the current study clashed with those of a previous study indicating that MLS128 down-regulated IGF-IR in 180 cells (*5*).

3.5. *Effects of MLS128 treatment on 110 kDa GP levels in colon cancer cell lines*

The right panel in Figure 4 shows the MLS128 IB of cell lysates prepared from LS180, LS174T, and HT29 cells after treatment with MLS128 or 1H7 for 1, 2, and 3 days. Although disappearance of the 110 kDa GP band was not as apparent as was seen in IGF-IR IB, partial disappearance of the 110 kDa GP was noted in all three colon cancer cell lines when they were treated for 3 days with MLS128. Of the three colon cancer cell lines examined, however, HT29 cells treated with MLS128 had the most significant reduction in 110 kDa GP, suggesting that MLS128 binding to 110 kDa GP is possibly involved in inhibiting HT29 cell growth. In contrast, the 110 kDa GP in cells treated with 1H7 remained at the same levels as non-treated control cells (Figure 4, right panel), suggesting that 1H7-treatment had no effect on 110 kDa GP levels.

3.6. *Various levels of growth-related molecules are expressed in colon cancer cell lines*

Since previous studies by the current authors and others suggested that the growth of colon cancer cells may be mediated by IGF-IR, EGFR, and c-Met (*5,19-21*), expression of those proteins was measured in three colon cancer cell lines and control MCF-7 breast cancer cells. Western blotting analyses were carried out using two concentrations of colon or breast cancer cell lysates. Typical results of Western blots with antibodies against six molecules are shown in Figure 5.

Three colon cancer cell lines expressed the IGF-IR protein. LS174T and HT29 cells expressed the protein at a level similar to that found in MCF-7 breast cancer cells but the level of expression in LS180 cells appeared to be lower than that in other cell lines (Figure 5A). When blotted with MLS128, all three colon cancer cell lines had similar levels of 110 kDa GP but 110 kDa GP was not detected in MCF-7 cells, which instead expressed MLS128-detectable bands with higher molecular masses (Figure 5B). The MLS128 IB of cell lysates in this study confirmed previous results for MLS128 IB when cell membranes were prepared from LS180, HT29, and MCF-7 cells (*5*). All three colon cancer cell lines contained similar levels of EGFR and c-Met but MCF-7 breast cancer cells almost no expression of EGFR and c-Met (Figure 5C and D). These results suggest that different signaling pathways are used to promote growth in colon and breast cancer cells.

In addition, IB experiments were carried out using anti-E-cadherin and CD44 antibodies since E-cadherin and CD44 were reported to have a size close to that of 110 kDa GP, which MLS128 binds to (*22,23*). Interestingly, LS180 cells did not express E-cadherin whereas two other colon cancer and MCF-7 breast cancer cell lines expressed similar levels of E-cadherin (Figure 4E). Although the three colon cancer cell lines had comparable patterns of anti-CD44 reactive bands corresponding to splice variants of > 80 kDa, LS180 cells appear to express higher levels of those molecules than LS174T and HT29 cells (Figure 4F). In contrast, MCF-7 breast cancer cells expressed almost no anti-CD44 reactive bands. The results of the IB experiments are summarized in Table 1, which clearly shows that (*i*) colon and breast cancer cells express vastly different sets of growth-related molecules, and (*ii*) the three colon cancer cell lines have somewhat similar but nonetheless distinctive patterns of these molecules.

4. Discussion

This study used three established colon cancer cell lines in an effort to reveal the potential roles of IGF-IR signaling in the growth of colon cancer cells and its possible interaction with MLS128-induced inhibition of growth. The three colon cancer cell lines expressed IGF-IR. Treatment of cells with 1H7 caused inhibition of their growth and down-regulation of IGF-IR. 1H7 inhibited cell growth by 30-40%, indicating that 60-70% of cell growth must be mediated by signaling pathways other than IGF-IR signaling. MLS128 did not affect the phosphorylation of downstream signaling molecules stimulated by IGF-I such as IRS-1, MAPK, and Akt. These results suggest that MLS128-induced inhibition of the growth of colon cancer cells is independent of IGF-IR signaling. Colorectal cancer is the world's third most common cancer, and further studies are needed

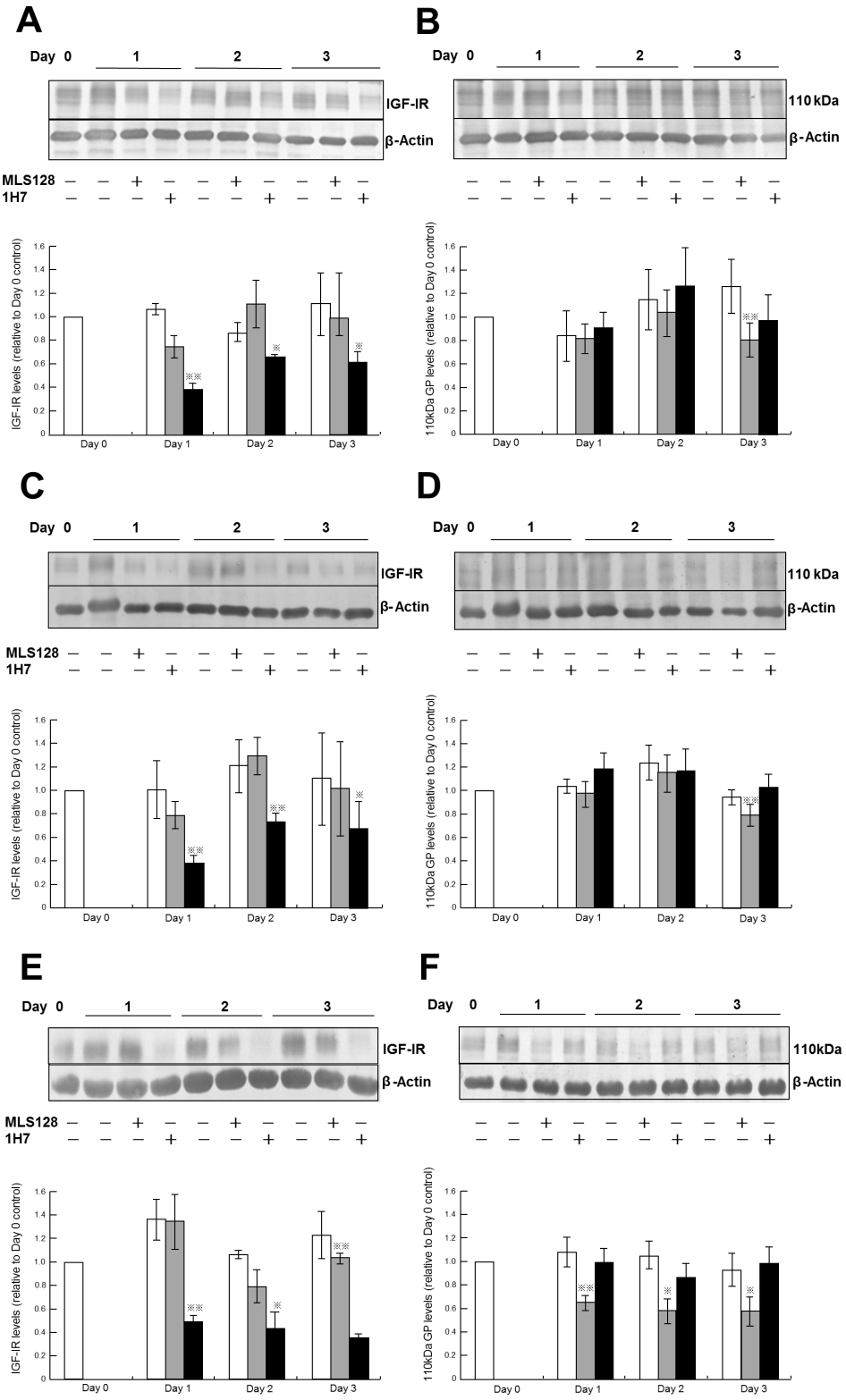


Figure 4. Effects of 1H7 or MLS128 treatment on cellular levels of IGF-IR (left panel) and 110 kDa GP (right panel). Experiments were carried out as described in the Methods, Briefly, LS180, LS174T, and HT29 colon cancer cells were cultured in respective media containing 1% FBS in the presence or absence of MLS128 (25 μg/mL) or 1H7 (0.36 μg/mL) for 24 (Day 1), 48 (Day 2) and 72 h (Day 3). Cell lysates (2 μg protein per lane) were subjected to Western blotting using primary antibodies, anti-IGF-IR β subunit, MLS128, and anti-β-actin and then subjected to color development using biotin-labeled secondary antibodies and the alkaline phosphatase kit. Shown are six panels (**A**~**F**), each of which consists of a typical immunoblot from 3-4 experiments (upper panel) and average values of IGF-IR or 110 kDa GP levels of 3 or 4 immunoblots (lower panel). The IGF-IR or 110 kDa levels were normalized to β-actin leveles. White bars represent controls, which are IGF-IR or 110 kDa GP levels from cells untreated with mAb. The grey and black bars represent IGF-IR levels (left panel) and 110 kDa GP levels (right panel) in cells treated with MLS128 and 1H7, respectively. Average values of these protein levels on Days 1, 2, and 3 were calculated with respect to Day 0. Average ± S.D. (n = 3-4) of IGF-IR (left panel) and 110 kDa GP (right panel) are shown for LS180 (**A** and **B**, respectively), LS174T (**C** and **D**, respectively), and HT29 (**E** and **F**, respectively). * $p < 0.05$; ** $p < 0.01$.

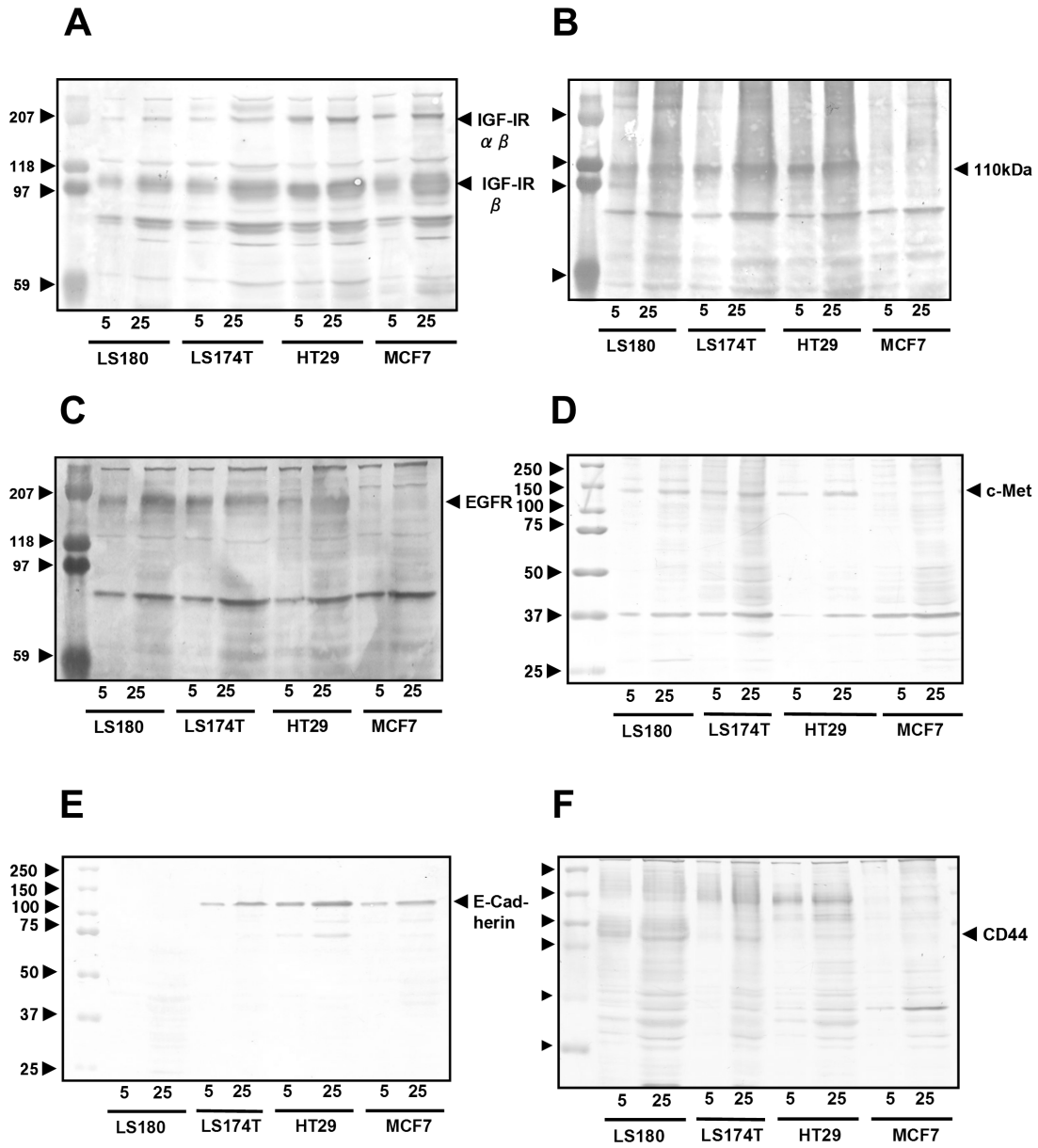


Figure 5. Different sets of growth-related molecules are expressed in colon and breast cancer cells. Expression of IGF-IR, MLS128 binding protein (110 kDa GP), and four other proteins was compared using Western blotting with 5 and 25 μg protein each of LS180, LS174T, HT29, and MCF7 cell lysates as described in the Methods. Primary antibodies used are anti-IGF-IR (**A**), MLS128 (**B**), anti-EGFR (**C**), anti-c-Met (**D**), anti-E-cadherin (**E**), and anti-CD44 (**F**). Bound primary antibodies were detected using biotin-labeled secondary antibodies for **A**, **B**, and **C**. Alternatively, bound primary antibodies were detected with HRP-conjugated secondary antibodies and color development using Ez West blue (**D**, **E**, and **F**). In contrast to relatively abundant proteins shown in A~C, proteins shown in **D**~**F** were not readily detected with biotin-labeled secondary antibodies due to the existence of non-specific 125, 75, and 73 kDa proteins functioning as endogenous biotin-containing enzymes in cells. Thus, an alternative method was used to detect sparse proteins (**D**, **E**, and **F**).

Table 1. Expression of growth-related cell surface molecules in LS180, LS174T, and HT29 colon cancer cells in comparison to MCF-7 breast cancer cells

Items	LS180	LS174T	HT29	MCF-7
IGF-IR	+	++	++	++
110 kDa GP	+	+	+	±
EGFR	+	+	+	±
c-Met	+	++	+	±
E-Cadherin	-	+	++	+
CD44	++	+	+	±

Levels of each protein are summarized from the results shown in Figure 5.

to determine the mechanisms by which its growth is inhibited. This study did not extensively examine levels of expression of growth-related molecules in colon cancers, but Figure 5 provides insight into growth signaling to each colon cancer cell line. Transcriptomes and/or proteomics could be used to examine other molecules and develop a profile of each colon cancer cell line. Such work could eventually provide a model for development of cancer therapeutics. As far as *in vivo* studies are concerned, a very recent genome-scale analysis of 276 human colon and rectal cancer samples

discovered amplification of IGF-II in addition to the expected mutations (*24*). This new finding is consistent with the results of the present study, which found that growth of colon cancer cells is IGF-dependent but only to a degree.

Western blotting analyses were carried out in an effort to narrow down other the growth signaling pathways that MLS128 may interact with (Figure 5). EGFR was expressed in all colon cancer cell lines, indicating that their growth is likely to be mediated by this receptor as well. LS180 cell growth was found to be EGF-dependent (Zamri *et al*. unpublished findings). c-Met signaling is reportedly involved in the growth of HT29 cells (*21,22*), c-Met expression in colon and breast cancer cells was determined using Western blotting. Three colon cancer cell lines expressed c-Met. These results suggest that growth of colon cancer cell lines is likely to be mediated by IGF-I, EGF, and HGF, the ligand for c-Met. Unlike the colon cancer cells, MCF-7 breast cancer cells did not express either EGFR or c-Met. The results described here are thus significant in that two colon cancer cell lines may use growth signaling similar to that in HT29 cells, which most signaling studies have thus far reported. However, their role in the growth of each cell line has to be carefully investigated since only three cell lines were compared but subtle differences were clearly noted. In fact, preliminary data indicate that the growth of LS180 cells was not stimulated by HGF (Oura *et al*. unpublished findings). More studies with established cell lines would shed light on the mechanisms of the growth of colon cancer cells and could eventually lead to the design and development of cancer therapeutics.

This study revealed one significant aspect of the nature of colon cancer cells. Namely, MLS128 binding to the 110 kD GP on colon cancer cell lines apparently resulted in inhibition of growth whereas MCF-7 cells expressed GPs with higher molecular masses as MLS128 binding proteins. In a previous study, those differences were revealed by Western blotting using membrane fractions (*5*). The current study used cell lysates for Western blotting and noted the same differences, thus suggesting that quantitative analyses of receptors and other signal molecules can be easily carried out using cell lysates instead of cell membranes. Given this, two sets of experiments were conducted as shown in Figures 4 and 5. Figure 4 shows that anti-IGF-IR antibody caused down-regulation, or more accurately speaking, degradation of the receptors in three colon cancer cell lines. In contrast, MLS128 did not down-regulate IGF-IR in colon cancer cell lines. This result rules out the original hypothesis that the down-regulation of IGF-IR is one possible mechanism by which MLS128 inhibits LS180 cell growth (*5*).

Treating three colon cancer cell lines with MLS128 for 3 days resulted in inhibition of their growth and obvious disappearance of the 110 kDa band as indicated by immunoblotting with MLS128. The disappearance of the110 kDa band was clearly evident in HT29 cells on Day 1, 2, and 3 after the mAb treatment but the disappearance of the 110 kDa band was seen only on Day 3 after the treatment of LS180 and 174T cells. The disappearance of the 110 kDa band could be explained by either the loss of the Tn-antigen epitopes on the protein backbone or degradation of the protein backbone by itself. Antibodies recognizing the protein backbone of 110 kDa GP are needed to identify whether protein degradation has occurred or Tn-antigen epitopes have been lost once colon cancer cells have been treated with MLS128. Identification of the 110 kDa GP is the next important step in producing antibodies against the 110 kDa GP and understanding MLS128's inhibition of cell growth.

In summary, growth of colon cancer cells depends in part on IGF-IR signaling as indicated by anti-IGF-IR mAb treatment inhibiting cell growth *via* IGF-IR down-regulation. The present study found that MLS128 bound specifically to 110 kDa GP in the three colon cancer cell lines examined and that IGF-IR signaling was not associated with MLS128's inhibition of the growth of LS180 cells. Results, however, suggested a possible link between IGF-IR- and 110 kDa GP-mediated growth signaling pathways in HT29 cells. The original working hypothesis regarding the interaction between two receptors has been tested further using HT29 colon cancer cells (manuscript in preparation).

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