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in Breast Cancer

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## INTRODUCTION

Muc4, also called sialomucin complex (SMC), the rat homolog of human MUC4, is a heterodimeric glycoprotein complex consisting of a peripheral O-glycosylated mucin subunit, ASGP-1, tightly but noncovalently linked to a N-glycosylated transmembrane subunit, ASGP-2. The complex is expressed in a number of normal, vulnerable epithelial tissues including mammary gland, uterus, colon, cornea, and trachea. Muc4/SMC is also overexpressed or aberrantly expressed on a number of human tumors, including breast tumors. Overexpression of Muc4/SMC has been shown to block cell-cell and cell-matrix interactions, protect tumor cells from immune surveillance, and promote metastasis. In addition, Muc4/SMC acts as a ligand for the receptor tyrosine kinase ErbB2/HER2. Thus, the receptor ErbB2/HER2 and the membrane mucin MUC4 are tightly associated at the cell surfaces on cells in which both are present. The hypothesis behind this proposal is that overexpression of the membrane mucin MUC4 in breast cancer cells reduces their response to the effects of the therapeutic monoclonal antibody Herceptin. Although the mechanism of action of Herceptin on breast cancer cells is still uncertain, its effects require that it bind to the receptor ErbB2/HER2 on the cells. Our model is that this association of the huge rigid MUC4 molecule with ErbB2/HER2 should drastically reduce access to the extracellular domain of the ErbB2/HER2, the region targeted by Herceptin. Thus, cells which express MUC4 should be more resistant to the effects of Herceptin than those which do not. We have recently shown that MUC4 is expressed in up to 70% of highly aggressive breast cancers, but less highly expressed in less aggressive tumors. The studies supported by this grant have tested the hypothesis that MUC4 can block Herceptin binding using tumor cell lines which express ErbB2/HER2 and which have been transfected with MUC4. The MUC4 gene in these cells was under the control of a tetracycline-regulatable promoter, which allows MUC4 expression to be turned on or off.

## BODY

### *Expression of Muc4/SMC in Human Breast Cancer*

Cancer progression can be associated with aberrant expression of glycoproteins on tumor cell surfaces. Muc4/SMC is overexpressed on the surface of the highly malignant metastatic 13762 rat mammary adenocarcinoma, with levels 100-fold higher than normal lactating mammary gland and 10,000-fold higher than normal virgin rat mammary gland (1,2). By immunohistochemistry and immunoblot analysis we have shown that MUC4 is expressed in a minority of solid breast tumors and is overexpressed in the majority of more aggressive tumor cells from effusions of breast cancer patients (3). To further investigate MUC4 expression in human breast cancer, we performed immunohistochemical staining of breast tumors from breast cancer patients. Paraffin-embedded infiltrating carcinoma specimens were tested for MUC4 expression by staining with anti-ASGP-1 monoclonal antibody 15H10. In these tumor specimens MUC4 stained throughout the ductal epithelium with more intense staining towards the luminal surface (data not shown). Moreover, there was strong staining of cells invading the lumen of the duct and the surrounding tissue. About 30% of these thirty-four tumor samples was strongly positive for MUC4 (Table I). As shown previously, breast cancer samples from breast cancer patient effusions showed an even higher level of positivity (Table I). To verify the staining of MUC4, we performed immunoblots on a strongly positive breast cancer sample compared to a

negative sample (Fig. 1). As positive controls, we show samples from the 13762 ascites cells and Muc4/SMC-transfected A375 cells grown with or without tetracycline to turn Muc4/SMC OFF or ON, respectively (Fig. 1). Taken together with our previous data, these observations suggest a role for Muc4/SMC in human breast tumor progression (3).

#### *Overexpression of Muc4/SMC inhibits antibody binding to ErbB2/HER2 on human melanoma cells*

Since Muc4/SMC and ErbB2/HER2 are overexpressed in a number of human breast tumors, we investigated whether overexpression of Muc4/SMC on human tumor cells would cause reduced antibody binding to ErbB2/HER2. We used A375 cells stably transfected with a tetracycline-responsive inducible expression system. Induction of Muc4/SMC expression in these cells leads to loss of cell-cell/cell-matrix adhesion, protection from immune killing and increased metastatic potential (3,4,5). One concern about ErbB2/HER2 antibody binding studies in cells that do or do not express Muc4/SMC is that overexpression of Muc4/SMC itself or the effects of Muc4/SMC overexpression may affect overall levels of ErbB2/HER2 and thus complicate the results. To determine if overexpression of Muc4/SMC has any effect on the expression levels of ErbB2/HER2, A375 cells were cultured for 72 hours in the presence or absence of tetracycline to repress or induce, respectively, Muc4/SMC expression. Cells were harvested, lysed, and 5 µg total cell protein was subjected to immunoblot analysis with anti-ASGP-2 and anti-ErbB2/HER2 antibodies. As expected, Muc4/SMC levels were undetectable in the presence of tetracycline, and in the absence of tetracycline, Muc4/SMC levels were very high. ErbB2/HER2 levels, on the other hand, were equivalent whether or not Muc4/SMC was expressed (Fig. 2A). Thus, overexpression of Muc4/SMC in the A375 cells does not affect ErbB2/HER2 levels.

To determine what effect overexpression of Muc4/SMC has on antibody binding to ErbB2/HER2, A375 cells were cultured as described above to induce or repress expression of Muc4/SMC. Cells were harvested in enzyme-free cell dissociation buffer and analyzed for ErbB2/HER2 by flow cytometry. The antibodies used for this analysis were Neomarkers antibody 2 (at 2 µg/ml) and Calbiochem antibody 5 (at 1 µg/ml), both anti-ErbB2/HER2 IgG1 antibodies directed against the extracellular domain of ErbB2/HER2. In the absence of Muc4/SMC, Neomarkers anti-ErbB2/HER2 antibody 2 stained approximately 45% of the cells, while Calbiochem anti-ErbB2/HER2 antibody 5 stained approximately 80% of the cells (Fig. 2B). When Muc4/SMC was overexpressed Neomarkers antibody 2 only stained approximately 15% of the cells, while Calbiochem antibody 5 only stained approximately 55% of the cells. This represents a 30 – 60% decrease in antibody binding to ErbB2/HER2. Since we have demonstrated that overexpression of Muc4/SMC has no effect on overall ErbB2/HER2 levels in these cells, these data suggest that overexpression of Muc4/SMC represses antibody binding to ErbB2/HER2.

An antibody titration was performed to investigate whether using more antibody would lead to more ErbB2/HER2 staining in the presence or absence of Muc4/SMC. A375 cells were cultured as described above and analyzed by flow cytometry with Neomarkers antibody 2 (at 4 µg/ml, 2 µg/ml, and 0.4 µg/ml dilutions) and Calbiochem antibody 5 (at 2 µg/ml and 0.2 µg/ml) as described above. With either antibody, the amount of ErbB2/HER2 staining increased when higher antibody concentrations were used for staining (Fig. 3). However, when Muc4/SMC was overexpressed, ErbB2/HER2 staining was always lower (by approximately 30-75%) than when

Muc4/SMC expression was low.

These studies were designed to address the question of the effect of Muc4/SMC overexpression on antibody-based tumor therapies such as Herceptin. Thus, A375 cells were cultured in the presence or absence of tetracycline for 72 hours. Cells were harvested in enzyme-free cell dissociation buffer and subjected to flow cytometry analysis using the humanized anti-ErbB2/HER2 antibody Herceptin. Three different antibody concentrations were used for this study – 100 µg/ml, 10 µg/ml, and 1 µg/ml final concentration. The highest concentration used here is the average patient serum concentration of Herceptin after 16 weeks of Herceptin therapy. The lowest concentration was chosen because this was the final concentration of the antibodies used for flow cytometry in the studies described above. When Muc4/SMC levels were low, almost 100% of the cells stained positively with any of the antibody concentrations used (Fig. 4). When Muc4/SMC was overexpressed, almost 100% stained when 100 µg/ml or 10 µg/ml Herceptin was used. However, when 1 µg/ml Herceptin was used for the analysis, only about 50% of the cells stained when Muc4/SMC was overexpressed. The final serum concentration of an antibody does not necessarily represent the final local concentration of the therapeutic antibody at the site of the tumor. Thus, these data suggest that overexpression of Muc4/SMC may block therapeutic antibody binding, providing a mechanism for resistance to these types of therapies.

*Effect of Muc4/SMC overexpression on antibody binding to ErbB2/HER2 on human breast cancer cells*

The A375 cells are human melanoma cells, not breast cancer cells, and Herceptin is currently only approved as a treatment for metastatic breast cancer. Thus, to determine if overexpression of Muc4/SMC blocks ErbB2/HER2 antibody binding on breast tumor cells, stably transfected MCF-7 cells were analyzed in a manner similar to that described for the A375 cells. As with the A375 cells, we first studied whether Muc4/SMC overexpression affects ErbB2/HER2 expression. Transfected MCF-7 cells were cultured in the presence or absence of tetracycline for 72 hours as described for the A375 cells. Cells were then lysed and 5 µg of total protein were subjected to immunoblot analysis with anti-ASGP-2 monoclonal antibody 4F12 or anti-ErbB2/HER2 monoclonal antibodies. As expected, in the presence of tetracycline, Muc4/SMC levels are undetectable, but in the absence of tetracycline, Muc4/SMC is induced to a very high level (Fig. 5A). MCF-7 cells express similar modest levels of ErbB2/HER2 whether or not Muc4/SMC is expressed (Fig. 5A). Thus, Muc4/SMC overexpression does not affect overall ErbB2/HER2 levels in human MCF-7 breast cancer cells.

To determine what effect Muc4/SMC overexpression has on Herceptin binding to ErbB2/HER2 on the surface of breast cancer cells, MCF-7 cells were cultured in the presence or absence of tetracycline for 72 hours as described above. Cells were harvested in enzyme-free cell dissociation buffer and analyzed by flow cytometry with Herceptin (at a 100, 10, or 1 µg/ml dilution). As with the A375 cells, there was reduced Herceptin binding when MCF-7 cells expressed high levels of Muc4/SMC. However, unlike the A375 cells, MCF-7 cells expressing high levels of Muc4/SMC showed a 25-40% reduction in Herceptin binding compared to MCF-7 cells not expressing Muc4/SMC, regardless of the concentration of Herceptin used for staining (Fig. 5B). These results suggest that for breast cancer cells, overexpression of Muc4/SMC may provide a block to antibody-based therapies even at the higher therapeutic doses.

### *Effect of antibody isotype on antibody binding to A375 Cells*

Overexpression of Muc4/SMC blocks cell-cell and cell-matrix interactions by non-specific steric hindrance (4). Part of this demonstration was that when Muc4/SMC was overexpressed, cell adhesion to a number of different ECM components was inhibited, and the degree of inhibition was dependent on the expression level of Muc4/SMC and the number of mucin repeats the Muc4/SMC molecules contained. So far we have demonstrated that Muc4/SMC overexpression inhibits IgG1 isotype antibody binding to ErbB2/HER2 only, and we have previously reported that Muc4/SMC and ErbB2/HER2 can form a complex. Thus, the inhibition of anti-ErbB2/HER2 antibody binding by Muc4/SMC overexpression may be from steric hindrance due to the formation of the Muc4/SMC-ErbB2/HER2 complex. To test this idea, we measured cell surface antibody binding with a different, unrelated antibody in the presence or absence of Muc4/SMC expression. A375 cells were cultured for 72 hours in the presence or absence of tetracycline as described. Cells were harvested and stained with either anti-Fas IgG or anti-Fas IgM isotype antibodies. When stained with larger, more bulky anti-Fas IgM antibodies, antibody binding is reduced by approximately 60% when Muc4/SMC is overexpressed (Fig. 6A). However, when stained with anti-Fas IgG antibodies, cell surface antibody binding is similar whether or not Muc4/SMC is expressed (Fig. 6B). These data suggest that inhibition of antibody binding is dependent on the class of antibody and suggest that inhibition of ErbB2/HER2 antibody binding may not be due entirely to nonspecific steric hindrance but instead from steric hindrance due to the formation of a Muc4/SMC-ErbB2/HER2 complex.

### *Co-immunoprecipitation of Muc4/SMC and ErbB-2 from A375 cells*

We have previously demonstrated that Muc4/SMC and ErbB-2 can form a complex in co-infected insect cells, normal mammary epithelial cells, and 13762 mammary tumor cells. To determine if there is some interaction between ErbB-2 and Muc4/SMC in the transfected A375 cells that may interfere with antibody binding to ErbB-2, a co-immunoprecipitation was performed. A375 cells expressing Muc4/SMC were lysed and immunoprecipitated with either anti-ASGP-2, anti-C-pep, or anti-ErbB-2 antibodies, and immunoprecipitates were subjected to immunoblot analysis with anti-ASGP-2 mAb 4F12. Muc4/SMC was readily detected in the anti-ErbB-2 immunoprecipitates but not in the non-immune rabbit serum control suggesting that Muc4/SMC and ErbB-2 form a complex in these cells (Fig. 7). The co-immunoprecipitation was also performed with transfected MCF7 cells expressing Muc4/SMC with similar results (data not shown). These data suggest that there is an interaction between Muc4/SMC and ErbB-2 that may interfere with antibody binding to ErbB-2.

### *Effect of capping on antibody binding to ErbB2/HER2 on A375 cells*

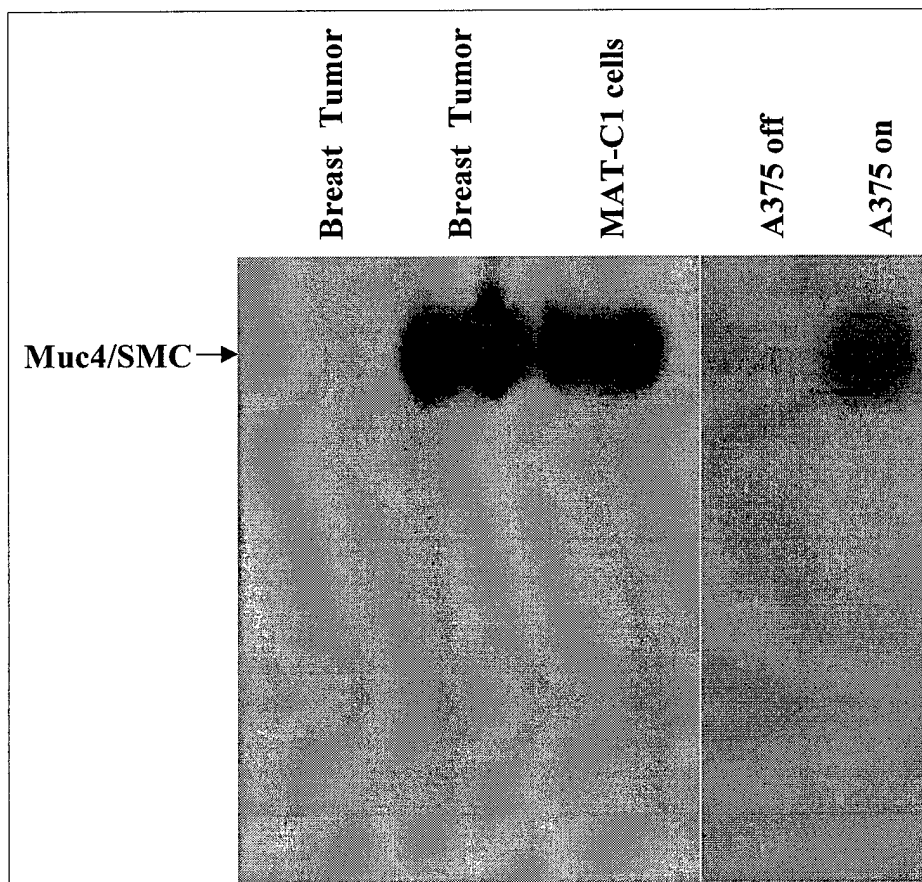
In previous studies we showed that capping of Muc4/SMC enhanced binding of antibody to intercellular adhesion molecule (ICAM), providing further evidence for non-specific steric hindrance as a mechanism for Muc4/SMC anti-adhesive effects (3). However, we envision that inhibition of anti-ErbB2/HER2 binding is due to specific steric hindrance from the formation of a Muc4/SMC-ErbB2/HER2 complex. Therefore, we performed a similar capping study. A375 cells cultured as described above were harvested in enzyme-free cell dissociation buffer and incubated with anti-ASGP-2 antibodies for thirty minutes to induce capping of the Muc4/SMC. Cells were then stained with anti-ErbB2/HER2 and analyzed by flow cytometry as described

above. As expected, when Muc4/SMC expression is low, capping has no effect on ErbB2/HER2 staining (Fig. 8). However, when Muc4/SMC is overexpressed, capping actually reduces antibody binding to ErbB2/HER2. If Muc4/SMC and ErbB2/HER2 were not in a complex, capping Muc4/SMC should relieve the block in antibody binding to ErbB2/HER2. The fact that capping of Muc4/SMC caused a greater block to anti-ErbB2/HER2 antibody binding is consistent with the idea that formation of a complex between Muc4/SMC and ErbB2/HER2 provides specific steric hindrance to antibody binding.

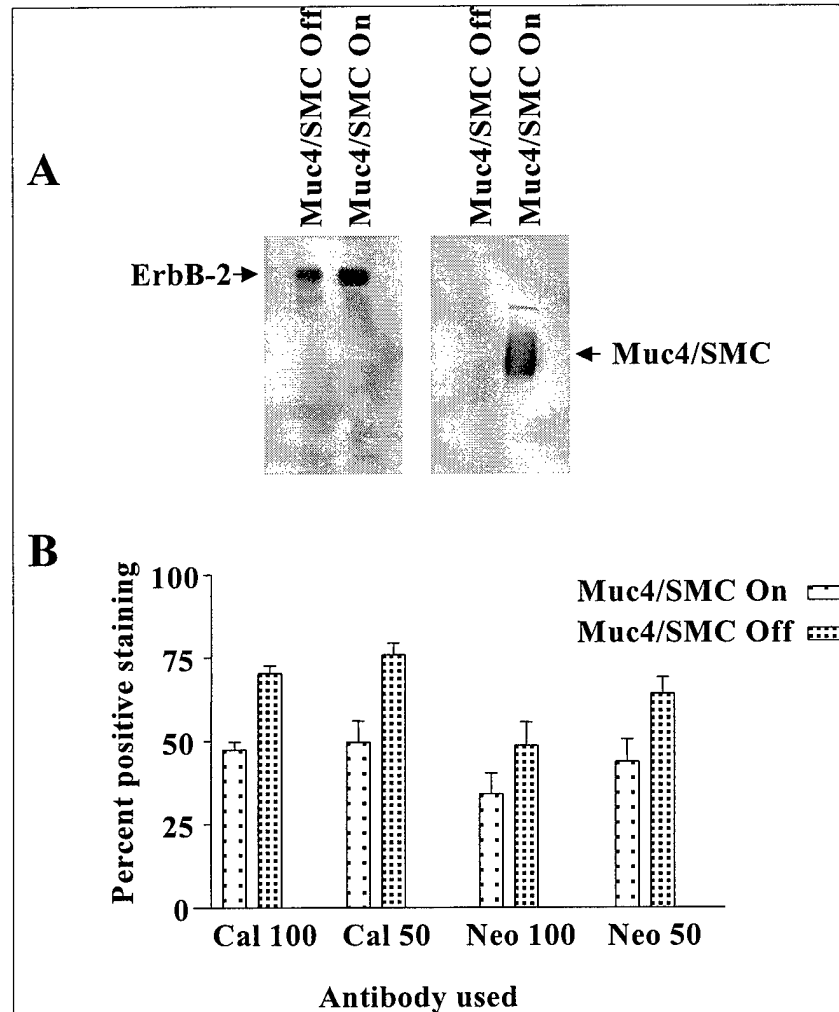
**Table I- IMMUNOCYTOCHEMICAL ANALYSES OF BREAST PATHOLOGY SAMPLES**

<b>Solid nonmalignant breast and tumors</b>		<b>Effusions from breast cancer patients</b>		
<b>Sample</b>	<b>MUC4 +</b>	<b>Sample</b>	<b>ErbB2 +</b>	<b>MUC4 +</b>
Ductal carcinoma	5/12	Pleural	8/8	5/8
Mucoid carcinoma	1/1	Ascites	5/5	4/5
Invasive lobular carcinoma	0/1			
Unknown histology	2/8			

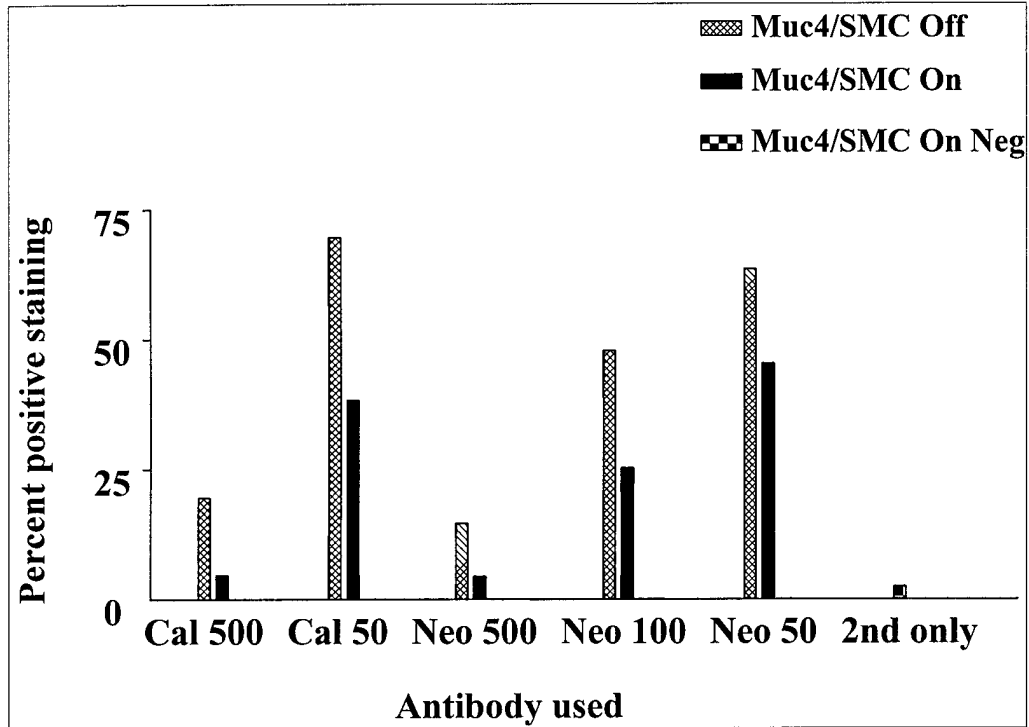
FIGURES



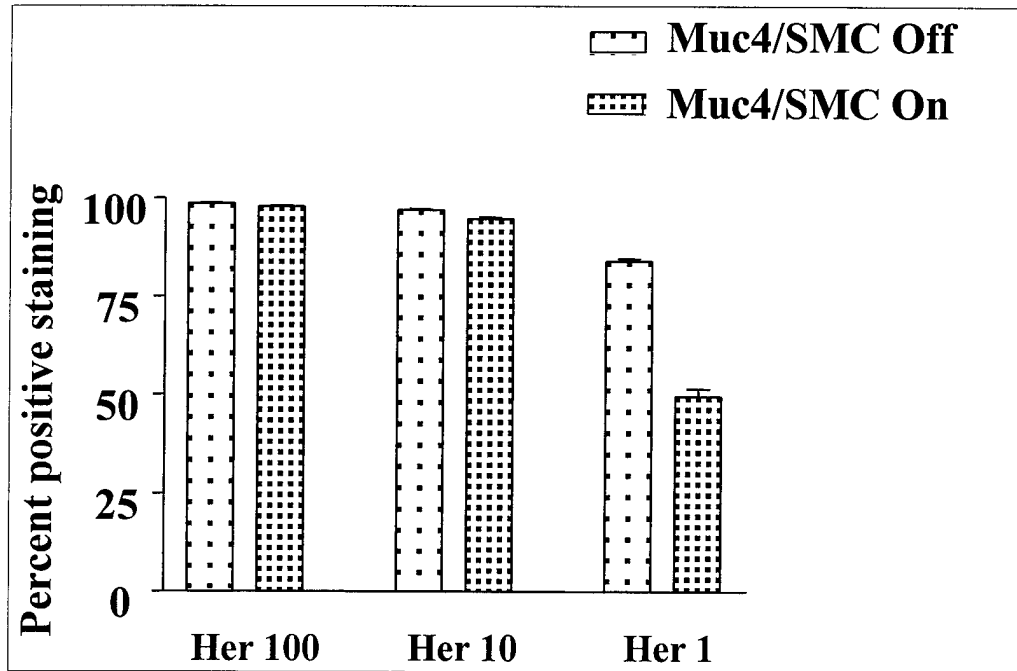
**Figure 1-** Immunoblot demonstration of the expression of MUC4 in human breast tumors. Both positively and negatively staining breast tumors are shown, along with samples of A375 melanoma cells with Muc4/SMC turned ON and OFF, and 13762 ascites cells.



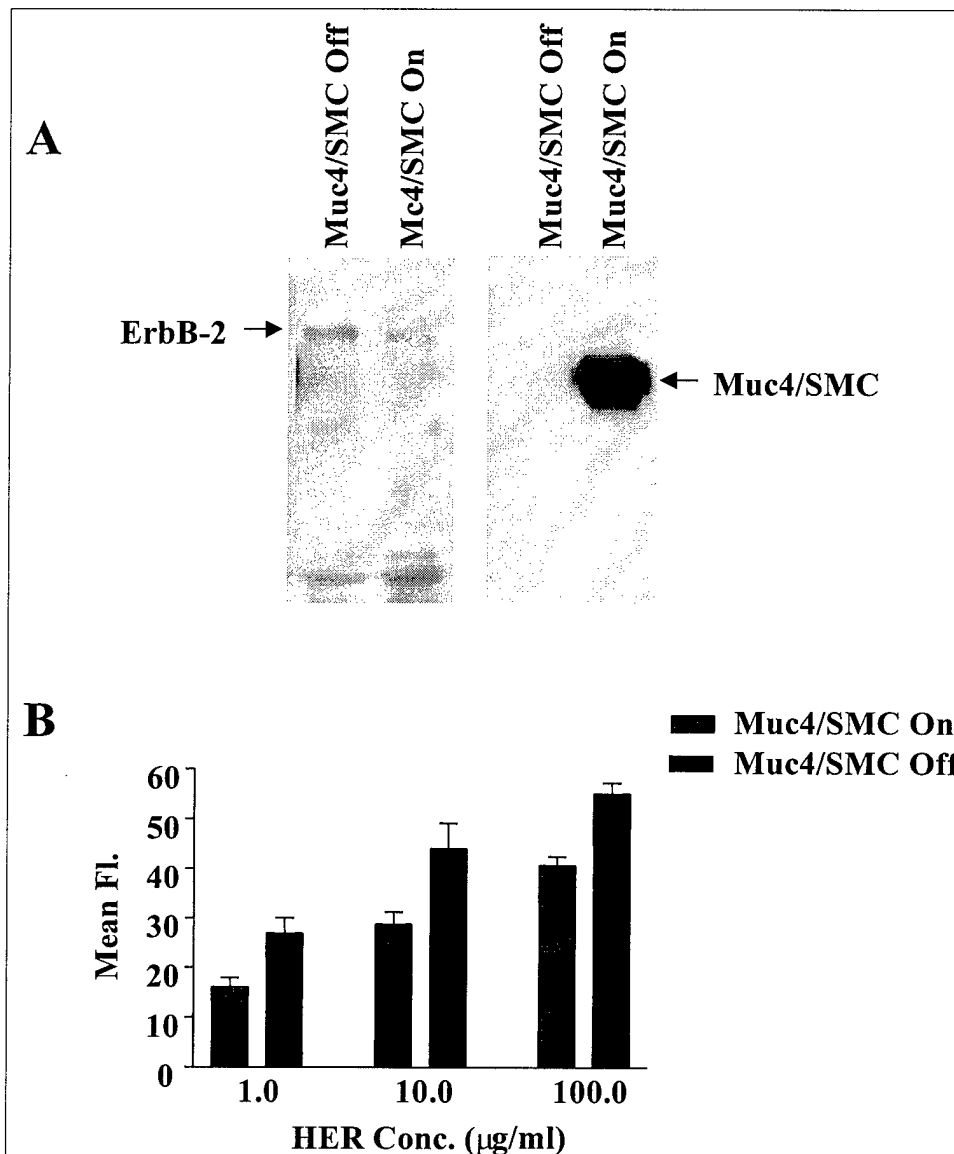
**Figure 2-** Effect of Muc4/SMC expression on ErbB2 expression and antibody binding in A375 human melanoma cells. A375 human melanoma cells stably transfected with Muc4/SMC under control of a tetracycline regulatable promoter were cultured in the presence or absence of tetracycline for 72 hours. A) Cells expressing or not expressing Muc4/SMC were harvested, lysed and subjected to immunoblot analysis with anti-ErbB2 (left panel) and anti-ASGP-2 (right panel) monoclonal antibodies. B) FACS analysis of ErbB2 antibody binding to A375 cells expressing or not expressing Muc4/SMC. A375 cells were harvested in enzyme-free cell dissociation buffer and analyzed by FACS using monoclonal antibodies (CalBiochem Ab5, NeomarkersAb2) against the extracellular domain of ErbB2. As indicated in the figure, 100 and 50 indicate the dilution of antibody used - 1:100 or 1:50. These data are representative of three experiments.



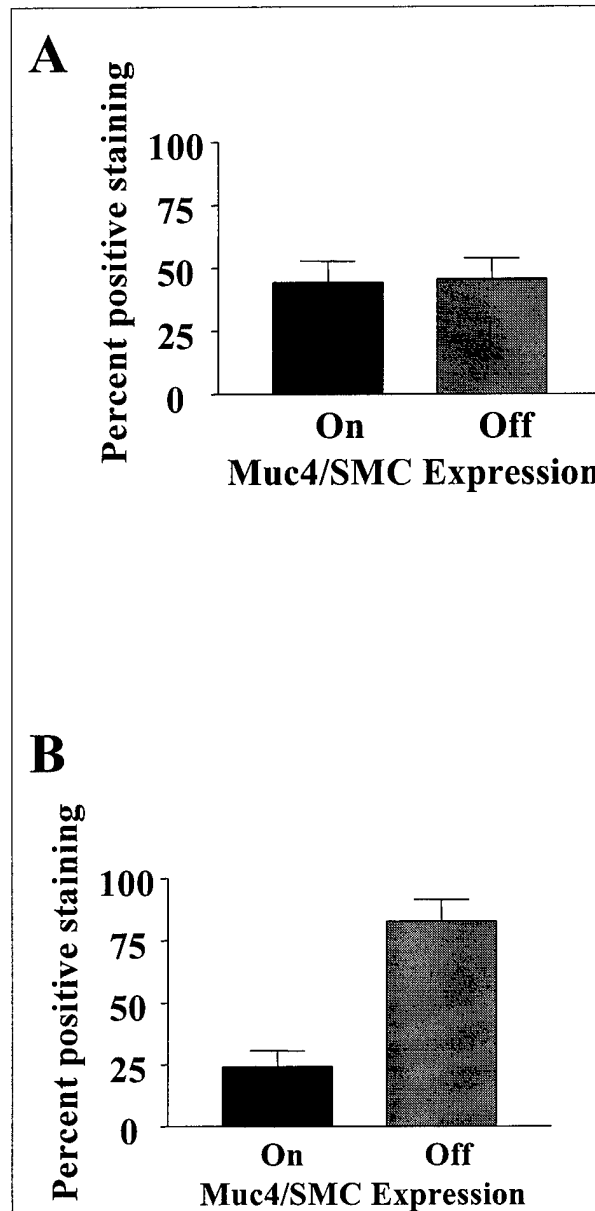
**Figure 3-** Effect of anti-ErbB2 antibody concentration on binding of anti-ErbB2 to A375 human melanoma cells expressing or not expressing Muc4/SMC. A375 human melanoma cells stably transfected with Muc4/SMC under control of a tetracycline regulatable promoter were cultured in the presence or absence of tetracycline for 72 hours. Cells were harvested and stained with Calbiochem anti-ErbB2 antibody 5 or NeoMarkers antibody 2 at the levels indicated at the bottom of the figure. As indicated in the figure, 50, 100, and 500 indicate the dilution of antibody used - 1:50, 1:100, or 1:500, respectively. 2nd only indicates absence of primary antibody in staining protocol.



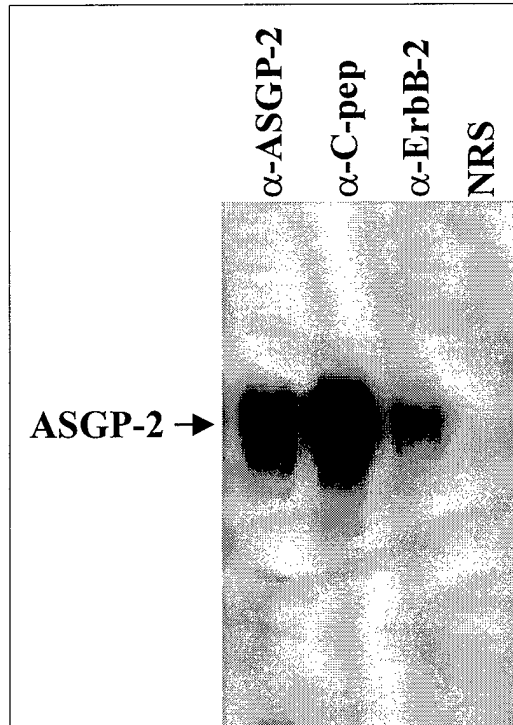
**Figure 4-** Effect of Muc4/SMC expression on Herceptin binding in A375 cells. A375 human melanoma cells stably transfected with Muc4/SMC under control of a tetracycline regulatable promoter were cultured in the presence or absence of tetracycline for 72 hours. Cells were harvested in enzyme-free cell dissociation buffer and analyzed by FACS using Herceptin, a humanized monoclonal antibody against the extracellular domain of ErbB2, at concentrations of 100  $\mu\text{g/ml}$ , 10  $\mu\text{g/ml}$ , or 1  $\mu\text{g/ml}$ . These data are representative of three experiments.



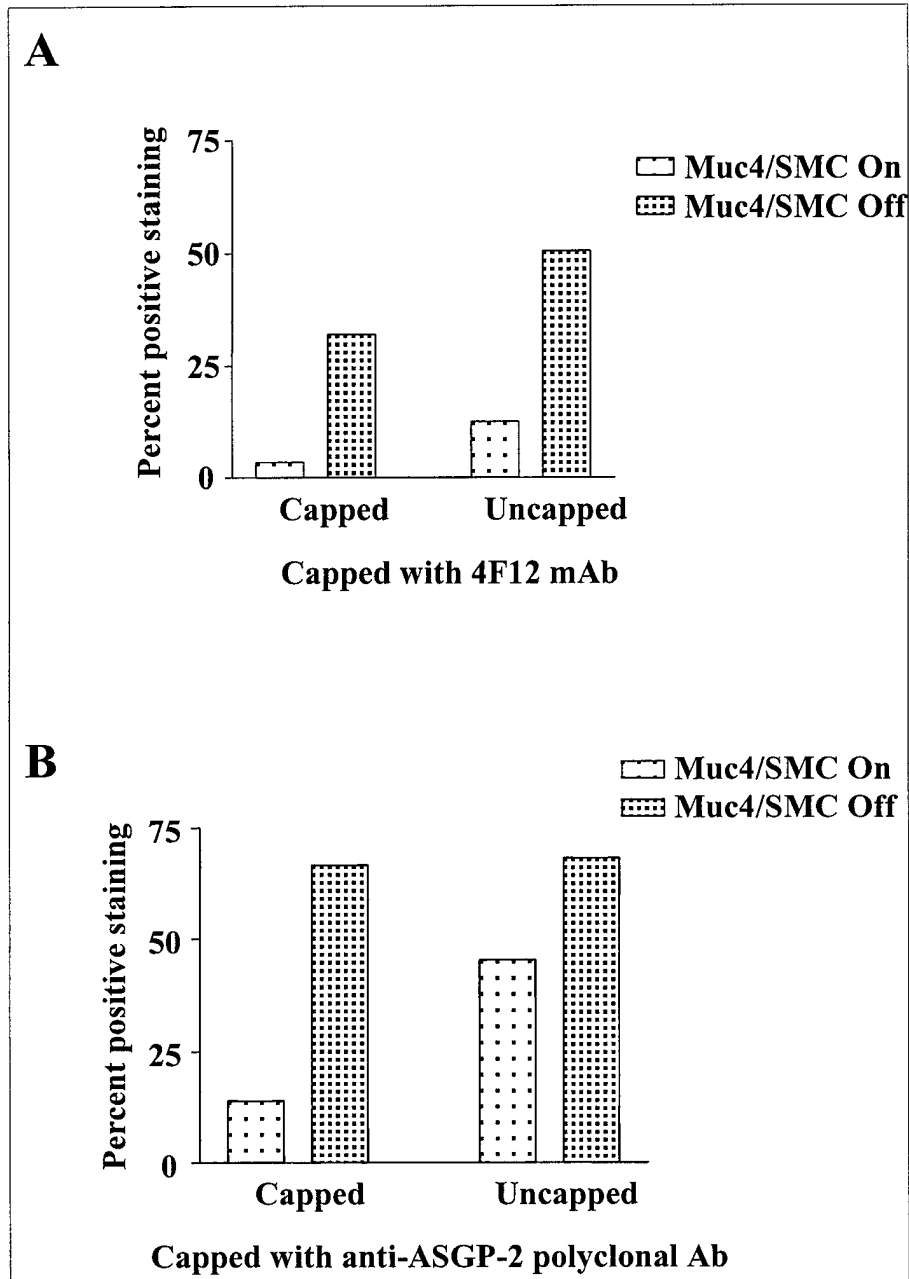
**Figure 5-** Effect of Muc4/SMC expression on anti-ErbB2 antibody binding in MCF-7 human breast cancer cells. MCF-7 cells stably transfected with Muc4/SMC under control of a tetracycline regulatable promoter were cultured in the presence or absence of tetracycline for 72 hours. A) Cells expressing or not expressing Muc4/SMC were harvested, lysed and subjected to immunoblot analysis with anti-ErbB2 and anti-ASGP-2 monoclonal antibodies. B) FACS analysis of ErbB2 antibody binding on MCF7 cells expressing or not expressing Muc4/SMC. MCF-7 cells were harvested in enzyme-free cell dissociation buffer and analyzed by FACS using Herceptin (HER) at concentrations of 100 µg/ml, 10 µg/ml, or 1 µg/ml. These data are representative of three experiments.



**Figure 6-** Effect of Muc4/SMC expression on cell surface binding of antibodies of different isotypes. A375 cells expressing high or low levels of Muc4/SMC were stained with anti-Fas IgG or anti-Fas IgM antibodies and analyzed by flow cytometry. A) A375 cells stained with anti-Fas IgG antibodies. B) A375 cells stained with anti-Fas IgM antibodies. These data are representative of three experiments.



**Figure 7-** Co-immunoprecipitation of Muc4/SMC and ErbB-2 from A375 cells. A375 cells expressing Muc4/SMC were solubilized in RIPA buffer and cleared lysates were immunoprecipitated with anti-ASGP-2, anti-C-pep, anti-ErbB-2, or non-immune rabbit serum as indicated at the top of the figure. Immunoprecipitates were subjected to immunoblot analysis with mAb 4F12.



**Figure 8-** Effect of capping Muc4/SMC on anti-ErbB2 antibody binding in A375 cells. A375 cells expressing high or low levels of Muc4/SMC were treated with anti-ASGP-2 monoclonal or polyclonal antibodies to cap Muc4/SMC. After capping, cells were stained with anti-ErbB2 antibodies and analyzed by flow cytometry. **A)** Anti-ErbB2 staining of A375 cells capped with anti-ASGP-2 mAb 4F12. **B)** Anti-ErbB2 staining of A375 cells capped with anti-ASGP-2 polyclonal antibody.

## KEY RESEARCH ACCOMPLISHMENT

Demonstration that Muc4 overexpression in ErbB2/HER2-expressing cells will reduce Herceptin binding to the cells, those providing one mechanism by which Herceptin resistance can be explained

## REPORTABLE OUTCOME

Shari A. Price-Schiavi, Scott Jepson, Peter Li, Maria Arango, Philip S. Rudland, Lisa Yee and Kermit L. Carraway. Rat Muc4 (Sialomucin Complex) Reduces Binding of Anti-ErbB2 Antibodies to Tumor Cell Surfaces, a Potential Mechanism for Herceptin Resistance. Manuscript in preparation.

## CONCLUSION

The ErbB2/HER2 ligand Muc4 can reduce binding of the therapeutic antibody Herceptin to breast cancer cells expressing ErbB2/HER2 and may be one cause of the low efficacy of Herceptin.

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List of Personnel

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Maria Carvajal

Scott Jepson

Victoria Ramsauer

Maria Carvajal was original requested on the budget for this research project. Ms. Carvajal left the University of Miami and therefore Scott Jepson and Victoria Ramsauer were involved in finishing the research involved.