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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 Muc1 (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 and signaling in MMTV-PyV MT tumorigenesis. We have found that MUC1 and c-Src interact in PyV MT-induced mammary tumors. Our data provide insights into the possible mechanism for the significant delay in tumor progression that is observed in the absence of Muc1. We suggest that the interaction of Muc1 with c-Src, a key player in PyV MT transformation, promotes the binding of c-Src to its downstream targets and influences its intracellular localization.

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 (MUC1 in humans, Muc1 in mice) is the founding member of a large and evolutionarily conserved family of proteins known as mucins [1, 2]. Mucins are broadly defined as proteins with extensive O-linked glycosylation, which constitutes 50% of the total molecular mass. MUC1 is a transmembrane mucin with a large, heavily glycosylated extracellular domain and a highly conserved cytoplasmic tail [2]. It is normally expressed on the apical surface of most simple secretory epithelia and several hematopoietic cell lineages [2]. MUC1 was initially cloned from mammary carcinomas as a tumor antigen [3]. More than 90% of human breast cancers overexpress aberrantly glycosylated MUC1 [4]. In cancer cells, the pattern of MUC1 expression is altered such that it is no longer restricted to the apical surfaces, instead it is expressed intracellularly and around the entire cell surface [5]. While MUC1 has been recognized as an important tumor antigen, the identification of a functional role for that overexpression has been elusive. Transgenic and knockout mice have provided insight into the role of MUC1 in tumor development, with the loss of Muc1 resulting in a reduction in tumor progression. Transgenic mice expressing the polyomavirus middle T antigen (PyV MT) under control of the mouse mammary tumor virus (MMTV) promoter exhibit a significant delay in tumor growth and a trend towards decreased metastasis when bred onto a Muc1 knockout background [6]. Also, lack of Muc1 expression causes a significant delay in tumor onset in the MMTV-Wnt-1 model of breast cancer [7]. Mammary tumors of MMTV-PyV MT and MMTV-Wnt-1 transgenic mice overexpress Muc1, and as such recapitulate the overexpression of human MUC1 observed in most breast carcinomas. Together, these studies indicate that MUC1 is not merely overexpressed in breast cancer, but also plays an important role in the progression towards invasive carcinoma. Accumulating evidence suggests a functional role for MUC1 in signal transduction. The 72 amino acid tail of MUC1 contains 7 tyrosines, six of which are 100% conserved across mammalian species [8]. The cytoplasmic tail of MUC1 is phosphorylated on tyrosine residues in epithelial cell lines and mouse mammary gland and contains potential docking sites for SH2 containing proteins in addition to a number of possible kinase recognition sites [8-11]. Indeed, MUC1 cytoplasmic tail interacts with a variety of proteins involved in neoplasia and cell adhesion such as EGFR, erbB2, erbB3, erbB4, c-Src, PKCδ, Grb2, β-catenin, GSK3β, and $p120^{ctn}$ [11-17].

We investigated the mechanisms underlying the delay in tumor progression in the absence of Muc1 in the MMTV-PyV MT mouse model of breast cancer in an effort to dissect the signaling role of Muc1 and its contribution to transformation. In the MMTV-PyV MT mammary tumors, activation of the intracellular kinase c-Src is pivotal to the PyV MT-induced transformation [18]. Following c-Src binding and activation, PyV MT modulates the activities of a variety of cellular proteins that function as regulators of cell proliferation such as protein phosphatase 2A, phosphatidylinositol 3-kinase (PI3K), Shc, 14-3-3 proteins and phospholipase C-γ1 [19]. As mentioned above, c-Src is among the signaling molecules known to associate *in vitro* with the cytoplasmic tail of MUC1. c-Src phosphorylates the MUC1 cytoplasmic domain at the YEKV sequence and is involved in regulating the interaction of MUC1 with GSK3β and β-catenin [12]. We hypothesize that MUC1, through its interaction with c-Src, is an important contributor to PyV MT-induced

transformation and functions as a scaffold for oncogenic kinases and signaling proteins. Our data suggest that Muc1 functions to promote PyV MT signaling mediated by c-Src activation.

In addition to determining the effect of Muc1 expression on oncogenic signaling in the MMTV-PyV MT model, we examined the effect of MUC1 overexpression in the mammary gland. We observed that the overexpression of human MUC1 in the mouse mammary gland driven by the MMTV promoter resulted in frank neoplasia accompanied by a failure of multiparous glands to dedifferentiate and involute. We have determined that this lack of involution can be observed in non-tumor bearing mammary glands as well, in that post-lactational involution is delayed 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

To understand the mechanism by which Muc1 affects PyV MT-induced tumorigenesis, we examined the effect of Muc1 expression on c-Src. Virgin female transgenic mice that express the PyV MT oncogene, driven by the MMTV promoter, develop multifocal mammary tumors by 2 months of age and by 4 months greater that 50% of the mice develop lung metastases [20]. The intracellular tyrosine kinase, c-Src, plays a pivotal role in mediating the oncogenic effects of the PyV MT antigen. As described in Table 1, the mouse models used in this study include MMTV-PyV MT transgenic mice either on a $Muc1^{+/+}$ wildtype background (MT) or a $Muc1^{-/-}$ knockout background (MTK). A detailed description of the $Muc1^{-/-}$ knockout mice was previously published [6]. We also crossed the MMTV-PyV MT mice with mice expressing the human MUC1 transgene to generate MMT bitransgenics. The MUC1 transgenic mice express the entire human MUC1 gene sequence including the 1.5 5kb upstream of the MUC1 gene to drive its expression [21]. Normal mammary glands from age-matched wildtype mice were used as controls. As described in the May 2003 report, we have established that Muc1 physically interacts and colocalizes with c-Src in MT and MTK tumors by co-immunoprecipitation and immunofluorescence studies. This falls in line with previous *in vitro* studies that described the interaction between Muc1 and c-Src and thus substantiates a physiological relevance for the association.

Previous findings have demonstrated that lack of Muc1 expression in mice causes a significant delay in PyV MT-induced mammary tumorigenesis. In the PyV MT tumors, we observed an interaction between Muc1 and c-Src, which is a key molecule in PyV MT transformation. We next investigated the effect of the Muc1/c-Src interaction on PyV MT-mediated transformation. We proposed two mechanisms by which Muc1 could promote tumorigenesis. One is that Muc1 may directly influence c-Src kinase activity and promote c-Src activation by inducing conformational changes or recruiting c-Src activating molecules. Alternatively, Muc1 may influence c-Src signaling by promoting its association with downstream signaling molecules.

We investigated the effect of Muc1 expression on c-Src kinase activity. c-Src was immunoprecipitated from lysates of MT, MMT, MTK tumors and normal wildtype 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 $^{32}\gamma$ P-ATP incorporated into a c-Src substrate peptide. The rational behind those experiments was to determine whether the interaction between Muc1 and c-Src was allowing c-Src to assume a more active state possibly through a change in c-Src conformation or through the indirect association of Src with an activating kinase or phosphatase mediated by binding to the cytoplasmic tail. As expected we observed elevates Src kinase activity in the MMTV-PyV MT tumors compared to wild-type mammary glands. In order to address the question of whether Muc1 modulates c-Src kinase activity, we compared c-Src activity in the MT tumors with the MTK tumors lacking Muc1. As reported previously, preliminary results suggested that tumors lacking Muc1 (MTK) exhibited reduced c-Src kinase activity when compared with MT tumors that express Muc1. However, analysis of increased mouse numbers did not substantiate these results. At that point we had analyzed full-grown tumors from MT and MTK animals. We thought 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 MT and MTK glands at an earlier time point. To this end, we have harvested and analyzed mammary glands from both MT and MTK mice at 8 weeks of age, at which point the mammary glands would be hyperplastic. We did not observe a significant difference in c-Src kinase activity between the MT and MTK hyperplastic glands (Figure 1). This result is perhaps not surprising, given previous results that showed that the associated kinase activities in mammary tumors decoupled from either the Shc or the PI3K signaling pathway were comparable to that of wildtype PyV MT [22]. The failure to observe a significant difference in c-Src activity in the absence of Muc1 could be attributed to the heterogeneity of the tumor samples and the elevated Src kinase activity in the tumors, whereby subtle changes in c-Src activity could pass undetected. These observations suggest that the delayed tumorigenesis was not a consequence of the inability to functionally activate c-Src tyrosine kinase. As such, we ruled out the first suggested mechanism for the role of Muc1 in PyV MT signaling.

We determined the effect of Muc1 on several downstream signaling pathways of c-Src. We directed our efforts towards analysis of c-Src downstream signaling pathways in the presence or absence of Muc1. Among the c-Src substrates that we investigated is β-catenin. β-catenin is a component of the E-cadherin mediated cell-cell junctions. β-catenin is also a signaling component of the Wnt pathway, where it functions as a transcriptional co-activator for mitogenic genes such as c-myc and cyclin D1. In initial experiments looking at the tyrosine phosphorylation of proteins that immunoprecipitate with c-Src , we detected increase tyrosine phosphorylation of a band close to the molecular mass of β-catenin. We then investigated the association between c-Src and β-catenin in the MT and MTK hyperplastic glands. MT and MTK samples were immunoprecipitated with an anti-c-Src antibody and blotted with an anti-β-catenin antibody. An increased association between c-Src and β-catenin was observed in the presence of Muc1 in the MT samples compared with the MTK samples that lack Muc1

(Figure 2). As such, Muc1 may function as a scaffolding protein bringing c-Src in close proximity to its substrates, such as β-catenin. Based on previously published *in vitro* findings, MUC1 is thought to compete with E-cadherin for β-catenin binding and is therefore involved in disrupting cell-adhesion complexes [15]. β-catenin interacts with Muc1 at an SXXXXXSSL site present in the cytoplasmic tail of MUC1 and that this interaction is enhanced by the tyrsoine phosphorylation of MUC1 cytoplasmic tail by c-Src [15]. Since c-Src has been shown to interact with Muc1 and β-catenin in PyV MT-induced tumors, we were interested in determining whether Muc1 expression would influence the association between β-catenin and E-cadherin. To address that question we immunoprecipitated β-catenin from MT, MMT and MTK tumors and immunoblotted for E-cadherin to assay for β-catenin/E-cadherin association. According to the model proposed by Kufe *et al.* we expected to see a decrease in β-catenin/E-cadherin association in the MT tumors that have overexpression of Muc1 compared with MTK tumors that lack Muc1. However, analysis of MT and MTK tumors did not reveal a substantial change in the amount of β-catenin in association with E-cadherin (Figure 3), suggesting an alternate mechanism for weakening cadherin-dependent adhesions in PyV MT tumors.

We also observed increased levels of activated pStat3, a downstream target of c-Src activation, in the presence of Muc1 (Figure 4). Stat3 is involved in promoting cell-cycle progression, cellular transformation and preventing apoptosis [23-26]. Some PyV MT tumors that lack Muc1 exhibit a decrease in Erk2 activation (*data not shown*). Activation of MAPK in association with MUC1 has been demonstrated *in vitro* and in the mammary glands of MMTV-MUC1 transgenic mice. [11, 27]. Increases in Stat3 and Erk2 phosphorylation could provide a mechanism for increased progression of Muc1-expressing PyV MT tumors. Muc1 expression may be enhancing the activation of Stat3 and Erk2, possibly by its association with EGFR and c-Src. Interestingly, analysis of MT and MTK hyperplastic glands suggested a decrease in EGFR tyrosine phosphorylation in MTK glands lacking Muc1 (*data not shown).*

The p85 subunit of phosphatidylinositol 3-kinase (PI3K) is among the key signaling molecules that associate with the PyV MT antigen [28, 29]. Activation of PI3K signaling influences cell proliferation, growth, survival and cytoskeletal organization [30-32]. We investigated whether the presence of Muc1 influenced the interaction of Src with p85. The interaction between c-Src and the p85 subunit of PI3K kinase was evident only in the MT hyperplastic glands that express Muc1 but not in the MTK glands that lack Muc1 (Figure 5). In a previously published study, transgenic mice expressing a mutated form of PyV MT decoupled from p85 binding develop mammary epithelial hyperplasias that are highly apoptotic, suggesting that PyV MT binding to p85 affects cell survival [22]. The analysis of MT and MTK glands did not reveal changes in the activation of Akt, a downstream target of PI3K signaling affecting cell growth and cell survival. Also, MT and MTK hyperplasias and tumors did not exhibit a difference in the rate of apoptosis, suggesting that this may not be the major pathway affected by the lack of Muc1 expression (unpublished data). In addition to influencing growth and survival, PI3K activation mediates activation of the Rac GTP binding protein, which plays a major role in remodeling the actin cytoskeleton [33]. The p85 subunit of PI3K is also among the well-characterized c-Src interacting partners. The association between c-Src and the p85

subunit of PI3K is thought to modulate the localization of activated c-Src at the cell membrane [34]. As such, the presence of Muc1 may be influencing the localization of c-Src at the cell membrane in PyV MT-induced tumors.

The alterations in the association of Src with its downstream effector molecules suggested a possible difference in the cellular localization of Src. In order to address that question we generated primary cell cultures from the mammary epithelial cells derived from MMTV-PyV MT glands on a wildtype or Muc1 null background. In the Muc1 null cells, c-Src is more commonly localized to the perinuclear region as opposed to being localized at the cell membrane in close proximity to its downstream targets (Figure 6B). This suggests that Muc1 is directly or indirectly influencing c-Src localization at the cell membrane. We demonstrate a novel association between Muc1 and a known Src substrate, FAK, in the PyV MT tumors (Figure 6A), which potentially promotes the peripheral targeting of activated c-Src. FAK is as a nonreceptor protein-tyrosine kinase localized within focal adhesions where the cytoskeleton interacts with proteins of the extracellular matrix via integrin receptors. FAK plays an important role in integrin-mediated signaling and modulating processes such as cell growth, differentiation, survival and migration [35]. Following activation by c-Src phosphorylation, FAK interacts with a number of downstream signaling molecules including the adapter protein Grb2 and the p85 subunit of PI3K [36]. In malignant cells MUC1 expression is no longer restricted to apical surfaces but is found throughout the entire cell surface. The binding of MUC1 to Src may help localize the kinase at the cell periphery where it can be in close proximity to target molecules such as β-catenin, FAK and p85. The interaction between MUC1 and FAK has not been described previously. Further investigation into the regulation of this association will shed light on the role of MUC1 on invasion and metastases in the context of integrin-mediated cell-matrix interactions.

While addressing the question of Muc1 signaling in the PyV MT induced tumors, we observed that transgenic mice overexpressing human MUC1 transgene developed mammary tumors. MMTV-MUC1 transgenic mice from two different founders (#9 and #15) were continuously bred for twelve months and then monitored as they aged. Cytoplasmic domain-deleted MUC1 transgenic mice (MMTV-MUC1ΔCT, #23) and wildtype controls were housed similarly. Tumors were observed in five out of eight MMTV-MUC1 mice and none were observed in the eight age-matched wildtype mice. MMTV-MUC1 transgenic mice from both lines develop mammary tumors. As such tumor incidence in the MMTV-MUC1 transgenic mice was 62% higher than in the wildtype mice ($p=0.03$, 95% CI=0.17 to 0.91). Statistical significance was determined using the Fisher Exact Test. While primary mammary gland tumors were observed in both of the MMTV-MUC1 lines, no tumors were observed in either the MMTV-MUC1ΔCT transgenic line (3 mice sacrificed and analyzed at 8 months and 3 mice at 20 months of age) or the wildtype control lines (analyzed at 20 months). Molecular analysis of these tumors shows a tumor-specific co-immunoprecipitation between MUC1 with β-catenin. Examination of the contralateral glands in MMTV-MUC1 transgenics demonstrates that the development of frank carcinomas is accompanied by a failure of multiparous glands to undergo post-lactational involution. Furthermore, uniparous MMTV-MUC1 transgenic mice display decreased postlactational apoptosis, elevated

WAP expression and aberrant pErk2 activation. These findings are the first to determine that MUC1 overexpression promotes *in vivo* transformation of the mammary gland. A detailed study of the MMTV-MUC1 tumors and involuting mammary glands was accepted for publication in the journal Oncogene. The publication entitled "MUC1 overexpression results in mammary gland tumorigenesis and prolonged alveolar differentiation" is appended in the report.

Table 1

MT	**MMTV-PyV MT/Muc1$^{+/+}$**
MTK	**MMTV-PyV MT/Muc1$^{-/-}$**
MMT	**MMTV-PyV MT/MUC1$^{+/0}$**

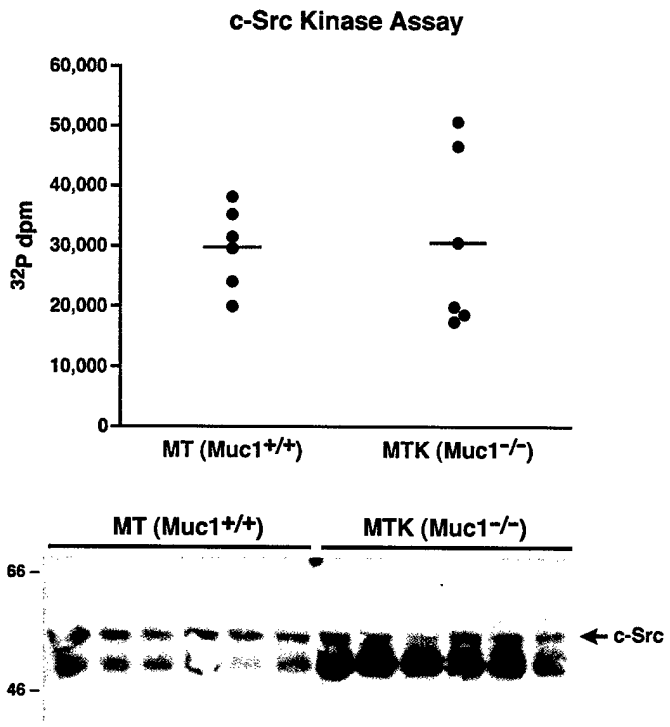

Figure 1. MT and MTK mice exhibited similar levels of c-Src kinase activity. c-Src was immunoprecipitated from lysates of MT and MTK tumors and normal wildtype mammary glands. **A**, kinase activity was measured by the amount of γ ^{32}P ATP incorporated in a c-Src substrate using a kit from Upstate Biotechnology (Lake Placid, NY). **B**, as a control for the amount of c-Src, duplicate immunoprecipitations were carried out and assayed by immunoblotting for c-Src. We compared c-Src kinase activity in MT and MTK samples at the initial stages of tumorigenesis when mice are at 8 weeks of age. There was no significant difference in the mean of c-Src activity between MT and MTK animals.

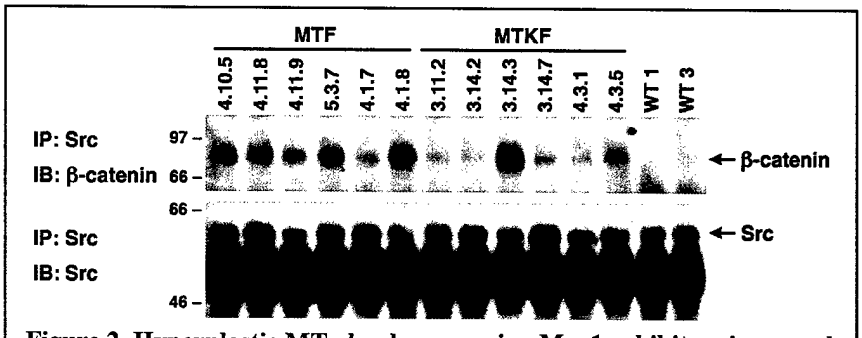

Figure 2. Hyperplastic MT glands expressing Muc1 exhibit an increased association between β-catenin and c-Src than MTK glands lacking Muc1. MT and MTK 8-week old glands (1 mg) were immunoprecipitated with anti-Src (sc-18) and immunblotted for β-catenin (Santa Cruz, sc- 7199), *upper panel* or for c-Src (sc-18), *lower panel*. The data are representative of the pattern observed in 11 samples studied. The high levels of β-catenin in MTK 3.14.3 is attributed to mouse to mouse variation

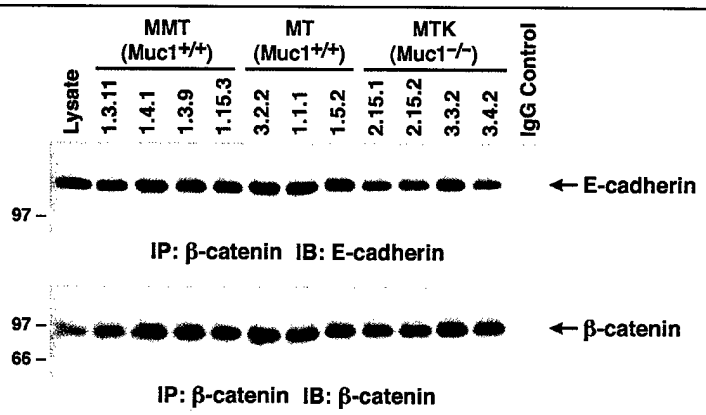

Figure 3. The presence of Muc1 does not influence the binding of β-catenin to E-cadherin. MT and MTK tumor lysates (1 mg) were immunoprecipitated with anti-β-catenin antibody and immunoblotted for E-cadherin. No differences in the amount of β-catenin binding to E-cadherin were observed between MT and MTK tumors.

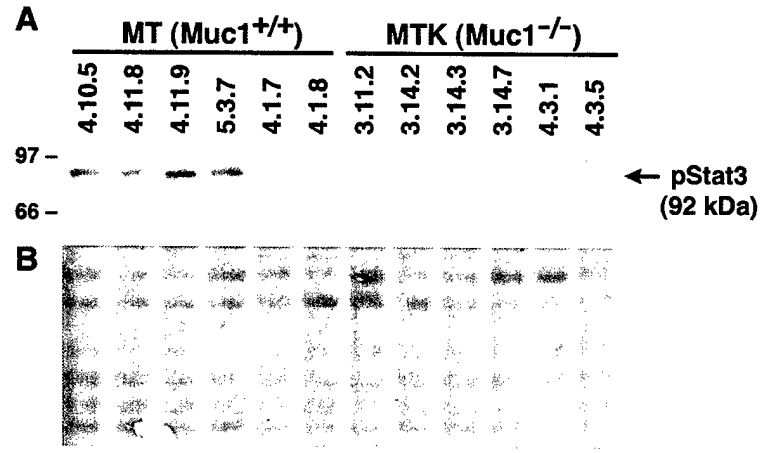

Figure 4. Hyperplastic MT glands expressing Muc1 have higher levels of activated Stat3 than MTK glands lacking Muc1. A, MT and MTK mice were analyzed at 8 weeks of age when the mammary glands are hyperplastic but not frankly tumorigenic. Lysate (100 μg) from MT and MTK tumors were analyzed by western blot for activated Stat3 using an anti-phospho-stat3 (Y705) antibody (Upstate). **B**, Equal protein loading was determined using Ponceau S staining.

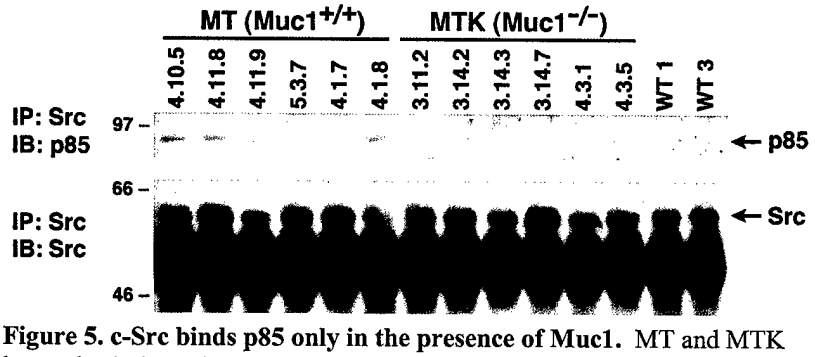

Figure 5. c-Src binds p85 only in the presence of Muc1. MT and MTK hyperplastic 8-week old glands (1 mg) were immunoprecipitated with anti-Src (sc-18) and immunoblotted for p85 (Upstate), *upper panel* or for c-Src (sc-18), *lower panel.*

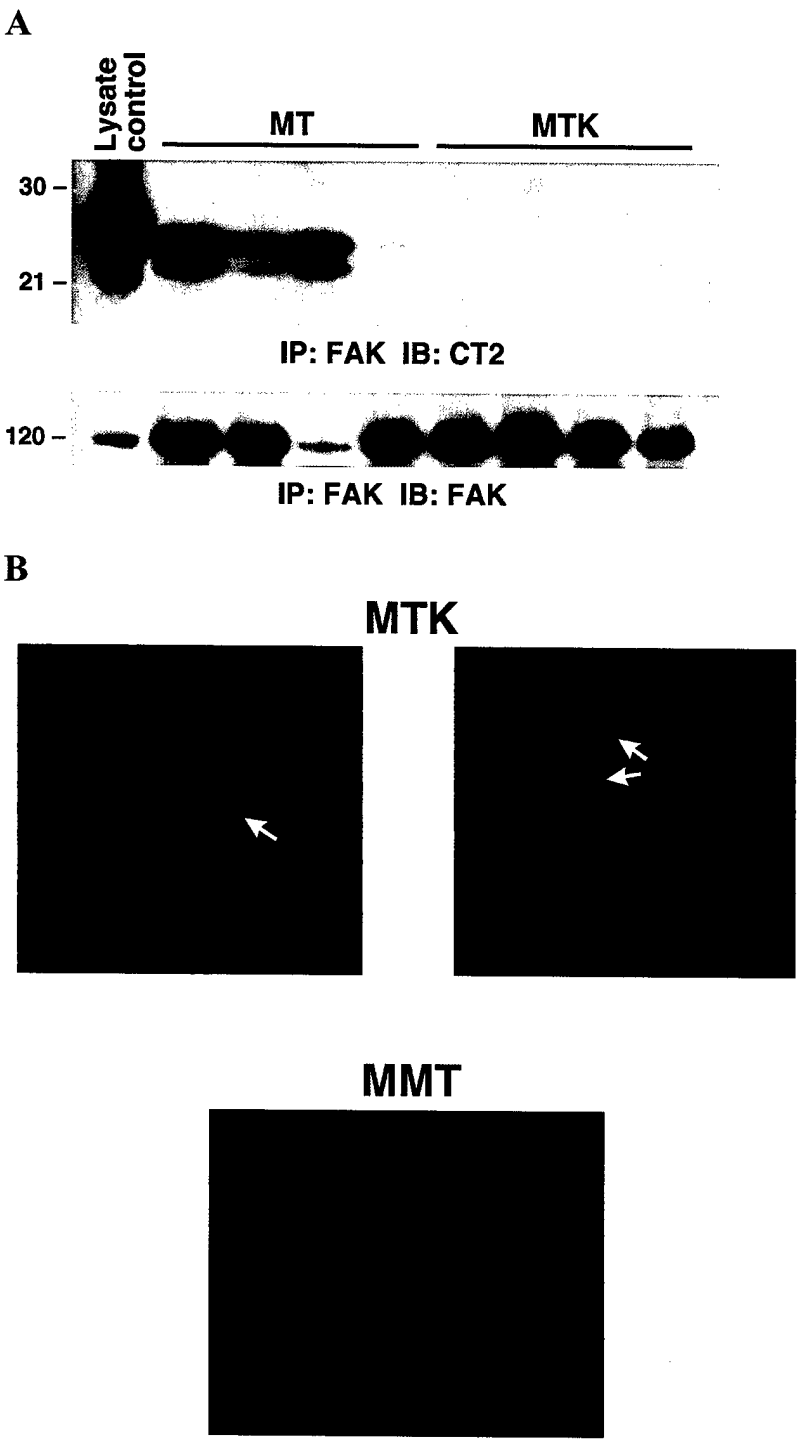

Figure 6. A,Muc1 interacts with FAK in MT tumors and potentially influences c-Src cellular localization. FAK was immunoprecipitated from MT and MTK tumors (1 mg) and immunoblotted for CT2 (*upper panel*) or for FAK (*bottom panel*). MT tumor lysate (100 ug) was used as positive control for Muc1 and FAK expression. **B, Muc1 interacts with FAK in MT tumors and potentially influences c-Src cellular localization.** c-Src localization was determined by immunofluorescence. MT and MMT primary cells were stained with anti-c-Src (2-17) and the nuclear TO-PRO 3 stain and Alexa 546 anti-mouse (red) secondary antibody. Sections were examined at 250X magnification using a Zeiss 510 laser scanning microscope.

KEY RESEARCH ACCOMPLISHMENTS

- c-Src levels and activity were higher in MT and MMT tumors than in normal mammary glands.
- Muc1 co-localizes and physically interacts with c-Src in MT and MMT tumors, corroborating previous *in vitro* observations and thus substantiating a physiological relevance for the association.
- Muc1 influences PyV MT signaling by promoting the association of c-Src with downstream signaling molecules without a direct effect for Muc1 on c-Src kinase activity.
- Muc1 expression promotes the association between c-Src and β-catenin in MT hyperplastic glands.
- Muc1 expression does not decrease the association of β-catenin and E-cadherin.
- Hyperplastic MT glands expressing Muc1 have higher levels of activated Stat3 than MTK glands.
- c-Src associates with the p85 subunit of PI3K only in the presence of Muc1.
- Muc1 directly associates with FAK and can influence c-Src cellular localization.
- MUC1 overexpression in MMTV-MUC1 transgenic mice results in the stochastic formation of mammary gland tumors.
- MUC1 overexpression results in sustained glandular differentiation in multiparous MMTV-MUC1 animals.
- MUC1 and β-catenin interact in MMTV-MUC1 tumors and hyperplasis and in MMTV-MUC1 lactating glands.
- Increased pErk1 and pErk2 activation in the MMTV-MUC1 mammary glands.
- MMTV-MUC1 transgenics exhibit delayed post-lactational involution correlating with the presence of large secretory ducts as determined by whole mount and histological analysis.
- MMTV-MUC1 transgenic glands exhibit elevated WAP expression at day 6 of post-lactational involution.
- Involuting MMTV-MUC1 glands display decreased levels of apoptosis compared with control wildtype glands.
- MMTV-MUC1 glands exhibit increased levels of pErk2 activation at day 8 of post-lactational involution.
- Involuting MMTV-MUC1 glands display increased levels of Erk1/2 activation at day 2 of involution and persistent Erk2 activation at day 8 of involution.

REPORTABLE OUTCOMES

PUBLICATIONS

- 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.* Accepted for publication. Oncogene, Papers in Press.

- Azzah Al Masri and Sandra J. Gendler. *Muc1 interacts with c-Src and affects PyV MT Signaling in the mouse mammary gland.* Manuscript submitted for publication.

POSTERS AND ORAL PRESENTATIONS

- 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. **I gave an oral presentation of the data at the AACR meeting.**

- Azzah Al Masri and Sandra J. Gendler. *Understanding the role of Muc1 in PyV MT induced tumorigenesis.* Abstract and poster at the FASEB meeting: Growth Factor Receptor Tyrosine Kinases in Mitogenesis, Morphogenesis and Tumorigenesis. Tucson, AZ. 8/2/2003-8/7/2003. **I gave an oral presentation of the data at the FASEB meeting.**

CELL LINES AND MOUSE MODELS

- Generation of PyV MT transgenics lacking Muc1 on the FVB background in addition to bitransgenics expressing the mutant form of MUC1ΔCT and PyV MT on a Muc1 knockout backgound.

- Generation of MT, MMT and MTK cell lines derived from transgenic mouse mammary glands.

INTELLECTUAL TRAINING

- Attendance and participation in weekly lab journal clubs (gave 5 presentations/year).
- Attendance and participation in weekly lab meetings in addition to weekly one-on-one meeting with my advisor, Dr. Sandra Gendler, and a biweekly one hour meeting with the lab protein interaction group.
- Attendance and participation in weekly Biochemistry and Molecular Biology departmental seminars.
- Attendance and participation in weekly departmental workshop presentations by graduate students teleconferenced to Mayo Clinic Rochester and Jacksonville (gave 1 presentation/year).
- Attendance and participation at biweekly Biomedical Research Forum (gave 1 presentation, May 2003).
- Participation in seminars by visiting scientists that take place biweekly.
- Application for postdoctoral training with Dr. Dietrich Stephan at TGen, Phoenix, AZ.
- Application for postdoctoral training with Dr. Rafael Fonseca, Mayo Clinic Scottsdale, AZ.

CONCLUSIONS

The MMTV-PyV MT mouse model was used to develop a better understanding of the signaling role of Muc1 in tumorigenesis. The MMTV-PyV MT model has proven invaluable in dissecting the signal transduction pathways that are altered during mammary gland transformation. In the MMTV-PyV MT tumors, Muc1 expression is dramatically increased [37]. Additionally, the significant delay in tumor progression in the MMTV-PyV MT x Muc1$^{-/-}$ defined a functional role for Muc1 in PyV MT-induced tumorigenesis [6]. We analyzed MMTV-PyV MT mammary tumors on a wildtype background compared with tumors of MMTV-PyV MT mice on a Muc1 null background to specifically delineate the effect of Muc1 on PyV MT-induced mammary tumorigenesis. We identified an *in vivo* association between Muc1 and c-Src in PyV MT-induced tumors. We proposed two mechanisms by which Muc1 could promote tumorigenesis. Our findings rule out the first mechanism by which Muc1 may directly influence c-Src kinase activity and promote c-Src activation by inducing conformational changes or recruiting c-Src activating molecules. Instead our data indicate that Muc1 contributes to PyV MT signaling by influencing c-Src signaling in promoting its association with downstream signaling molecules. Muc1 promotes the association of c-Src with signaling targets such as β-catenin and the p85 subunit PI3K. Additionally, an increase in the levels of activated Stat3 and in some animals activated Erk2 in the PyV MT tumors was observed in the presence of Muc1. We demonstrate a novel association between Muc1 and a known Src substrate FAK, which potentially promotes the peripheral targeting of activating c-Src. In primary cell cultures from mammary epithelial cells derived from MT and MTK mice, we observed that in the absence of Muc1, Src is commonly found at the perinuclear region of the cell in contrast to the cytoplasmic and peripheral localization of Src in the presence of Muc1. These findings provide insights into the possible mechanism for the significant delay in tumor progression that is observed in the absence of Muc1. We suggest that the interaction of Muc1 with c-Src, a key player in PyV MT transformation, promotes the binding of c-Src to its downstream targets and influences its cellular localization. Our understanding of the critical role of Muc1 signaling in PyV MT-induced oncogenesis will be invaluable in delineating the effect of MUC1 overexpression in human breast cancer.

The question of whether MUC1 overexpression is a cause or a consequence is at the heart of understanding the role of MUC1 in cancer. We now show that the overexpression of MUC1 in the MMTV-MUC1 mouse model is directly involved in the *in vivo* transformation of the mammary gland. We have found that prolonged expression of MUC1 in the mammary gland results in the stochastic development of unifocal tumors. Coincident with the development of frank neoplasia, prolonged expression of MUC1 inhibited post-lactational involution by the inhibition of apoptosis. As such, increased MUC1 expression is not merely a byproduct of transformation, but can itself promote transformation in physiological context. We demonstrate a decrease in apoptosis in the involuting MMTV-MUC1 transgenic glands in comparison to the wildtype glands. We also observed elevated Erk activation in correlation with MUC1 overexpression.

Our understanding of the biology of MUC1 and its contribution to malignant transformation is constantly evolving. The complexity of the structure of MUC1 mirrors the complexity in the function of this molecule. Burgeoning evidence indicates that MUC1 overexpression is not merely a byproduct of transformation. It contributes to the survival of tumor cells, promoting proliferation and invasion. The fact that overexpression of MUC1 can itself promote transformation in a physiological context sheds new light on the biology of this molecule. MUC1 is considered a therapeutic target in immunotherapy studies and MUC1-based therapy is currently at different stages of clinical testing. However, the interactions of this molecule with key signaling proteins serve as targets for future drug development that potentially influence the differentiation, proliferation and migration of tumor cells.

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ORIGINAL PAPER

MUC1 overexpression results in mammary gland tumorigenesis and prolonged alveolar differentiation

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MUC1 is a transmembrane mucin that was initially cloned from malignant mammary epithelial cells as a tumor antigen. More than 90% of human breast carcinomas overexpress MUC1. Numerous studies have demonstrated an interaction between MUC1 and other oncogenic proteins such as β-catenin, erbB receptors and c-Src, but a functional role for MUC1 in transformation has not been identified. We previously reported the development of transgenic mice that overexpress human MUC1 in the mouse mammary gland (MMTV-MUC1). Analysis of these transgenic mice at an early age demonstrated the ability of MUC1 to potentiate EGF-dependent activation of MAP kinase signaling pathways in the lactating mammary gland. We now report that multiparous MMTV-MUC1 transgenic mice stochastically develop unifocal mammary gland carcinomas late in life. Molecular analysis of these tumors shows a tumor-specific coimmunoprecipitation between MUC1 and β-catenin. Examination of the contralateral glands in MMTV-MUC1 transgenics demonstrates that the development of frank carcinomas is accompanied by a failure of multiparous glands to undergo postlactational involution. Furthermore, uniparous MMTV-MUC1 transgenic mice display decreased postlactational apoptosis, elevated whey acidic protein expression and aberrant pErk2 activation. These findings are the first to determine that MUC1 overexpression promotes *in vivo* transformation of the mammary gland.

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Keywords: mucin; involution; apoptosis; breast cancer; mammary gland

Introduction

MUC1 is a transmembrane mucin that was initially cloned from malignant mammary epithelial cells as a tumor antigen (Gendler *et al.*, 1987). MUC1 contains both a heavily glycosylated extracellular domain and a highly conserved cytoplasmic domain (Gendler *et al.*, 1990; Lan *et al.*, 1990; Ligtenberg *et al.*, 1990; Wreschner *et al.*, 1990). While it is normally expressed on the apical surfaces of most glandular epithelia, MUC1 is expressed over the entire surface of transformed epithelial cells, displaying an altered pattern of glycosylation.

While MUC1 has been identified as aberrantly expressed in greater than 90% of human breast carcinomas (Zotter *et al.*, 1988; Girling *et al.*, 1989), the identification of a functional role for that overexpression has been elusive. Transgenic and knockout mice have provided insight into the role of MUC1 in tumor development, with the loss of Muc1 resulting in a reduction in tumor progression. Transgenic mice expressing the polyomavirus middle T antigen (PyV MT) under control of the mouse mammary tumor virus (MMTV) promoter exhibit a significant delay in tumor growth and a trend towards decreased metastasis when bred onto a Muc1 knockout background (Spicer *et al.*, 1995b). Also, lack of Muc1 expression causes a significant delay in tumor onset in the MMTV-Wnt-1 model of breast cancer (Schroeder *et al.*, 2003). Mammary tumors of MMTV-PyV MT and MMTV-Wnt-1 transgenic mice overexpress Muc1, and as such recapitulate the overexpression of human MUC1 observed in most breast carcinomas. Together, these studies indicate that MUC1 is not merely overexpressed in breast cancer but also plays an important role in the progression towards invasive carcinoma.

Numerous observations point to a functional role of MUC1 in signal transduction. The 72 amino-acid tail of MUC1 contains seven tyrosines, six of which are 100% conserved across mammalian species. The cytoplasmic tail of MUC1 contains potential docking sites for SH2-containing proteins in addition to a number of possible kinase recognition sites (Spicer *et al.*, 1995a). Indeed, MUC1 cytoplasmic tail interacts with a variety of proteins involved in neoplasia and cell adhesion such as epidermal growth factor receptor (EGFR), erbB2,

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erbB3, erbB4, c-Src, protein kinase C delta (PKCδ), Grb2, β-catenin, GSK3β and p120^{ctn} (Pandey *et al.*, 1995; Yamamoto *et al.*, 1997; Li and Kufe, 2001; Li *et al.*, 2001a, b; Schroeder *et al.*, 2001, 2003; Ren *et al.*, 2002). *In vitro* studies show that a defined sequence in the cytoplasmic tail of MUC1 (SXXXXXSSLS) interacts with β-catenin, and can compete with E-cadherin at adherens junctions for binding of β-catenin when expressed at high levels (Yamamoto *et al.*, 1997). The serine/threonine kinase GSK3β can also bind the cytoplasmic domain of MUC1, and binding to this kinase competes away binding between MUC1 and β-catenin in breast cancer cell lines (Li *et al.*, 1998). Studies in our lab determined that MUC1 and β-catenin biochemically interact in a tumor-specific manner in MMTV-Wnt-1 transgenic mice (Schroeder *et al.*, 2003). Finally, both c-Src and the erbB receptor tyrosine kinases phosphorylate MUC1, as well as the serine/threonine kinase PKCδ, and these phosphorylation events promote interactions between β-catenin and MUC1 (Li *et al.*, 2001a, b; Schroeder *et al.*, 2001; Ren *et al.*, 2002). These observations indicate that the cytoplasmic domain of MUC1 may provide a proto-oncogenic signal.

We have previously reported the generation of transgenic mouse models overexpressing various forms of MUC1 in the mouse mammary gland (Schroeder *et al.*, 2001). Using the MMTV promoter, we directed the expression of either full-length, cytoplasmic domain-deleted or tandem repeat-deleted human MUC1 to the mammary gland in an effort to understand the effects of MUC1 overexpression in this tissue. These studies allowed us to determine a variety of signaling partners and downstream effects of MUC1 overexpression (including interacting with erbB receptors and potentiation of Erk1 and Erk2 activation). An expansion of our analysis of the MMTV-MUC1 transgenics included a long-term study of MUC1 overexpression, which resulted in the observation that MUC1 overexpression results in frank neoplasia accompanied by a failure of multiparous glands to dedifferentiate and involute. We have determined that this lack of involution can be observed in nontumor-bearing mammary glands as well, in that postlactational regression is suppressed by MUC1 overexpression. These data show, for the first time, that MUC1 can act as a promoter of transformation in the mammary gland. Furthermore, these data indicate that the mechanism for MUC1-induced tumorigenesis may be through the inhibition of normal glandular involution.

Results

MUC1 overexpression results in the stochastic formation of mammary gland tumors

We previously described the analysis of three MUC1 transgenic mouse models, one overexpressing full-length human MUC1 (MMTV-MUC1), a second overexpressing human MUC1 lacking the cytoplasmic domain (MMTV-MUC1ΔCT) and a third overexpressing human MUC1 lacking the tandem repeat extracellular domain (MMTV-MUC1ΔTR) (Schroeder *et al.*, 2001). The initial characterization of these transgenic mice focused on signal transduction initiated by MUC1 overexpression, resulting in the determination that MUC1 interacts with erbB receptors, and can modify the activation of Erk1/2 mitogen-activated protein kinases (MAPK) in an EGF-dependent manner. An expansion of our previous study included determining the effects of MUC1 overexpression over the lifetime of the mouse mammary gland. MMTV-MUC1 transgenic mice from two different founders (#9 and #15) were continuously bred for 12 months and then monitored as they aged. Cytoplasmic domain-deleted MUC1 transgenic mice (MMTV-MUC1ΔCT, #23) and wild-type controls were housed similarly. Tumors were observed in five out of eight MMTV-MUC1 mice and none were observed in the eight age-matched wild-type mice. As such, tumor incidence in the MMTV-MUC1 transgenic mice was 62% higher than in the wild-type mice ($P=0.03$, 95% CI = 0.17–0.91). Statistical significance was determined using the Fisher's exact test. While primary mammary gland tumors were observed in both of the MMTV-MUC1 lines, no tumors were observed in either the MMTV-MUC1ΔCT transgenic line (three mice killed and analysed at 8 months and three mice at 20 months of age) or the wild-type control lines (analysed at 20 months). Pathological analysis of the tumors indicated a wide range of tumor subtypes in the MMTV-MUC1 transgenics, including papillary carcinoma (Figure 1a), solid carcinoma (Figure 1b), adenosquamous carcinoma (Figure 1c), microacinar carcinoma (Figure 1d) and lobular hyperplasia (Figure 1e) (M.D. Robert Cardiff, , U.C. Davis, personal communication).

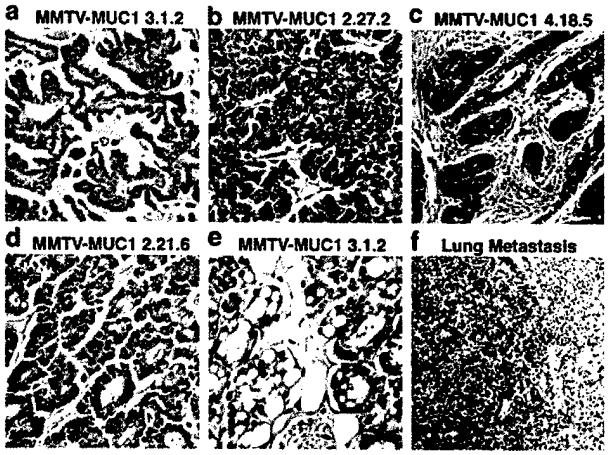

Figure 1 MUC1 overexpression results in the stochastic formation of mammary tumors. MMTV-MUC1 induced tumors, stained by hematoxylin and eosin, included a wide range of tumor subtypes including: papillary (**a**), solid carcinoma (**b**), adenosquamous carcinoma (**c**) microacinar carcinoma (**d**) and lobular hyperplasia (**e**). The expression of the human MUC1 transgene was detected in the lung metastasis of an MMTV-MUC1-induced tumor using an antibody to the FLAG epitope of the MUC1 transgene (**f**). Images (**a**–**f**) were captured at a magnification of $\times 200$

In addition, we observed tumors in the lungs of four of five MMTV-MUC1 transgenic mice with mammary gland tumors. The lung tumors express the MUC1 transgene as determined by immunohistochemical detection of the FLAG epitope present in the transgenic construct (Figure 1f). While the FVB strain is prone to the development of primary lung tumors (at 14 months, 26% of FVB females develop spontaneous lung tumors; Mahler *et al.*, 1996), the percentage of pulmonary tumor formation in the presence of the MMTV-MUC1 transgene was substantially higher. These data indicate that mice transgenic for MMTV-MUC1 can develop primary mammary gland carcinomas as well as pulmonary carcinomas.

MUC1 overexpression results in sustained differentiation in multiparous animals

All mice described above (whether tumor bearing or normal) were killed by 20 months of age and analysed for changes in the normal mammary gland architecture. The mammary glands of MMTV-MUC1ΔCT and wild-type females displayed the normal architecture expected from involutional regression (Figure 2c and d). However, contralateral mammary glands from the MMTV-MUC1 animals were not regressed, but instead maintained a fully differentiated phenotype, although none of the animals were pregnant at the time of being killed or had been pregnant for at least 4 months prior to being killed (Figure 2a and b). This ability to sustain a differentiated phenotype suggested that MUC1 overexpression may either inhibit apoptotic regression that normally follows the end of lactation or may promote hyperproliferation of epithelial cells.

To determine the rate at which the transformed phenotype was observable in the mammary gland of transgenic animals, we isolated mammary glands from 8-month-old lactating MMTV-MUC1 transgenic animals and wild-type littermates. In tumor-free MMTV-MUC1 animals, mammary glands were analysed for changes in normal architecture. In the MMTV-MUC1 transgenics but not in the age-matched wild-type females, we observed regions of the mammary gland that appeared histologically atypical from the normal lactating gland (Figure 3a, c). Hyperplastic epithelial cells were surrounded by adipose tissue and some secretory droplets were present despite the absence of full lactational development in those areas of the gland. In addition, we analysed a multiparous MMTV-MUC1 mammary gland that was not pregnant or lactating at the time of being killed. Histological analysis of this particular MMTV-MUC1 mammary gland revealed hyperplasia and abnormal architecture (Figure 3b) (M.D. Thomas Lidner, Mayo Clinic Scottsdale, personal communication).

MUC1 and β-catenin interact in MMTV-MUC1 tumors and hyperplasia and in MMTV-MUC1 lactating glands

β-catenin interacts with the cytoplasmic tail of MUC1 in a tumor-specific manner (Schroeder *et al.*, 2003). Through coimmunoprecipitation studies, we found that β-catenin interacts with the cytoplasmic tail of MUC1 in all MMTV-MUC1 tumors, but not in control wild-type mammary glands (Figure 4, upper panel). This association between MUC1 and β-catenin was also detected in the lactating glands of 8-month-old MMTV-MUC1 mice and in two out of four of the age-matched lactating wild-type mice (Figure 5b, upper panel). The interaction that occurs in the wild-type lactating gland could be attributed to the overexpression of Muc1 that occurs during lactation and the cellular changes associated with milk secretion. Note that while transgenic MUC1 was expressed (Figure 5a), overall levels of both β-catenin and endogenous Muc1 were similar in both the transgenic and wild-type mammary glands (Figure 5b, middle and

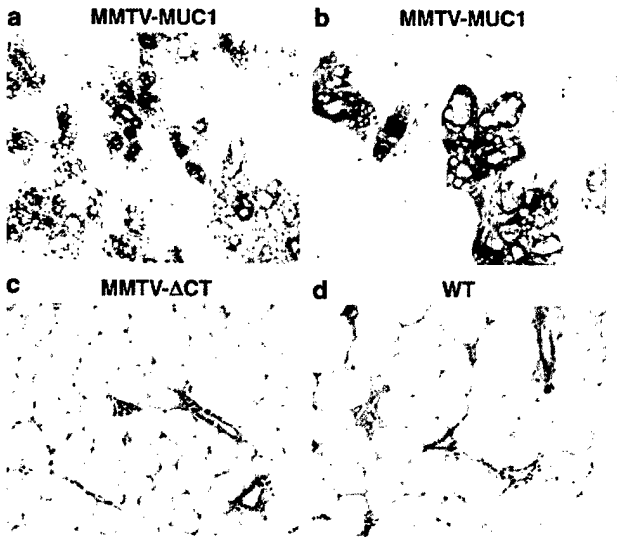

Figure 2 MUC1 overexpression results in sustained differentiation in multiparous females. Glands of MMTV-MUC1 multiparous females that had not undergone a pregnancy in greater than 4 months retained the appearance of pregnancy, with fully differentiated glands and alveoli (**a** and **b**) as opposed to fully regressed glands in the MMTV-ΔCT (**c**) and wild-type females (**d**), both stained by hematoxylin and eosin. The hyperplastic glands of MMTV-MUC1 transgenics show expression of the transgene upon immunohistochemical analysis, using an antibody (B27.29) that recognizes human MUC1 but not mouse Muc1. Images were taken at a magnification of ×200

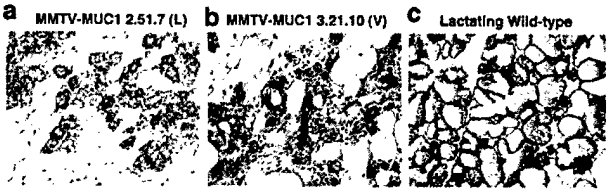

Figure 3 Mammary hyperplasia is evident in 8-month-old MMTV-MUC1 transgenic glands. The glands were stained by hematoxylin and eosin. Areas of hyperplasia were present in the 8-month-old MMTV-MUC1 lactating gland (**a**, (L) is for lactating) and similar areas of hyperplasia were observed in a nonpregnant and nonlactating gland of an 8-month-old MMTV-MUC1 female (**b**, (V) is for hyperplastic). (**c**) is a lactating wildtype gland

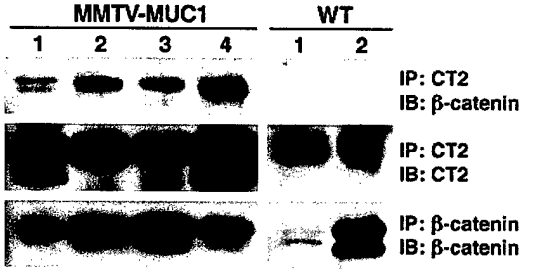

Figure 4 MUC1 associates with β-catenin in the MMTV-MUC1 tumors. Lysates (1 mg) of MMTV-MUC1 tumors and control wild-type (WT) mammary glands were immunoprecipitated with an anti-MUC1 antibody (CT2) and immunoblotted with anti-β-catenin, upper panel. The levels of MUC1 were determined by immoblotting with anti-MUC1 (CT2), middle panel. β-catenin was immunoprecipitated from normal and tumor lysates and detected by immunoblotting with anti-β-catenin, bottom panel. The level of β-catenin detected in WT2 appears high, which is apparently due to mouse-to-mouse variation

bottom panels). In addition, MUC1 was found to interact with β-catenin in a hyperplastic gland from a virgin 8-month-old nonpregnant nonlactating MMTV-MUC1 transgenic (Figure 5b, animal #3.21.10, upper panel).

Increased pErk1 and pErk2 activation in the MMTV-MUC1 mammary glands

Previous studies with the MMTV-MUC1 transgenics have demonstrated that high levels of MUC1 in the lactating mouse mammary gland strongly induced EGF-dependent activation of the extracellular signal-regulated protein kinases Erk1 (p44) and Erk2 (p42) (also known as MAPKs) (Schroeder *et al.*, 2001). Erk1 and Erk2 are two downstream effectors of the EGFR and subsequent Ras signaling pathway, and are commonly activated in EGFR-induced transformation (Grant *et al.*, 2002). Thus, we were interested in determining the effects of MUC1 overexpression on Erk1 and Erk2 activation. We analysed seven approximately 8-month-old transgenic and six wild-type glands, and observed increased levels of dual phosphorylated Erk1 and Erk2 in five out of seven of the MMTV-MUC1 lactating glands as well as in the hyperplastic MMTV-MUC1 3.21.10 gland, while total Erk levels were similar to wild-type glands. A representative image is shown in Figure 5c.

MMTV-MUC1 transgenics exhibit delayed postlactational involution

In an effort to understand the mechanism of prolonged glandular differentiation in the older, multiparous MMTV-MUC1 transgenics, we analysed young uniparous females for differences in postlactational involution. Following lactation, the mammary gland normally involutes via apoptosis, resulting in a defined series of gene transcriptional events, adipose tissue deposition and the loss of differentiated epithelial alveoli (Strange *et al.*, 2001). MMTV-MUC1 transgenic and wild-type females were allowed to lactate for 6–10 days followed

Figure 5 MUC1 associates with β-catenin in MMTV-MUC1 glands and increased Erk1/2 activity correlates with MUC1 overexpression. Mammary gland lysates (100 μg) of lactating (L) 8-month-old MMTV-MUC1 transgenic and wild-type mice were immunoblotted for the transgene using HMFG2 (**a**). MMTV-MUC1 3.21.10 (V) is a hyperplastic gland of a nonlactating and nonpregnant MUC1 transgenic. Lysates (1 mg) of 8-month-old transgenic and wild-type females were immunoprecipitated with anti-β-catenin and immunoblotted with CT2, an anti-MUC1 antibody (**b**, upper panel). The levels of β-catenin were determined by immunoblotting using anti-β-catenin antibody (Santa Cruz Biotechnology) (**b**, middle panel). CT2 detects the expression levels of both MUC1 transgene and endogenous Muc1 (**b**, bottom panel). Mammary gland lysates (100 μg) of 8-month-old MMTV-MUC1 transgenic and wild-type mice were immunoblotted for dpErk1/2 (**c**, upper panel) and Erk1/2 (**c**, lower panel)

by pup removal to initiate involution. The animals were then killed at various time points and their mammary glands were analysed 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 wild-type mice, and observed a delay in the postlactational involution of MMTV-MUC1 glands in comparison to the wild-type controls (Figure 6). At day 4 of involution, we noted the presence of lobuloalveolar structures in the mammary glands of both the MMTV-MUC1 transgenics (Figure 6a) and the wild-type controls (Figure 6d). By day 6 the collapse of lobulo-alveolar structures proceeded normally in the mammary glands of the wild-type females (Figure 6e). In contrast, the mammary glands of MMTV-MUC1 transgenics were still filled with lobuloalveolar structures (Figure 6b). At day 8 of involution, the wild-type glands had fully regressed (Figure 6f), while large,

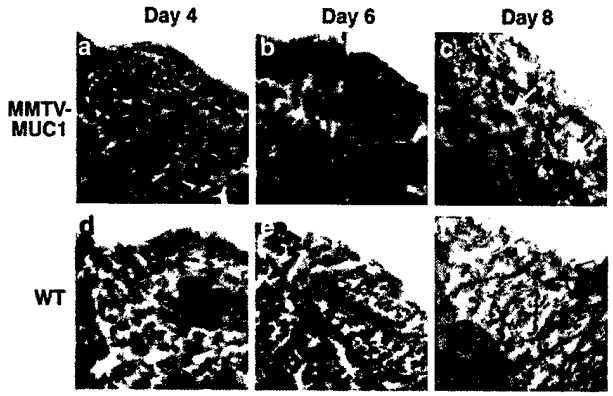

Figure 6 Mammary gland involution is delayed in the MUC1 transgenic mice. Representative images of whole mounts of involuting thoracic mammary glands from MMTV-MUC1 transgenic females (**a–c**) and wild-type controls (**d–f**) at day 4 of involution (**a** and **d**), day 6 of involution (**b** and **e**) and day 8 of involution (**c** and **f**). Arrows depict the large secretory ducts that are present in the MMTV-MUC1 glands at day 8 of involution. Images were captured at a magnification of $\times 250$

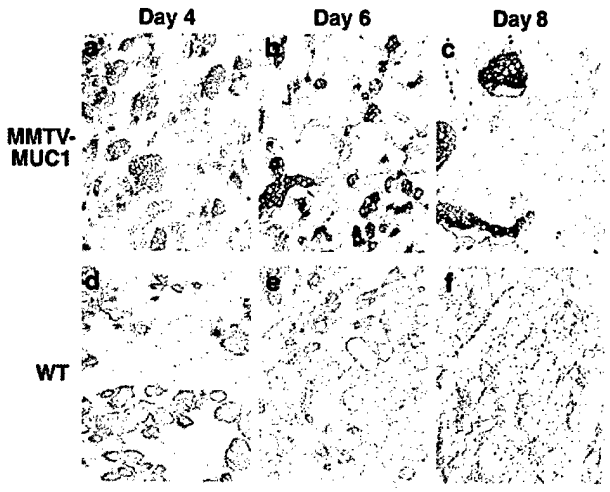

Figure 7 MUC1 is highly expressed within the alveolar lumens and secretory ducts of MUC1 transgenic mice. Paraffin-embedded inguinal glands from MUC1 transgenic females (**a–c**) and wild-type controls (**d–f**) at day 4 of involution (**a** and **d**), day 6 of involution (**b** and **e**) and day 8 of involution (**c** and **f**). 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). Wild-type glands (**d–f**) stained with CT2 (recognizes the last 17 amino acids of the cytoplasmic tail of both human and mouse Muc1). Wild-type glands stained with B27.29 were negative (data not shown). Images were captured at a magnification of $\times 100$

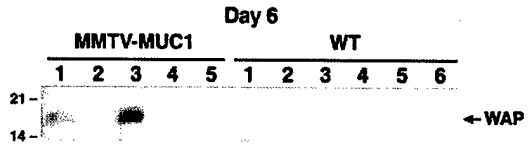

Figure 8 Increased WAP expression is observed in the mammary glands of MUC1 transgenics at day 6 of involution. Protein lysates (100 μg) from thoracic mammary glands of transgenic glands (MMTV-MUC1) and wild-type glands at day 6 of involution were examined by Western blot analysis for WAP expression. Increased WAP expression was observed in two out of five MUC1 transgenics, while it was completely absent in their wild-type counterparts at day 6 of involution

secretory ducts were still present in the MMTV-MUC1 glands (Figure 6c).

MMTV-MUC1 and control wild-type mammary glands obtained at days 4, 6 and 8 were also sectioned and stained with hematoxylin and eosin to examine gland differentiation histologically. We observed no significant differences between MMTV-MUC1 and wild-type glands at days 4 and 6. However, at day 8 MMTV-MUC1 transgenics, showed large secretory ducts that were no longer present in the wild-type controls (data not shown). This delayed involution at day 8 in the transgenic gland correlates with high expression levels of the MUC1 transgene within the large secretory ducts (Figure 7). We performed immunohistochemical analysis to examine MUC1 expression in MMTV-MUC1 and control wild-type glands at days 4, 6 and 8. Wild-type animals were stained for endogenous Muc1 (Figure 7d–f) and MMTV-MUC1 animals were stained for the expression of the MUC1 transgene using an antibody specific to human MUC1 (Figure 7a–c). Similar to wild-type Muc1, the MUC1 transgene was predominantly apically expressed within the alveolar lumens and secretory ducts. Figure 7f depicts the collapse of the lumenal spaces in the wild-type at day 8, while large lumenal spaces with appreciable amounts of MUC1 were present in the MMTV-MUC1 transgenics (Figure 7c).

Increased whey acidic protein (WAP) expression

WAP is normally highly expressed during lactation and its expression is downregulated during the early phase of involution (Burdon *et al*., 1991; Marti *et al*., 1999). We examined WAP expression by Western blot analysis in the transgenic and wild-type animals at days 2, 4, 6 and 8 of involution. As expected, WAP expression decreased in the wild-type mice from days 2 to 8. However, at day 6, a subset of MMTV-MUC1 glands (2/5) continued to express elevated levels of WAP when compared to wild-type mice (Figure 8). We studied the expression levels and activation of a number of key apoptotic and antiapoptotic markers at days 2, 4, 6 and 8 of involution. We saw no observable differences in pStat3, pStat5, pAkt and caspase-3 (data not shown).

Decreased apoptosis in the MMTV-MUC1 mammary glands

Apoptotic cell death accounts for the disappearance of the epithelial compartment during mammary gland involution. In order to determine the mechanism by which MUC1 overexpression resulted in delayed mammary involution, we performed TUNEL assays to quantitate differences in apoptosis at day 2 of involution. We chose to analyse the mammary glands at that time

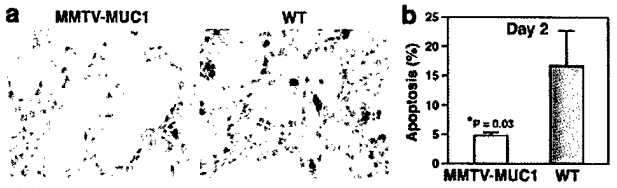

Figure 9 MMTV-MUC1 glands exhibit decreased levels of apoptosis at day 2 of involution. (**a**) Representative images of MMTV-MUC1 and wild-type glands at day 2 of involution analysed by the TUNEL assay. Brown staining depicts apoptotic nuclei. Images were captured at a magnification of ×400. (**b**) MMTV-MUC1 glands have a decreased percentage of apoptotic cells compared with wild-type glands at day 2 of involution

point since that is when the majority of apoptosis takes place (Walker *et al.*, 1989). Figure 9a shows representative examples of TUNEL analysis on paraffin-embedded mammary glands of transgenic and wild-type mice. Mammary glands of MMTV-MUC1 transgenic mice displayed a reduced rate of apoptosis compared to the wild-type glands. We found that the percentage of apoptotic cells was 16.7 (±6.1) for the wild-type glands and 4.8 (±0.5) for MMTV-MUC1 transgenics, $P=0.03$ from the two-sample *t*-test (Figure 9b).

Increased pErk2 activation in the MUC1 transgenic mammary glands

The MMTV-MUC1 transgenics display morphological inhibition of postlactational involution as detected by whole mount and histology (Figures 6 and 7), biochemical inhibition as determined by continued expression of the differentiation protein WAP (Figure 8) and are functionally inhibited as shown by the failure of MMTV-MUC1 glands to undergo wild-type levels of apoptosis (Figure 9). We next investigated if Erk1/2 was also aberrantly activated during involution. We examined mammary glands from both MUC1 transgenic and wild-type controls for their expression profile of Erk1 and Erk2 by Western blot analysis at days 2, 4, 6 and 8 of involution. While wild-type and transgenic glands displayed no difference in Erk1 and Erk2 activation at days 4 and 6 (data not shown), we detected increased Erk1/2 phosphorylation in the MMTV-MUC1 mammary glands when compared to their wild-type counterparts at day 2 (Figure 10a). In addition, there was increased Erk2 activation in the MMTV-MUC1 transgenics at day 8 of involution, compared to the wild-type glands (Figure 10b). This similarity between histological and biochemical alterations in the postlactational gland and glands contralateral to tumors may indicate that these alterations are requisite precursors to MUC1-induced transformation of the mammary gland.

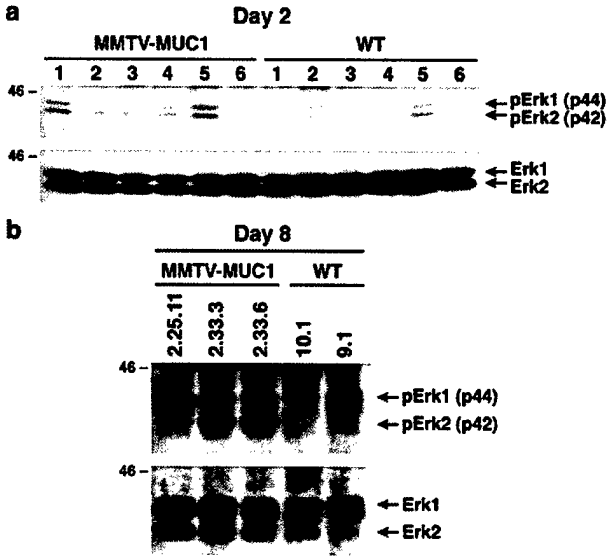

Figure 10 Erk1 and Erk2 activation is increased at day 2 of involution in MMTV-MUC1 glands and Erk2 activation persists at day 8 in MMTV-MUC1 transgenic mammary glands. Protein lysates (100 μg) from thoracic mammary glands of transgenic glands (MMTV-MUC1) and wild-type glands were examined by Western blot analysis for Erk1 and Erk2 expression and activation at day 2 (**a**) and day 8 of involution (**b**). Erk1/2 were detected with p42/44 (Cell Signaling) and antibodies specific to dual phosphorylated Erk1/2 were used to detect active Erks (Sigma). (**a**) Increased Erk1/2 activation is observed in MMTV-MUC1 glands at day 2 of involution (**a**, upper panel) and no differences were observed in the levels of Erk1/2 (bottom panel). (**b**) pErk2 is still present in the MMTV-MUC1 transgenic mammary glands at day 8 of involution, while pErk2 is absent in the wild-type counterparts (upper panel). There is corresponding decrease in Erk2 levels in the wild-type compared to MUC1 transgenics (lower panel)

Discussion

We have found that prolonged expression of MUC1 in the mouse mammary gland results in the stochastic development of unifocal tumors. Coincident with the development of frank neoplasia, prolonged expression of MUC1 inhibited postlactational involution by the inhibition of apoptosis. This is the first report to demonstrate that increased MUC1 expression is not merely a byproduct of transformation, but can itself promote transformation in a physiological context.

Frequently, these same mechanisms are observed as normal epithelium progresses to transformation, as is observed in the formation of hyperplastic alveolar nodules in precancerous mammary glands (Strange *et al.*, 2001). Mammary gland involution is a developmentally regulated process triggered upon cessation of lactation and consists of elimination of unneeded epithelial cells by apoptosis and subsequent tissue remodeling. It has been demonstrated that premalignant hyperplasias do not respond to apoptotic stimuli and fail to regress during postlactational involution (Strange *et al.*, 2001). It is likely that the sustained differentiation and the delay in involution in the MMTV-MUC1 mammary glands resulted in the persistence of a population of epithelial cells that are resistant to apoptosis and hormonal regulation. In turn, these cells could acquire additional mutations leading to transformation and ultimately the development of mammary tumors.

In our study, we observed elevated Erk activation in lactating MMTV-MUC1 glands and in an MMTV-MUC1 hyperplastic nonlactating gland. Consistent with our findings, previous studies have linked MUC1 to Erk1/2 activation. We have previously demonstrated that MMTV-MUC1 transgenic mice undergoing lactation have the ability to activate Erk1/2 in response to EGF, whereas wild-type mammary glands do not (Schroeder *et al.*, 2001). In a separate study, Meerzaman *et al.* (2001) demonstrated the ability of CD8/MUC1 chimeric receptors to activate Erk2 phosphorylation in COS cells. Erk1 and Erk2 are serine/threonine protein kinases that belong to the highly conserved family of MAPKs that function in mediating cellular responses to a variety of extracellular signals (Kolch, 2000). Overexpression of Erk1 and Erk2 has been detected in breast cancer cells with a five- to 10-fold increase in activity over benign conditions. The overexpression of Erk1 and Erk2 was detected in epithelial cells of primary breast cancer as well as at distant metastases (Sivaraman *et al.*, 1997). A recent study found that Erk1 and Erk2 are not only overexpressed but also hyperactivated in human breast tumors compared with normal tissue. Interestingly, Erk2 expression levels were higher than Erk1 in most tumor samples (Mueller *et al.*, 2000). Not much is known about the independent functions of Erk2 as opposed to Erk1; however, it has been reported that Erk2 plays a critical role in mediating EGF-stimulated proliferation in MCF-7 breast cancer cells (Flury *et al.*, 1997). The specific downstream effects of Erk1 and Erk2 activation in the mammary epithelial cells are still not defined. However, a recent report indicates that inhibition of Erk1 and Erk2 activation results in increased apoptosis after BRCA1 expression in MCF-7 breast cancer cells (Yan *et al.*, 2002). This suggests that the increases in Erk1 and Erk2 activation associated with MUC1 overexpression could provide a mechanism for the failure to eliminate cells by apoptosis, resulting in premalignant hyperplasia that contributes to mammary tumors.

The current question pertaining to understanding the role of MUC1 in cancer is delineating which domain of MUC1 is necessary to drive tumorigenesis. Much research has focused on studying the cytoplasmic domain of MUC1, which contains seven conserved tyrosine residues and a number of consensus binding motifs. MUC1 cytoplasmic tail interacts with a variety of proteins involved in neoplasia and cell adhesion such as EGFR, erbB2, erbB3, erbB4, c-Src, PKCδ, Grb2, β-catenin, GSK3β and p120^{ctn} (Pandey *et al.*, 1995; Yamamoto *et al.*, 1997; Li and Kufe, 2001; Li *et al.*, 2001a, b; Schroeder *et al.*, 2001, 2003; Ren *et al.*, 2002). c-Src and erbB receptor tyrosine kinases and the serine/threonine kinase PKCδ are among the kinases that are known to phosphorylate the cytoplasmic domain of MUC1. The phosphorylation of the cytoplasmic tail of MUC1 is thought to regulate its association with two antagonistic proteins, β-catenin and GSK3β. MUC1, through its various cytoplasmic tail interactions, may act as a scaffolding protein to enhance growth-promoting and oncogenic signals. It will be interesting to determine whether the lack of the cytoplasmic tail of MUC1 will alter the delayed involution phenotype observed in the MMTV-MUC1 mice.

Previously, our lab has demonstrated that tumor-specific MUC1/β-catenin complexes are detected in primary breast tumors and metastatic lesions (Schroeder *et al.*, 2003). We speculated that these complexes may promote metastatic invasion by directing β-catenin to the leading edge of the cell and facilitating its interaction with fascin. Recently published studies described the association between the cytoplasmic tail of MUC1 and β-catenin in the nucleus of human pancreatic cancer cell lines and in the nucleus of multiple myeloma cells (Li *et al.*, 2003a; Wen *et al.*, 2003). In a recently published report, stable expression of human MUC1 in rat 3Y1 fibroblasts resulted in anchorage-independent growth of the cells and tumor formation upon injection into nude mice (Li *et al.*, 2003b). Analysis of the tumor sections depicted the presence of the cytoplasmic tail of MUC1 in the nucleus in a complex with β-catenin (Li *et al.*, 2003b). In fact, the cytoplasmic tail has been shown to activate transcription of cyclin D1, a β-catenin target gene. Mutation of Y46, a critical residue within the cytoplasmic tail of MUC1 that regulates its association with β-catenin, was found to attenuate the function of MUC1 as a transcriptional activator of Wnt target genes and also abrogate its transforming capability (Huang *et al.*, 2003). In the current study, we have shown that the association between β-catenin and MUC1 exists in the MMTV-MUC1 induced tumors but not in the normal mammary gland. We also observe this association in the hyperplastic glands of the MMTV-MUC1 transgenic mice. This suggests that MUC1/β-catenin complex formation may be an early event in mammary transformation.

MUC1 has long been recognized as a tumor-associated antigen. More than 90% of human breast cancers overexpress MUC1. MUC1 overexpression in cancer is thought to influence adhesion, invasion and immune surveillance. Recent work has highlighted a role for the cytoplasmic domain of MUC1 in cell signaling that contributes to malignant transformation of epithelial cells. Similar to the human breast tumors, mouse models of breast cancer such as the MMTV-PyV MT and MMTV-Wnt-1 transgenic mice exhibit overexpression of Muc1. The lack of Muc1 expression causes a significant delay in tumor onset in the MMTV-Wnt-1 mice, suggesting that Muc1 is involved in the initiation stages of cancer in that β-catenin-dependent model. Recent *in vitro* studies demonstrate the ability of MUC1 to transform cells. We now show that the overexpression of MUC1 is directly involved in the *in vivo* transformation of the mammary gland.

Materials and methods

Transgenic animals

Transgenic mice that express the 42 tandem repeat human MUC1 cDNA (referred to as MMTV-MUC1) or human MUC1 lacking the cytoplasmic tail (referred to as MMTV-MUC1ΔCT) have been described previously (Schroeder *et al.*, 2001). Transgenic mice were maintained on an inbred FVB

background and wild-type littermates were used as controls. Two separate MMTV-MUC1 lines (MMTV-MUC1#9 and #15), line #23 of MMTV-MUC1ΔCT and wild-type littermates were bred continuously for 12 months and then monitored for tumor development. Human MUC1 and mouse Muc1 are distinguished as such.

Induction of involution

Uniparous wild-type or transgenic MMTV-MUC1 female mice were allowed to lactate for 6–10 days to allow for full development of lactation, and the day the pups were removed was counted as day 0 of involution. Only mice having litters of >5 pups were included in the study. The animals were killed at 2, 4, 6 or 8 days after pup removal and the mammary glands were harvested.

Whole mounts

Whole mounts of thoracic and inguinal glands were prepared by spreading the glands on a glass slide and fixing in a 1:3 solution of glacial acetic acid and absolute ethanol for 1 h. Following fixation, the glands were washed with 95% ethanol for 15 min, followed by a wash with absolute ethanol for 15 min. The glands were defatted overnight through four changes of acetone. The glands were then rehydrated and stained overnight in 0.2% carmine and 0.5% aluminum potassium sulfate. Destaining was performed by washing for 15 min each in 70% ethanol then 95% ethanol followed by absolute ethanol and then 95% ethanol and finally 70% ethanol. The slides were then stored in glycerol.

Protein analysis

Thoracic glands were prepared for Western blot analysis as described previously in Schroeder *et al.* (2001). Protein concentrations were determined by BCA (Pierce). Protein lysates (100 μg) were separated by SDS–PAGE and transferred to polyvinylidene difluoride membrane (Immobilon). Immunoprecipitations were performed with 1 mg of protein lysate using recombinant protein G–agarose conjugate (Invitrogen). Antibodies for immunoblotting were obtained from the following sources: CT2, a hamster monoclonal antibody developed in house to the cytoplasmic tail of MUC1. CT2 recognizes the cytoplasmic tail of both human MUC1 and mouse Muc1. HMFG-2 and B27.29 are mouse monoclonal antibodies that recognize the tandem repeat of human MUC1. HMFG-2 was the kind gift from Dr Joyce Taylor-Papadimitriou (ICRF, London, UK). B27.29 was kindly provided by Biomira (Edmonton, Alberta, Canada). β-catenin H-102 (10 μg) was used for immunoprecipitation and β-catenin C-18 was used for immunoblotting, both from Santa Cruz; Erk1/2 (Cell Signaling, #9102), dpErk1/2 (Sigma Chemical Company, M8159) and WAP (Santa Cruz Biotechnology, M16) were also used. For immunoblotting, the antibodies were used at the following dilutions: CT2 (1:500), HMFG-2 (1:20), B27.29 (1:1000), β-catenin (1:1000), Erk1/2 (1:1000), dpErk1/2 (1:10 000) and WAP (1:500). HRP-conjugated secondary antibodies were from Pierce and Jackson Laboratories and used at 1:25 000.

Immunohistochemistry

Inguinal mammary glands were fixed in methacarn (methanol:chloroform:acetic acid, 6:3:1) or formalin and processed as described previously (Schroeder *et al.*, 2001). Dilutions for antibodies are as follows. anti-MUC1, B27.29 (1:500), anti-Muc1 or anti-MUC1, CT2 (1:400). Lung metastases were stained with an antibody to the FLAG epitope tag, M5 (1:100, Sigma).

TUNEL

The Apoptag Plus Peroxidase *In Situ* Apoptosis Detection kit (Serologicals Corporation) was used to identify apoptotic nuclei within the mammary glands. Formalin-fixed inguinal mammary glands (at day 2 of involution) from three separate MMTV-MUC1 and three wild-type controls were analysed. The protocol outlined by the manufacturer was modified as described by Garrity *et al.* (2003). We stained duplicate sections and a minimum of 1000 cells was counted per section covering 20 randomly chosen fields (×400). The number of apoptotic cells was calculated as a percentage of total cell count. Statistical significance was determined using the two-sample *t*-test.

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