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13. ABSTRACT (Maximum 200 Words) The overall aim of the research is to develop a better understanding of the role of MUC1 in breast cancer. Loss of Mucl (mouse homologue of MUC1) significantly reduces tumor progression in polyomavirus middle T antigen (PyV MT)-induced mammary tumors. The high transforming activity of the PyV MT antigen depends on c-Src, which has been shown to phosphorylate the cytoplasmic tail of MUC1. Our aim is to identify specific proteins that associate with MUC1 and induce signaling that potentiates tumorigenesis, specifically the modulation of c-Src activity in MMTV-PyV MT tumorigenesis. We have found that MUC1 and c-Src interact in PyV MT-induced mammary tumors. Preliminary findings suggest decreased c-Src kinase activity in tumors lacking Mucl. We saw no effect of lack of Mucl expression on E-cadherin/ β -catenin association. We are in the process of analyzing the effect of Mucl expression on various downstream targets of c-Src. It is likely that MUC1 functions to recruit c-Src and other signaling molecules that are essential for PyV MT-induced transformation. Other studies in the lab have shown that overexpressed MUC1 induces mammary gland tumors. We found that overexpressed MUC1 also inhibits mammary gland involution. These results suggest that MUC1 functions as a weak oncogene in the mammary gland.				
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INTRODUCTION

MUC1 is a large, heavily O-glycosylated membrane mucin that is normally expressed on the apical surface of most simple secretory epithelia and several hematopoietic cells [1-3]. More than 90% of human breast carcinomas and metastases overexpress aberrantly glycosylated MUC1 [4]. Moreover, in most adenocarcinomas MUC1 expression is not restricted to the apical surface [5]. MUC1 has been identified as an important tumor antigen, however the precise function of MUC1 in tumorigenesis and disease progression remains undefined. Numerous observations point to a role for MUC1 in signal transduction. The 72 amino acid tail of MUC1 contains 7 tyrosines, 6 of which are 100% conserved across the species [6]. The cytoplasmic tail of MUC1 has been shown to be phosphorylated on tyrosine residues in epithelial cell lines and mouse mammary gland [7-9]. The cytoplasmic tail of MUC1 contains potential docking sites for SH2 containing proteins and a number of possible kinase recognition sites [6]. Indeed, MUC1 has been shown to interact with a variety of proteins involved in neoplasia and cell adhesion (EGFR, erbB-2, erbB-3, erbB-4, c-Src, Grb2, β -catenin, GSK3 β , p120^{cas}) and shown to potentiate ERK1/2 activation [8, 10-14]. To elucidate the role of MUC1 in tumor progression and metastasis, we crossed MMTV-mTag transgenic mice (expressing the polyomavirus middle T antigen (PyV MT) under the transcriptional control of the mouse mammary tumor virus (MMTV) promoter) onto a Muc1 null background (mouse Muc1 is denoted in lowercase) [15]. Although MMTV-mTag transgenic mice develop multifocal mammary tumors that frequently metastasize to the lung [16], tumor progression is significantly delayed in Muc1-null/MMTV-mTag transgenics [15]. The high transforming activity of the PyV MT antigen is dependent on the interaction of PyV MT with the Src family of kinases (c-Src, c-Yes, and Fyn), and it has been shown that activation of c-Src is essential for the induction of mammary tumors in the mTag transgenic mice [17]. c-Src is a well characterized nonreceptor tyrosine kinase implicated in mammary tumorigenesis. As mentioned above, c-Src is among the proteins that interact with the cytoplasmic tail of MUC1. *In vitro* studies have shown that c-Src phosphorylates the MUC1 cytoplasmic domain at the YEKV motif. Phosphorylation of MUC1 by c-Src results in the binding of the c-Src SH2 domain, in turn regulating the interaction of MUC1 with GSK3 β and β -catenin [10]. The delayed mammary tumor progression in the mTag transgenic mice that lack MUC1 and the functional interaction between MUC1 and c-Src among other signaling molecules suggest a signaling role for the cytoplasmic domain of MUC1 in mTag tumorigenesis. We hypothesize that MUC1 mediates molecular interactions between oncogenic kinases and other signaling proteins that contribute significantly to transformation. Transgenic mouse models are used to investigate proteins that interact with the cytoplasmic tail of MUC1 and induce signaling that leads to oncogenic transformation.

MUC1 is not merely overexpressed in cancer, but functions to promote transformation. This has been corroborated by tumor progression studies in transgenic and knockout mice, in that lack of Muc1 results in a reduction in tumor progression. In addition to the above-mentioned delay in tumor progression in the MMTV-PyV MT transgenic mice bred onto Muc1^{-/-} background, lack of Muc1 expression cause a significant delay in tumor onset in the MMTV-Wnt-1 model of breast cancer. Interestingly, long-term analysis of MUC1 transgenic mice led to the finding that overexpression of full-length MUC1 but not the cytoplasmic domain-deleted MUC1 resulted in stochastic formation of mammary gland tumors that metastasized to the lungs in multiparous females. Tumor formation was accompanied by a failure of multiparous glands to dedifferentiate

and involute. Additionally, we have determined that this lack of involution can be observed in non-tumor bearing mammary glands as well, in that post-lactational regression is suppressed by MUC1 overexpression. These findings shed new light on the role of MUC1 in the mammary gland, suggesting that it functions as a weak oncogene.

BODY

The first specific aim for this project was to determine the effects of Muc1/MUC1 expression on c-Src activity in the mammary gland and tumorigenesis. We proposed to utilize Muc1 knockout mice (Muc1^{-/-}) as well as transgenic mice expressing either the human MUC1 or the cytoplasmic tail deleted form of MUC1 (MUC1 Δ CT). We have carried out the crosses required to get the different transgenic lines included in this study. We have harvested and analyzed mammary gland tumors from MTF, MMTF, MTKF and M Δ CTKF mice along with age matched wildtype FVB mice (refer to Table 1 for abbreviations). As expected, we observed an increase in Src levels in MTF and MMTF tumors compared with wildtype control mammary glands by western blot analysis (Figure 1). Initially we characterized the interaction between Muc1/MUC1 and c-Src in mammary tumors. Mammary gland tumor lysates from MTF mice were immunoprecipitated with antibodies to c-Src (sc-18, Santa Cruz) or to the cytoplasmic tail of Muc1 (CT2, hamster monoclonal). The immunoprecipitates were then analyzed by immunoblotting for c-Src (Figure 2A,B) and Muc1 (Figure 2C). Reciprocal co-immunoprecipitations confirmed an interaction between c-Src and Muc1 in the MTF mammary tumors (Figure 2B, C). The molecular weight of c-Src is 60 kDa and that of immunoglobulin heavy chain is around 55 kDa. In order to eliminate the possibility of contamination with immunoglobulin heavy chain, we carried out the Muc1 immunoprecipitations using an immobilized Muc1 antibody (Seize Primary Immunoprecipitation kit, Pierce). Immunoblotting for c-Src verified that c-Src formed a complex with Muc1 in MTF and MMTF bitransgenic tumor lysates (Figure 3). To further verify the interaction between c-Src and Muc1 and to determine the localization of this complex, we used confocal microscopy to analyze Muc1/c-Src co-localization. MTF mammary tumors were stained with an antibody that recognizes the cytoplasmic tail of Muc1. Muc1 was localized to the apical membrane. c-Src was localized throughout the cell membrane and throughout the cytoplasm. Dual staining for c-Src and MUC1 revealed that they are co-localized mainly at the apical membrane (Figure 4). Thus far we have established that Muc1 colocalizes and physically interacts with c-Src in MTF and MMTF tumors. The antibody to Muc1 efficiently pulls down endogenous Muc1 in the MTF and MMTF tumors, therefore we did not have to resort to the use of the biotinylated MUC1 peptide to pull down c-Src.

We next investigated the effects of Muc1 on c-Src activity. c-Src was immunoprecipitated from lysates of MTF, MMTF tumors and normal wild-type mammary glands. Kinase assays were performed to determine c-Src activity. The assays were performed using a Src kinase assay kit from Upstate Biotechnology (Lake Placid, NY). The assay measures the amount of γ^{32} P-ATP incorporated into a c-Src substrate peptide. c-Src activity in the MMTF bitransgenics was comparable to that in the MTF lysates. However, c-Src activity in the tumors was notably higher than that in the wild-type normal mammary gland lysates (Figure 5). In order to address the question of whether Muc1 modulates c-Src kinase activity, we compared c-Src activity in the

MTF tumors (n=3) with the MTKF tumors (n=3) lacking Muc1. Normal mammary glands from wildtype FVB and Muc1^{-/-} mice were used as controls. Preliminary findings indicate that c-Src kinase activity is higher in the MTF tumors than in MTKF tumors (Figure 6). This suggests that the presence of Muc1 positively influences c-Src activity. We plan to verify these findings by using more animals per group (at least n=6 to achieve statistical significance). The samples analyzed thus far are full grown tumors from MTF and MTKF animals. It may be that the presence of Muc1 plays a role at the initial stages of tumorigenesis; hence we might see a greater difference in c-Src kinase activity between MTF and MTKF at an earlier time point. To this end, we have harvested mammary glands from both MTF and MTKF mice at 8 weeks of age, at which point the mammary glands would be hyperplastic. c-Src kinase assays will be carried out as described above. Also, we will include the analysis of M Δ CTK animals to determine whether the cytoplasmic tail of Muc1 is functionally involved in influencing c-Src kinase activity. Immunoblotting with antibodies that are specific to activated c-Src (recognizing pTyr416) was not successful possibly due to the poor reaction of the antibody with primary mouse lysate. However, we are currently investigating downstream targets of c-Src such as p130 Cas and pp120 catenin and MAPKs to determine any differences resulting from lack of Muc1 expression.

It is believed that MUC1 competes with E-cadherin for β -catenin binding and therefore is involved in disrupting cell-adhesion complexes [11]. Previously, it has been shown that *in vitro* β -catenin interacts with MUC1 at an SXXXXXSSL site present in the cytoplasmic tail of MUC1[13]. Also, GSK3 β has been shown to bind directly to MUC1 at a DRSPY site adjacent to that for β -catenin, hence decreasing the interaction of MUC1 with β -catenin [11]. On the other hand, the interaction between MUC1 and c-Src results in phosphorylation of MUC1 at a YEKV motif located between sites involved in interactions with GSK3 β and β -catenin. Phosphorylation of MUC1 provides a binding site for c-Src SH2 domain, and in turn inhibits the interaction between MUC1 and GSK3 β [10]. Therefore, inhibition of the association of MUC1 with GSK3 β increases the binding of MUC1 and β -catenin. We have observed that c-Src interacts with β -catenin in the MTF, MMTF and MTKF tumors (data not shown) and this led us to determine whether c-Src's interaction with Muc1 affects the amount of β -catenin bound to E-cadherin. To address that question we immunoprecipitated β -catenin from MTF, MMTF and MTKF tumors and immunoblotted for E-cadherin to assay for β -catenin/E-cadherin association. According to the model proposed by Kufe *et al.* [11] we expected to see a decrease in β -catenin/E-cadherin association in the MTF tumors that have overexpression of Muc1 compared with MTKF tumors that lack Muc1. However, we saw no difference in the level of β -catenin/E-cadherin association between MTF and MTKF tumors (data not shown). An interaction between MUC1 and the p85 subunit of PI3K has been identified by co-immunoprecipitation in the breast cancer line T47D and studies are underway to characterize such an interaction in the MTF tumors given that p85 signaling is involved in the PyV MT-induced transformation. We are currently investigating the association between Muc1 and other signaling molecules that are involved in PyV MT induced tumorigenesis such as the adapter molecule Shc.

We had proposed to utilize phage display to identify novel proteins that interact with MUC1 cytoplasmic domain. The MUC1 cytoplasmic tail peptides were used as baits to screen a breast tumor cDNA T7 phage library (Novagen). We carried out several rounds of screening. However we were faced with the problem of non-specific binding and we failed to obtain true positive

interactions. We tried several strategies to reduce the non-specific binding first by changing the immobilization solid from 96 well plates to streptavidin beads then by switching to a more stringent panning protocol. Phage display is known to have a high false positive rate. These studies were not pursued further due to the high false positive rates and novel findings discussed below.

While addressing the question of Muc1 signaling in the PyV MT induced tumors, we observed that transgenic mice overexpressing the human MUC1 transgene developed mammary tumors. The offspring from two MUC1 transgenic lines expressing the full-length human MUC1 cDNA under the control of the MMTV promoter (MMTV-MUC1#9 and #15) were continuously housed as breeding pairs for up to a year. The females were then housed separately and monitored. Additionally, one cytoplasmic-deleted MUC1 transgenic line (MMTV- Δ CT) and an FVB control line were housed similarly. Approximately 60% of multiparous MMTV-MUC1 females developed mammary tumors with a long latency period that ranged from 6-24 months. Primary mammary gland tumors were observed in both of the MMTV-MUC1 lines; however, no tumors were observed in either the MMTV- Δ CT transgenic lines or the FVB control lines, indicating that the cytoplasmic domain of MUC1 is largely responsible for tumor formation. MMTV-MUC1 induced tumors encompassed a variety of types including: microacinar, papillary, solid carcinomas and adenocarcinomas with the presence of lobular hyperplasias in all tumor samples (Figure 7). We found that 90% of the MUC1 expressing tumors also developed lung metastases. Histological analysis of the MMTV-MUC1 mammary glands revealed a lack of glandular involution in the multiparous transgenic females. We pursued this further by analyzing the effect of MUC1 overexpression on mammary gland involution. Uniparous MMTV-MUC1 transgenic and wildtype females were allowed to lactate for 6-10 days followed by pup removal to initiate involution. The animals were then sacrificed at various time points and their mammary glands were analyzed at days 2, 4, 6 and 8 of involution. We performed whole mount analysis to assess any gross morphological differences between glands of MMTV-MUC1 transgenic and wildtype mice. Whole mount analysis (Figure 8) showed that involution in the MUC1 transgenic mammary glands was delayed in comparison to the wildtype controls. At day 4 of involution, we noted the presence of lobulo-alveolar structures in the mammary glands of both the MMTV-MUC1 transgenics and the wildtype controls. By day 6 the collapse of lobulo-alveolar structures proceeded normally in the mammary glands of the wildtype females. In contrast, the mammary glands of MMTV-MUC1 transgenics were still filled with lobulo-alveolar structures and little adipose tissue was observed. The delay in mammary gland involution in the MMTV-MUC1 transgenics was more pronounced at day 8 of involution. By day 8 of involution the wildtype glands had fully regressed while large, secretory ducts were still present in the MMTV-MUC1 glands. Hematoxylin and eosin staining of the mammary glands of MMTV-MUC1 transgenics and controls corroborated the results of the whole mounts (data not shown). We observed no significant differences between MMTV-MUC1 and wildtype glands at days 4 and 6. However, at day 8 MMTV-MUC1 transgenics showed large secretory ducts that were no longer present in the wildtype controls. Interestingly, the delayed involution by day 8 in the transgenic gland correlates with high expression levels of the MUC1 transgene (Figure 9). We performed immunohistochemical analysis to examine MUC1 expression in MMTV-MUC1 and control FVB glands at days 4, 6 and 8. Wildtype animals were stained for endogenous Muc1 and MMTV-MUC1 animals were stained for expression of the MUC1 transgene using an antibody specific to human MUC1. Similar to wildtype Muc1, the MUC1 transgene was predominantly

apically expressed within the alveolar lumens and secretory ducts. This correlated with the staining pattern of MMTV-MUC1 during lactation where it is thought that MUC1 is either shed or present at the plasma membrane during the release of milk proteins and fat into the lumens [8]. Figures 9F and 9C depict the collapse of the luminal spaces in the FVB at day 8 while large luminal spaces with appreciable amounts of MUC1 were present in the MMTV-MUC1 transgenics.

We next investigated differences between the involuting glands of the MMTV-MUC1 transgenics and controls at the molecular level by western blot analysis. We assayed for the level and activation of a number of apoptotic and anti-apoptotic markers at day 2, 4, 6 and 8 of involution. The proteins we analyzed are pStat3, pStat5, pAkt, caspase 3, pErk1/2 (MAPK), and WAP (whey acidic protein). At day 6, the levels of pStat3 were lower in the control mammary glands than in the MMTV-MUC1. pStat3 is a pro-apoptotic and its presence at higher levels in the MMTV-MUC1 transgenics indicates a delay in the first phase of involution comprising of the removal of epithelial cells by apoptosis (Figure 10A). This finding correlates with the delay in involution that is seen morphologically at day 6. We saw no observable differences in pStat5 levels at the various timepoints (data not shown). Both the antibodies for pAkt and caspase 3 did not yield a positive reaction. Other antibodies for these proteins are under investigation. We determined the activation of Erk1 and Erk2 by western blot analysis using antibodies specific to tyrosine phosphorylated Erk1 and Erk2 (Figure 10C). Interestingly, there was a significant difference in Erk2 activation between transgenics and wildtype at day 8 of involution. pErk2 was absent in wildtype animals while pErk2 was still present in the MMTV transgenic mammary glands. The levels of Erk1 and Erk2 were similar in transgenic and wildtype glands. WAP (whey acidic protein) is normally highly expressed during lactation and its expression is downregulated during involution. We examined WAP expression by western blot analysis in the transgenic and wildtype animals at days 2, 4, 6, and 8 of involution. As expected, WAP expression decreased in the wildtype mice from day 2 to day 8 (data not shown). However, at day 6 in 2 out of 5 transgenic glands, there was an appreciable increase in WAP levels when compared to wildtype (Figure 10B). This falls in line with the lack of glandular dedifferentiation in the MMTV-MUC1. In order to determine the mechanism by which MUC1 overexpression resulted in delayed mammary involution, we performed TUNEL assays to quantitate differences in apoptosis. Day 2 MMTV-MUC1 and wildtype mammary glands were stained and we are currently in the process of counting the number of apoptotic cells.

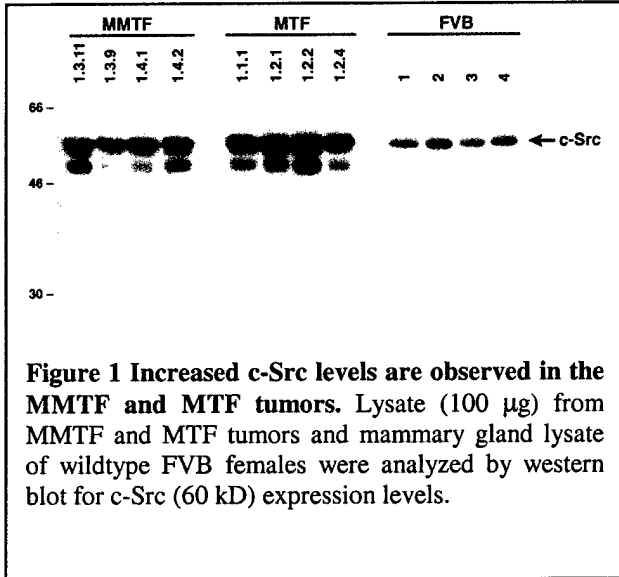


Figure 1 Increased c-Src levels are observed in the MMTF and MTF tumors. Lysate (100 μ g) from MMTF and MTF tumors and mammary gland lysate of wildtype FVB females were analyzed by western blot for c-Src (60 kD) expression levels.

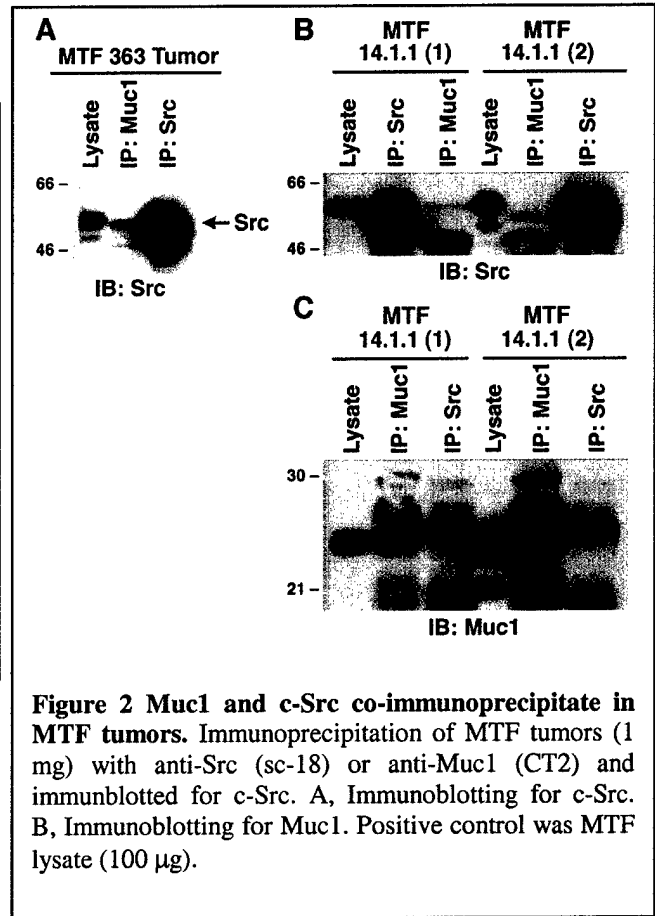


Figure 2 Muc1 and c-Src co-immunoprecipitate in MTF tumors. Immunoprecipitation of MTF tumors (1 mg) with anti-Src (sc-18) or anti-Muc1 (CT2) and immunoblotted for c-Src. A, Immunoblotting for c-Src. B, Immunoblotting for Muc1. Positive control was MTF lysate (100 μ g).

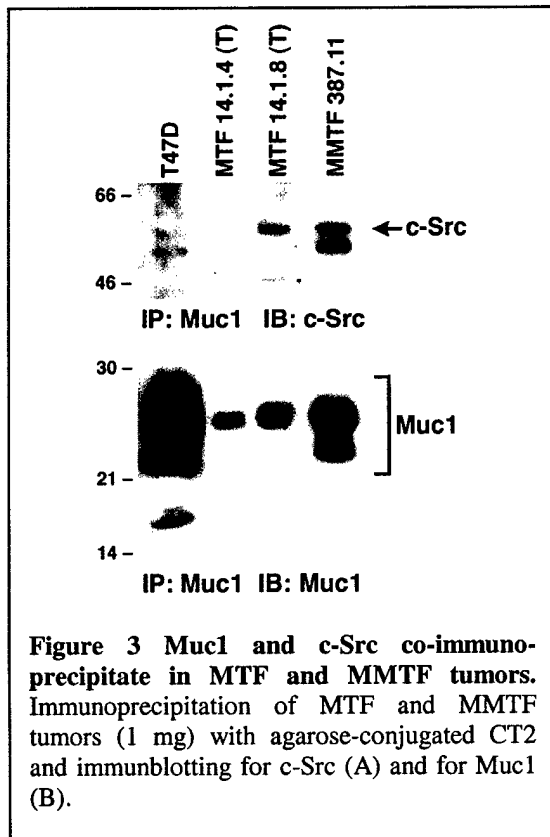


Figure 3 Muc1 and c-Src co-immunoprecipitate in MTF and MMTF tumors. Immunoprecipitation of MTF and MMTF tumors (1 mg) with agarose-conjugated CT2 and immunoblotting for c-Src (A) and for Muc1 (B).

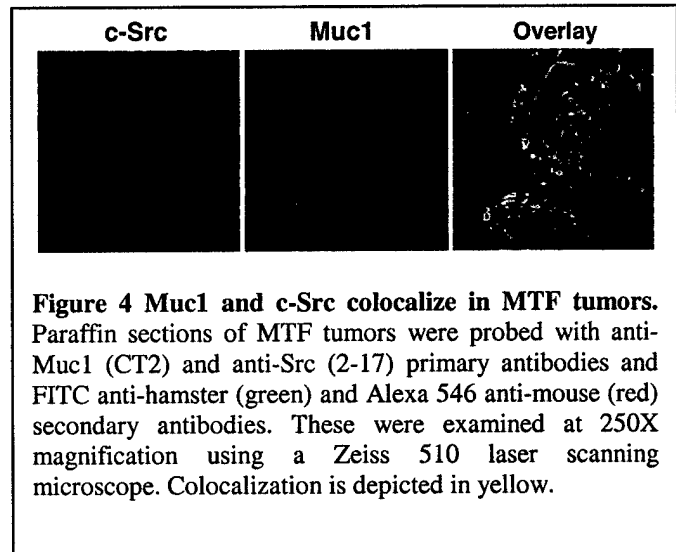
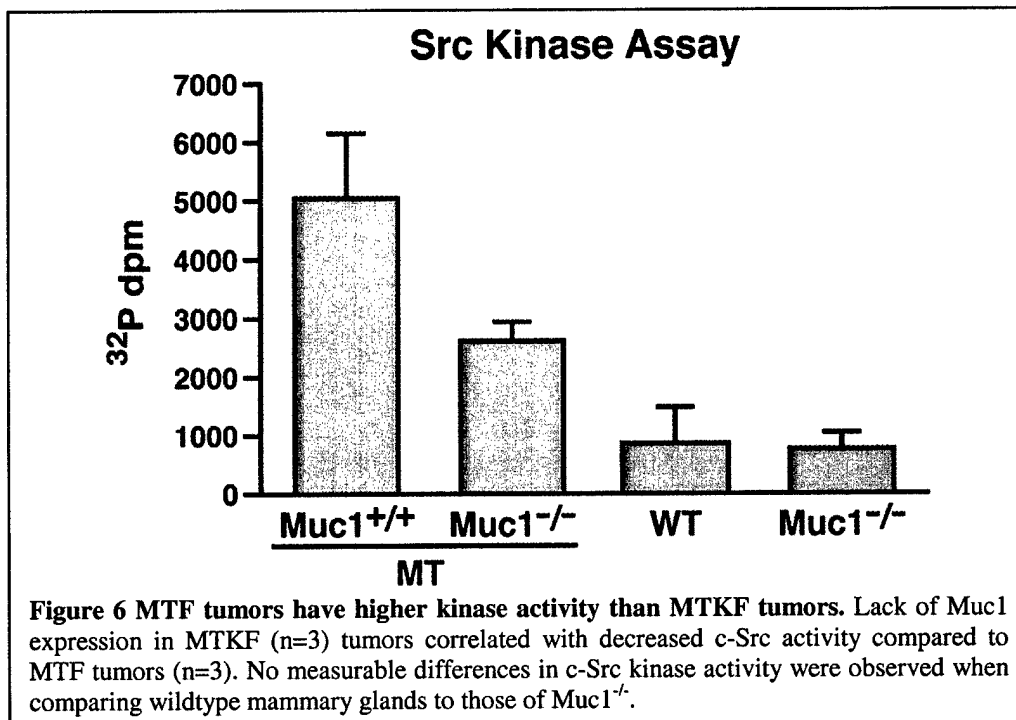
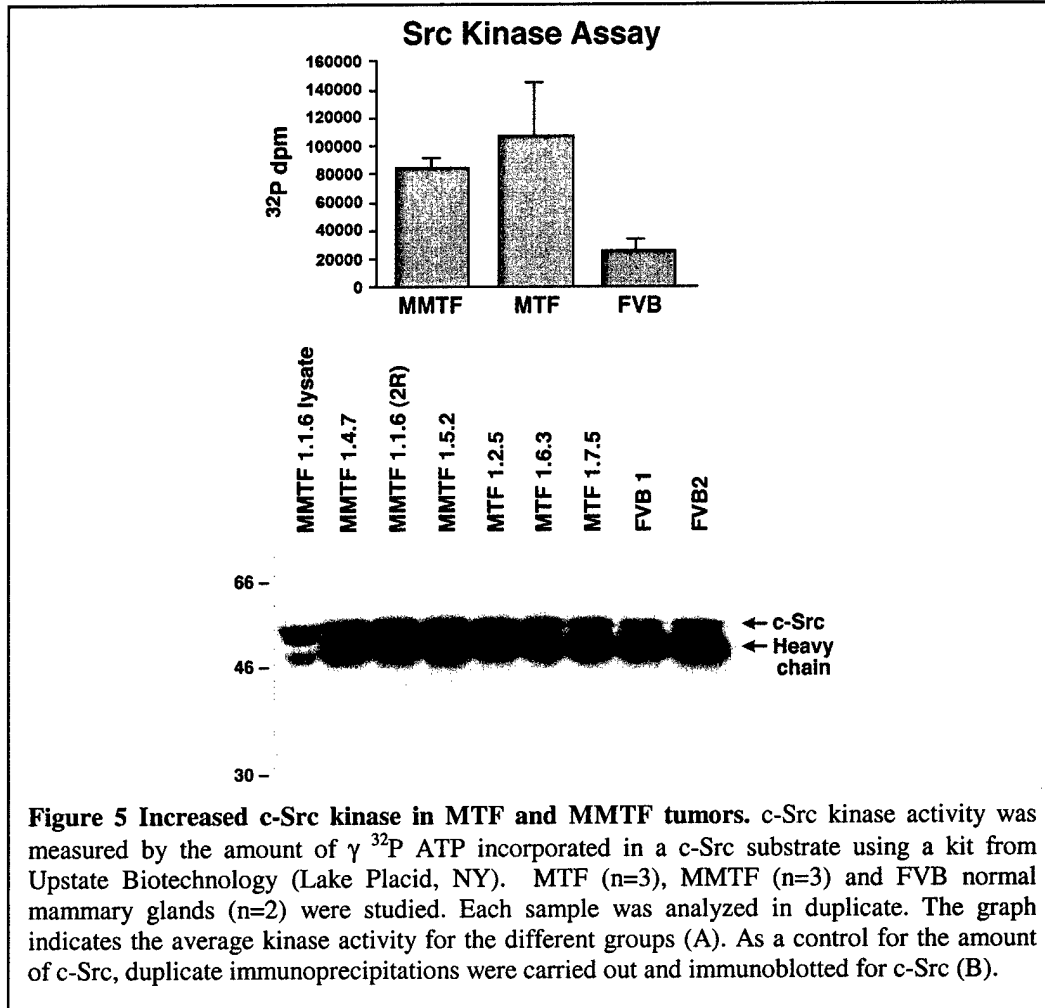


Figure 4 Muc1 and c-Src colocalize in MTF tumors. Paraffin sections of MTF tumors were probed with anti-Muc1 (CT2) and anti-Src (2-17) primary antibodies and FITC anti-hamster (green) and Alexa 546 anti-mouse (red) secondary antibodies. These were examined at 250X magnification using a Zeiss 510 laser scanning microscope. Colocalization is depicted in yellow.



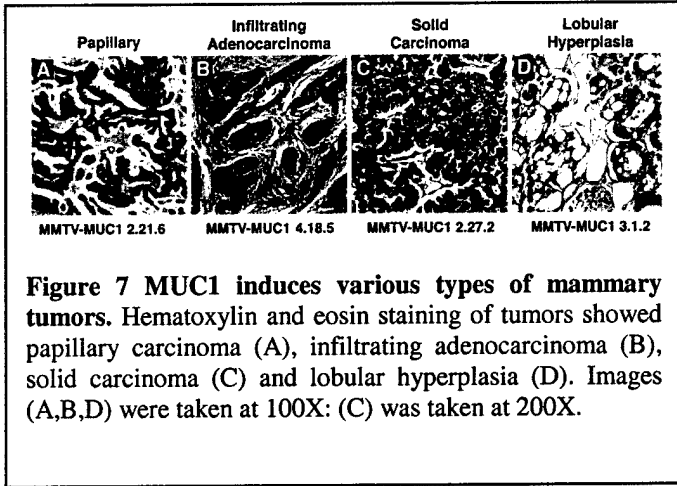


Figure 7 MUC1 induces various types of mammary tumors. Hematoxylin and eosin staining of tumors showed papillary carcinoma (A), infiltrating adenocarcinoma (B), solid carcinoma (C) and lobular hyperplasia (D). Images (A,B,D) were taken at 100X; (C) was taken at 200X.

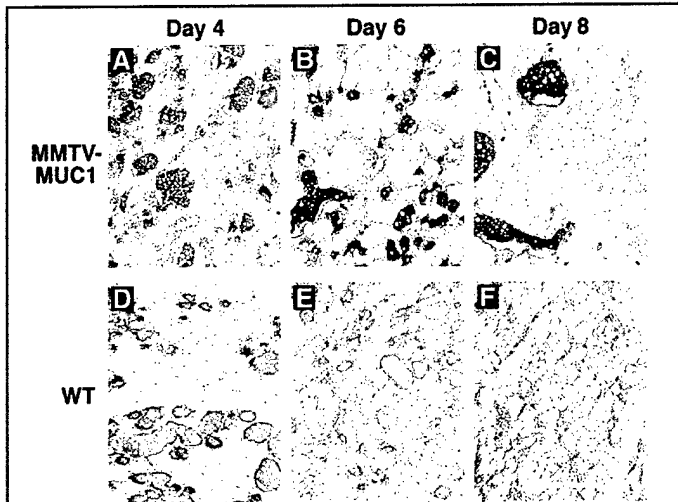


Figure 9 MUC1 is highly expressed within the alveolar lumens and secretory ducts of MUC1 transgenic mice. Immunohistochemical analysis of MUC1 transgenic mammary glands (A-C) using anti-MUC1 B27.29 (recognizes the PDTRPAP epitope in the tandem repeat of human MUC1). Wildtype glands (D-F) were stained with anti-Muc1 (recognizes the last 17 amino acids of the cytoplasmic tail of both human and mouse Muc1).

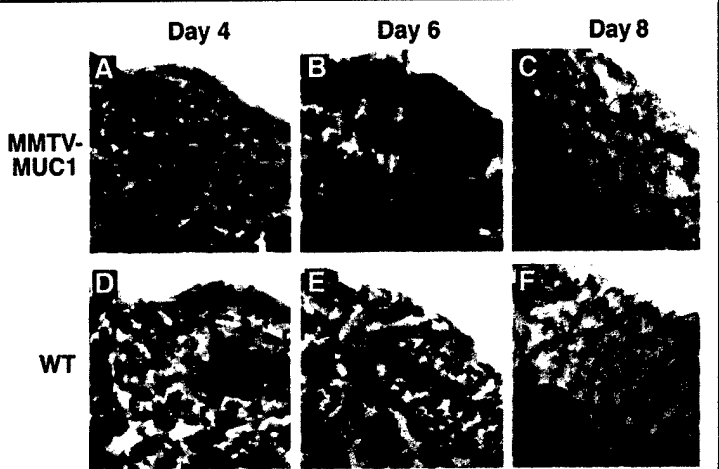


Figure 8 Mammary gland involution is delayed in the MUC1 transgenic mice. Representative images of whole mounts of involuting thoracic mammary glands from MMTV-MUC1 transgenic females (A-C) and wildtype controls (D-F) at day 4 of involution (A,D), day 6 of involution (B,E) and day 8 of involution (C,F). Images were taken at a magnification of 25X.

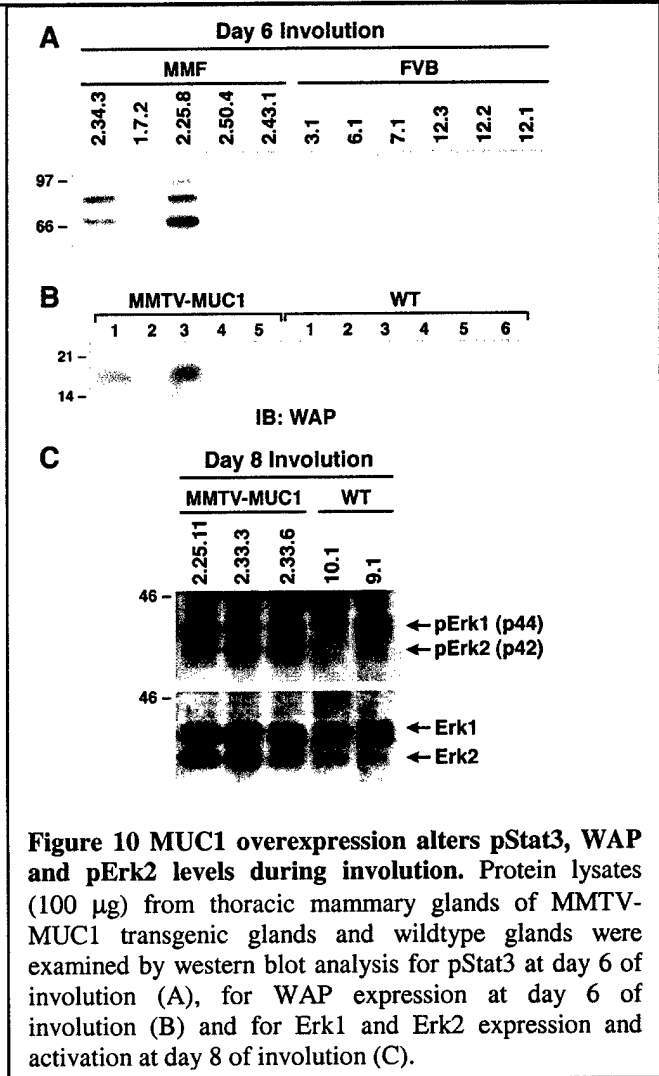


Figure 10 MUC1 overexpression alters pStat3, WAP and pErk2 levels during involution. Protein lysates (100 µg) from thoracic mammary glands of MMTV-MUC1 transgenic glands and wildtype glands were examined by western blot analysis for pStat3 at day 6 of involution (A), for WAP expression at day 6 of involution (B) and for Erk1 and Erk2 expression and activation at day 8 of involution (C).

Table 1

MMTV-PyV MT	MTF
MUC1.Tg x MMTV-PyV MT	MMTF
Muc1 ^{-/-} x MMTV-PyV MT	MTKF
Muc1 ^{-/-} x MUC1 Δ CT x MMTV-PyV MT	MT Δ CTKF

KEY RESEARCH ACCOMPLISHMENTS

- c-Src levels were higher in MTF and MMTF tumors than in normal mammary glands.
- Muc1 colocalizes and physically interacts with c-Src in MTF and MMTF tumors.
- Preliminary findings show increased c-Src activity in tumors expressing Muc1, MTF, when compared with tumors lacking Muc1, MTKF. This suggests that Muc1 positively influences c-Src activity.
- Muc1 expression does not seem to influence the interaction between β -catenin and E-cadherin.
- Multiparous transgenic mice that overexpress human MUC1 developed metastasizing mammary tumors with a long latency, suggesting that Muc1 functions as a weak oncogene.
- MMTV-MUC1 Δ CT did not develop tumors, indicating that the cytoplasmic tail of MUC1 is required for its oncogenesis potential.
- MMTV-MUC1 transgenic females displayed prolonged glandular differentiation.
- MUC1 overexpression caused a delay in mammary gland involution correlating with the presence of large secretory ducts and increased WAP levels and pErk2 activation

REPORTABLE OUTCOMES

- Azzah Al Masri, Joyce A. Schroeder, Melissa C. Adriance, Melissa C. Thompson, and Sandra J. Gendler. *MUC1 overexpression induced mammary gland tumors and delayed postlactational involution*. Abstract and poster at the AACR Mouse Models Meeting. Lake Buena Vista, FL. 2/19/2003-2/22/03.
- Azzah Al Masri, Joyce A. Schroeder, Melissa C. Adriance, Melissa C. Thompson, and Sandra J. Gendler. *MUC1 overexpression induced mammary gland tumors and delayed postlactational involution*. Abstract chosen for oral presentation at the AACR Mouse Models Meeting. Lake Buena Vista, FL. 2/22/03.
- Azzah Al Masri and Sandra J. Gendler. *Understanding the role of Muc1 in PyV MT induced tumorigenesis*. Abstract submitted, FASEB meeting: Growth Factor Receptor Tyrosine Kinases in Mitogenesis, Morphogenesis and Tumorigenesis. Tucson, AZ. 8/2/2003-8/7/2003.
- Generation of PyV MT transgenics lacking Muc1 on the FVB background in addition to bitransgenics expressing the mutant form of MUC1 Δ CT and PyV MT on a Muc1 knockout background.

CONCLUSIONS

We have demonstrated that Muc1 interacts with c-Src in the PyV MT induced tumors. c-Src plays a pivotal role in the PyV MT mediated transformation. Aberrant activation of c-Src kinase activity has been characterized in a large proportion of human breast carcinomas. Preliminary kinase assays showed increased c-Src activation in the tumors expressing Muc1 when compared with tumors lacking Muc1. This possibly provides a mechanism for the delay in tumorigenesis observed in the PyV MT mice on the Muc1^{-/-} background.

Long-term analysis of MUC1 transgenic mice led to the finding that overexpression of full-length MUC1 but not the cytoplasmic domain-deleted MUC1 resulted in stochastic formation of mammary gland tumors that metastasized to the lungs in multiparous females. Tumor formation was accompanied by a failure of multiparous glands to dedifferentiate and involute. Additionally, we have determined that this lack of involution can be observed in non-tumor bearing mammary glands as well, in that post-lactational regression is suppressed by MUC1 overexpression. We found that the delay in mammary involution in the MUC1 transgenics correlated with elevated WAP expression and pErk2 activation. Our findings indicate that MUC1 functions as a weak oncogene whereby the cytoplasmic tail of MUC1 acts as a scaffolding protein to coordinate and enhance multiple growth-promoting and oncogenic signals.

MUC1 has been identified as a tumor antigen and an indicator of disease prognosis. Our findings will bring us closer to understanding the role that MUC1 plays in breast cancer development and progression and in turn contribute to the improvement of the diagnosis and treatment of the disease.

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