

Transforming Growth Factor α -Dependent Cancer Progression Is Modulated by Muc1

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Abstract

Transforming growth factor α (TGF α) is a potent inducer of cellular transformation, through its binding and activation of the epidermal growth factor receptor (EGFR). Previous studies in our laboratory showed that EGFR could also be affected by the glycoprotein MUC1, which inhibits ligand-stimulated degradation of EGFR in breast epithelial cell lines. To determine the effect of Muc1 expression on TGF α /EGFR-dependent breast transformation, we crossed the WAP-TGF α transgenic mouse model of breast cancer onto a *Muc1*-null background. We found that the loss of Muc1 expression dramatically affects mammary gland transformation and progression. Although 100% of WAP-TGF α /*Muc1*^{+/+} mice form mammary gland tumors by 1 year, only 37% of WAP-TGF α /*Muc1*^{-/-} form tumors by this time. This difference is also associated with a delay in onset, with a doubling of onset time observed in the WAP-TGF α /*Muc1*^{-/-} compared with the WAP-TGF α /*Muc1*^{+/+} mice. Analysis of signal transduction pathways revealed that activation of cyclin D1 expression is significantly suppressed in tumors derived from WAP-TGF α /*Muc1*^{-/-} animals compared with those expressing Muc1. The loss of Muc1 expression also results in a significant inhibition in the formation of hyperplastic lesions during tumor progression. On the C57Bl/6 inbred background, pulmonary lesions were observed in 28 of 29 WAP-TGF α /*Muc1*^{+/+} animals (including one metastatic pulmonary adenocarcinoma and multiple perivascular lymphomas), although none were detected in the WAP-TGF α /*Muc1*^{-/-} animals. Together, these data indicate that Muc1 is an important modulator of TGF α -dependent tumor progression. [Cancer Res 2007;67(14):6591–8]

Introduction

The epidermal growth factor receptor (EGFR) family of tyrosine kinases are frequently deregulated in cancer, and commonly amplified and/or overexpressed in invasive carcinoma (reviewed in ref. 1). The family is comprised of four homologous receptors and multiple related ligands. The receptors are type 1 tyrosine kinase transmembrane glycoproteins and include erbB1 (EGFR/HER1), erbB2 (HER2/neu), erbB3 (HER3), and erbB4 (HER4). Ligands for the family include epidermal growth factor (EGF), transforming growth factor α (TGF α), amphiregulin, heparin-binding EGF, betacellulin, epiregulin, and epigen (reviewed in refs. 1, 2). Ligand-induced receptor homodimerization or heterodimerization results in

tyrosine kinase activation (with the exception of erbB3, which has an inactive kinase and relies solely on heterodimerization for activation) and transphosphorylation of tyrosine residues in the cytoplasmic domain. This leads to the recruitment of a variety of effector proteins including Src, phosphoinositide-3-kinase, Shc, PLC γ , STATs, Grb2, and cbl, resulting in proliferation, inhibition of apoptosis, differentiation, or degradation of endocytosed receptors (3–6). Studies have shown that the coexpression of EGFR and ligands such as TGF α predicts a worse prognosis in patients with breast cancer (7). Additionally, TGF α is overexpressed both at the mRNA and protein levels in ~70% of breast cancer patients analyzed (8, 9).

Although transgenic overexpression of EGFR has met with limited success as a tumor model (10), overexpression of its cognate ligand, TGF α , results in a strong induction of mammary gland tumors. Various transgenic mouse models have been described that overexpress TGF α , including metallothionein promoter-driven (MT-TGF α), mouse mammary tumor virus LTR-driven (MMTV-TGF α), and whey acidic protein promoter-driven (WAP-TGF α ; refs. 11–13). These models show that TGF α overexpression induces breast cancer with a variety of histotypes and onset, depending on the strength of the promoter (reviewed in refs. 1, 14). In the WAP-TGF α transgenic model, tumors were generally well-differentiated, continuing to express both keratin 18 and whey acidic protein, whereas also expressing high levels of cyclin D1 compared with nontransformed contralateral glands. Additionally, these tumors express high levels of Wnt-1 and Wnt3a, indicating a potential role for β -catenin stabilization in tumor progression (15). Although activation of EGFR is known to promote metastatic progression (16), the WAP-TGF α transgenic mice show no evidence of metastasis when generated on a mixed genetic background (13).

EGFR activity can be modulated by a number of proteins, including Src, cbl, and MUC1 (17–19). MUC1 is a member of the mucin family of heavily *O*-glycosylated proteins, found apically localized in normal ductal epithelium (reviewed in ref. 20). Alternatively, MUC1 is found underglycosylated and nonpolarized in many adenocarcinomas, including cancers of the breast, lung, pancreas, ovary, and prostate, in addition to being highly expressed in leukemias, myelomas, and lymphomas (21–23). Transgenic mouse models driving MUC1 (human) overexpression to the mouse mammary gland (MMTV-MUC1) develop breast cancer with incomplete incidence and ectopic expression of MUC1 in 3Y1 cells results in their transformation (24, 25). Through these and other studies, it was determined that MUC1 and EGFR formed biochemical complexes in the breast, and that MUC1 expression potentiates EGF-dependent extracellular signal-regulated kinase 1 and 2 mitogen-activated protein kinase signaling (19). Recent studies in our laboratory have shown that MUC1 expression enhances EGFR stability by inhibiting its down-regulation upon EGF stimulation (26). This occurs via a MUC1-dependent decrease in ubiquitination of EGFR upon EGF treatment and a concomitant increase in MUC1-associated recycling (26).

Note: Supplementary data for this article are available at Cancer Research Online (<http://cancerres.aacrjournals.org/>).

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doi:10.1158/0008-5472.CAN-06-4518

These studies prompted us to evaluate the effects of Muc1 (mouse) overexpression on EGFR-dependent transformation in the breast. To evaluate the role of Muc1, we generated WAP-TGF α mice on a *Muc1* wild-type and *Muc1*-null background and evaluated the effects of Muc1 expression on tumor progression and subsequent signaling cascades (13, 27). We report here that Muc1 expression has a dominant effect on TGF α -dependent transformation of the breast, affecting both onset and progression.

Materials and Methods

Mice. WAP-TGF α transgenic mice on C57Bl/6 \times SJL background were generously provided by David Lee. Genotyping was done by PCR analysis using the following primers against the rat *TGF α* cDNA:

(forward) 5'-CAGTATTGTTCCATGGGACCTGC-3'
(reverse) 5'-TCTCGTGTCTGCAGACGAGGGCAC-3'.

This reaction was amplified for 35 cycles of 60 s at 94°C, 60 s at 55°C, and 60 s at 72°C, which generates a 260-bp fragment. The *Muc1*^{-/-} mice were generously provided by Sandra Gendler. Genotyping was done by PCR analysis using the following primers against the *Muc1/LacZ* sequence used to generate the knockout:

(forward) 5'-CCTCACACACGGAGCGCCAGC-3'
(reverse-LACZ 3) 5'-TTCTGGTG-CCGGAACCAGGC-3'.

This reaction produced a product of 300 bp. Wild-type mice were confirmed using the same forward primer, but with the following reverse primer:

5'-TCCCCCTGGCACATACTG-GG-3'.

This reaction produced a product of 300 bp. Both of these reactions were amplified for 40 cycles of 30 s at 95°C, 30 s at 65°C, and 30 s at 72°C. All the mice were maintained under conditions specified by the Association for Assessment and Accreditation of Laboratory Animal Care International and the Institutional Animal Care and Use Committee. For tumor studies, animals were bred continuously and measurements were begun after the third pregnancy. Measurements were made with calipers and a tumor was considered established after it reached 0.5 cm and did not regress upon subsequent palpations. Tumors were allowed to progress to 10% of the

animal's body weight, upon which time animals were sacrificed and tissues taken. Statistical analyses (*t* test) of tumor onset comparing WAP-TGF α /*Muc1*^{+/+} and *Muc1*^{-/-} was done by the Biometry Shared Service at The University of Arizona Cancer Center.

Whole mounts. The thoracic and inguinal mammary glands were collected and the whole mounts were prepared by stretching the glands onto a glass slide and air-dried for 2 to 3 h. The glands were fixed in 1:3 solution of glacial acetic acid/100% ethanol for 1 h at room temperature and subsequently washed in 95% ethanol for 15 min and in 100% ethanol for another 15 min. Defatting of the mammary glands was done by using acetone and incubating for 4 days with changes of acetone every day. The glands were rehydrated by washing in 100% ethanol, then in 95% ethanol, and finally followed by 70% ethanol. The slides with whole mounts were rinsed in distilled water and stained in 0.2% carmine/0.5% aluminum potassium sulfate solution overnight. Destaining was done by washing the glands for 15 min in 70% ethanol, 95% ethanol, 100% ethanol, again in 95% ethanol, and followed by 70% ethanol. Images of the whole mounts were captured using a Leica MZFLIII dissection scope with an Optronics MagnaFire camera at various magnifications.

Immunoprecipitation and immunoblotting. Protein lysates of the thoracic mammary glands were prepared by harvesting and immediately homogenizing in protein lysis buffer containing protease and phosphatase inhibitors (described in ref. 26), followed by the removal of the insoluble fraction by centrifugation, and stored at -80°C until use. The protein concentrations were determined by bicinchoninic acid assay (Pierce). Immunoprecipitation and immunoblotting were done as described in ref. (26). EGFR (1005), cyclin D1 (M-20) antibodies, and cyclin D1 blocking peptide were purchased from Santa Cruz Biotechnologies. CT-2 (MUC1 cytoplasmic domain) and EGFR (Ab-17) antibodies were purchased from Neomarkers, Inc. Phosphorylated EGFR (Y992) was purchased from Cell Signaling Technology. The β -actin antibody was purchased from Sigma Co. Secondary antibodies used were anti-rabbit HRP (Pierce), anti-Armenian hamster horseradish peroxidase antibody (The Jackson Laboratory), and anti-mouse horseradish peroxidase (Pierce).

Quantification of Western blots and statistical analysis. Immunoblots were scanned as TIFF images and the density of the bands were analyzed by Scion Image (NIH). The density values for cyclin D1, EGFR (1005), and EGFR (Y992) were averaged for *Muc1*^{-/-} and *Muc1*^{+/+}. Statistical significance was determined by an ANOVA test between *Muc1*^{-/-} and *Muc1*^{+/+} values.

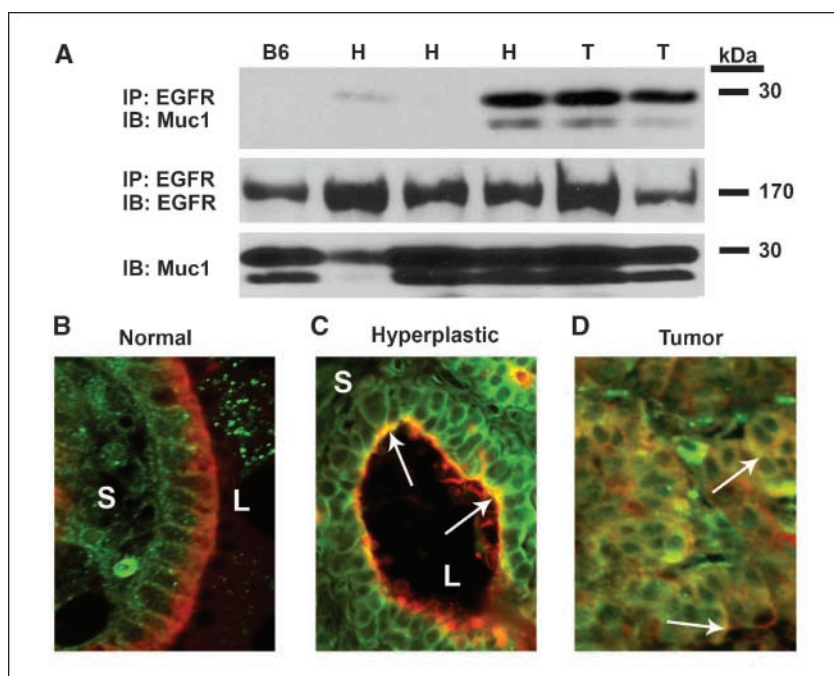
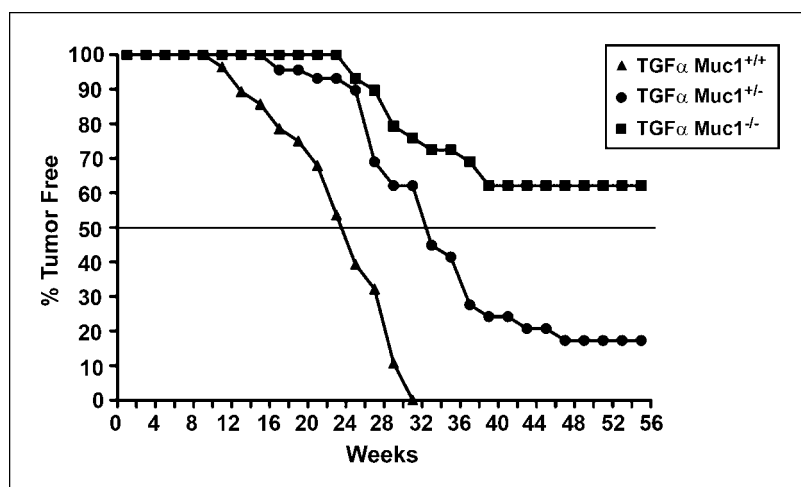


Figure 1. EGFR and Muc1 interact in tumors from WAP-TGF α transgenic mammary glands. **A**, tissue lysates isolated from a littermate control C57Bl/6 (*B6*) and WAP-TGF α mice [hyperplastic (*H*) and tumor (*T*)] were used to immunoprecipitate (*IP*) EGFR. These samples were then separated by SDS-PAGE and immunoblotted (*IB*) with anti-EGFR and anti-Muc1 antibodies (*top* and *middle*). Total levels of Muc1 in the samples (*bottom*). **B**, either a hyperplastic mammary gland or tumor from a WAP-TGF α transgenic mouse were sectioned and used for immunofluorescence [green, anti-pEGFR (Y992); red, anti-Muc1]. Arrows, areas of colocalization; S, stromal compartments; and L, ductal lumens. The normal duct and hyperplastic duct were both from the same tissue section. Magnification, $\times 630$.

Figure 2. Loss of Muc1 expression increases the latency and incidence of mammary tumorigenesis in WAP-TGF α transgenic mice. WAP-TGF α transgenic animals [*Muc1*^{+/+} (\blacktriangle), *Muc1*^{+/-} (\bullet), or *Muc1*^{-/-} (\blacksquare) background] were continuously bred to activate transgene expression and palpated weekly to detect tumor formation. WAP-TGF α /*Muc1*^{+/+} transgenics first form tumors by 12 wks after their first pregnancy versus 18 wks for WAP-TGF α /*Muc1*^{+/-} and 26 wks for WAP-TGF α /*Muc1*^{-/-} mice (*t* test, *P* = 0.0004 for *Muc1*^{+/+} versus *Muc1*^{-/-}). Only 37% of WAP-TGF α /*Muc1*^{-/-} mice develop tumors by 52 wks after their first pregnancy, whereas 100% of WAP-TGF α /*Muc1*^{+/+} formed tumors by 34 wks after their first pregnancy. Kaplan-Meier curve denotes time from first parturition to time of palpable 0.5 cm diameter tumor (nonregressing). *Solid line*, when 50% of animals were tumor-free. WAP-TGF α /*Muc1*^{+/+} (*n* = 28), WAP-TGF α /*Muc1*^{+/-} (*n* = 29), and WAP-TGF α /*Muc1*^{-/-} (*n* = 29).



Immunofluorescence. Inguinal mammary glands were fixed in methacarn for 2 h, changed to 70% ethanol, embedded in paraffin, and sectioned by the Tissue Acquisition and Cell Molecular Analysis shared service at the Arizona Cancer Center. The slides were rehydrated and immunostained with primary antibodies at 1:100 dilutions and secondary antibody at 1:200 concentration. The primary antibodies used were the same as that applied in the immunoblotting protocol. The secondary antibodies used were Texas red anti-hamster IgG (Jackson ImmunoResearch) and Alexa Fluor 488 anti-rabbit IgG (Invitrogen). The slides were analyzed using a Leica DMLB compound microscope.

Quantification of hyperplastic tissue. Animals were bred three, four, or five times, and were sacrificed >10 days after parturition (note that these animals did not lactate and pups were removed after birth). Mammary glands were whole-mounted and hyperplastic foci were counted under a Leica dissection microscope, including only foci of ~1.0 mm in size.

Results

Muc1 and EGFR interactions were specific to hyperplasia and transformation. We have recently established that MUC1 can inhibit ligand-mediated degradation of EGFR through decreased ubiquitination and increased recycling (26). We wanted to determine if this effect on EGFR stabilization would affect EGFR-dependent transformation. To investigate EGFR-dependent transformation, we used the WAP-TGF α mouse model of mammary gland carcinoma, which forms mammary gland tumors with complete incidence, accompanied by the formation of hyperplastic glands prior to tumor formation (13). In the WAP-TGF α transgenic mouse, TGF α was shown to be expressed abundantly in the alveolar and ductal epithelium of the mammary glands as well as in the mammary gland tumors of transgenic animals (13).

We first established the levels of Muc1 expression in the WAP-TGF α transgenic mouse model. WAP-TGF α mice were bred to induce tumor formation, and tissues were collected from mammary gland tumors and contralateral hyperplastic glands. In addition, we collected mammary glands from nontransgenic C57Bl/6 littermates as normal controls. Analysis of total protein levels showed similar levels of both EGFR and Muc1 expression in each of these tissues (Fig. 1).

We next examined the interactions between EGFR and Muc1 in the mammary glands of C57Bl/6 (B6) and WAP-TGF α transgenic mammary glands (both hyperplastic and tumors). Biochemical interactions between EGFR and Muc1 were not observed in the normal gland (Fig. 1A, B6). Alternatively, robust interactions were detected in WAP-TGF α mammary gland tumors and occasionally

in hyperplastic mammary glands (Fig. 1A, T and H, respectively). To determine a potential mechanism for this difference, we examined the localization of Muc1 and the phosphorylated form of EGFR (specific to the autophosphorylated tyrosine 992; refs. 28, 29), and we observed a significant difference in the localization of these proteins which varied by the state of transformation. We analyzed colocalization of pEGFR and Muc1 in WAP-TGF α transgenic mammary gland sections that contained normal and hyperplastic tissue within the same section and tumor sections (Fig. 1B-D). In the sections of the tissue in which apical/basolateral polarization was retained, Muc1 is found only at the apical surface, not interacting with basolateral pEGFR (Fig. 1B). As the gland transitions to hyperplasia, interactions between Muc1 and pEGFR can be observed beneath the apical domain of the ductal epithelium (Fig. 1C, arrows). In the fully transformed portion of the tissue, Muc1 and pEGFR are found colocalizing throughout the cytoplasm of the transformed cells (Fig. 1D, arrows).

Muc1 expression dramatically alters tumor incidence and latency in WAP-TGF α transgenic mice. To determine the effect of Muc1 expression and its interaction on EGFR-dependent transformation, we generated WAP-TGF α transgenic mice on *Muc1*^{+/+}, *Muc1*^{+/-}, and *Muc1*^{-/-} backgrounds and analyzed tumor progression. Previous studies by other groups using the WAP-TGF α model have been done on both a mixed (C57Bl/6 \times SJL) or inbred (FVB) background, but to perform our studies, we crossed our animals with an inbred C57Bl/6 background (N>5). This allowed us to cross the WAP-TGF α mice to the *Muc1*^{-/-} (C57Bl/6) mice without introducing any modifier genes (13, 30).

Animals were bred continuously to induce the activation of the WAP promoter, and mammary glands were palpated once a week (every week after their third pregnancy) for ~1 year to evaluate tumor progression. Tumors were considered established upon palpation of a 0.5 cm diameter solid mass that remained after subsequent palpations. As previously reported, we observed a 100% incidence of mammary tumor formation in WAP-TGF α mice on a *Muc1*^{+/+} background (29 of 29 animals developed tumors), with a mean onset of 6 months (Fig. 2; ref. 13). Alternatively, tumor formation was dramatically affected by the loss of Muc1 expression. In WAP-TGF α /*Muc1*^{-/-} mice, only 37% of the animals developed tumors by 52 weeks of age after their first pregnancy (11 of 30 animals developed tumors; Fig. 2). Not only did the ablation of Muc1 expression affect tumor formation, but the age of onset of tumor formation was significantly delayed as well.

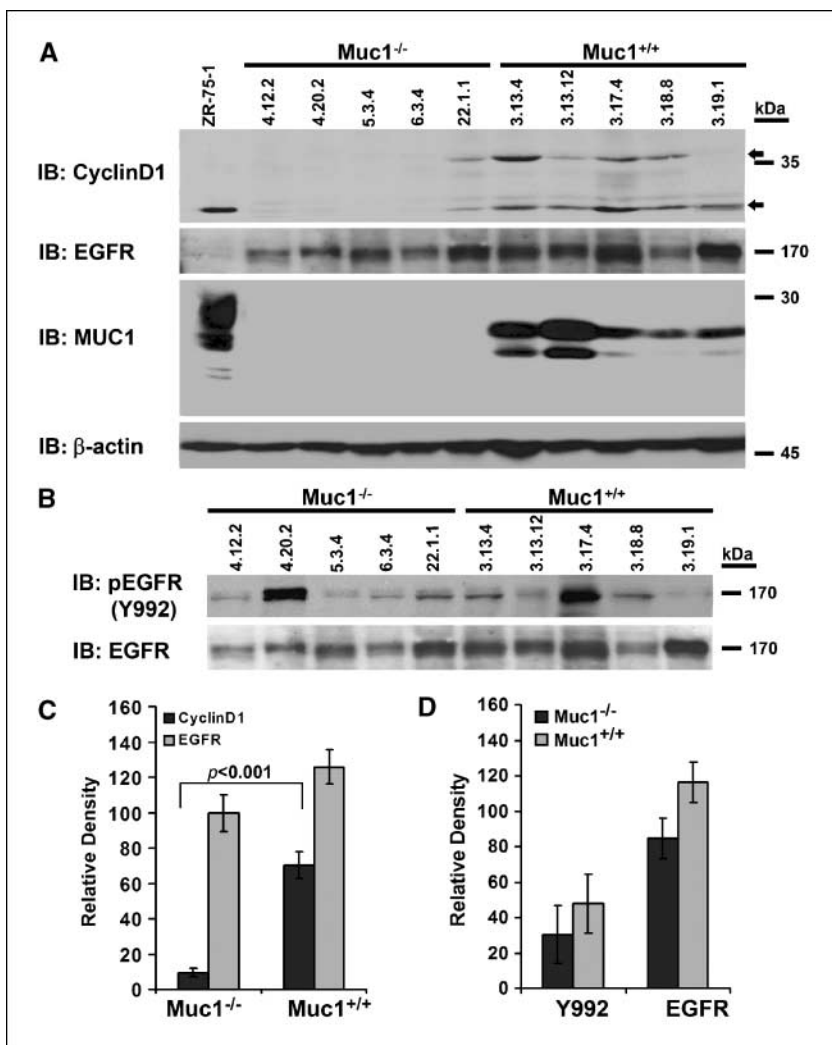


Figure 3. Cyclin D1 expression in tumors from WAP-TGF α transgenic mice correlates with Muc1 expression. *A*, protein lysates from tumors derived from WAP-TGF α mice on either a *Muc1*^{-/-} or *Muc1*^{+/+} background were separated by SDS-PAGE and immunoblotted (*IB*) with anti-cyclin D1, anti-EGFR, anti-Muc1, and anti- β -actin antibodies. ZR-75-1 human adenocarcinoma cell lysate was used as a positive control for cyclin D1 expression. Note the double bands (\blackleftarrow) detected in mouse lysates were confirmed to be cyclin D1 by using blocking peptide to cyclin D1 (data not shown). In addition, upon longer exposure, EGFR was detected in ZR-75-1. *B*, protein lysates from tumors derived from WAP-TGF α mice on *Muc1*^{-/-} and *Muc1*^{+/+} backgrounds were separated on SDS-PAGE and immunoblotted with anti-phosphorylated EGFR (Y992) and anti-EGFR. *Right*, molecular weights. *C*, densitometry showed that the protein levels of cyclin D1 were significantly elevated in Muc1 expressing tumors (ANOVA, $P < 0.001$). *D*, levels of EGFR and phosphorylated-EGFR (Y992) were not significantly different between *Muc1*^{-/-} and *Muc1*^{+/+} tumor lysates. *Bars*, SE.

Although tumors were observed in the WAP-TGF α /*Muc1*^{+/+} mice as early as 12 weeks, the first tumor in the WAP-TGF α /*Muc1*^{-/-} mice was not observed until 24 weeks ($P = 0.0004$). Although the WAP-TGF α /*Muc1*^{+/+} mice developed mammary tumors slightly later as compared with WAP-TGF α /*Muc1*^{+/+}, the difference in the latency period was not statistically significant (Fig. 2). Evaluation of Muc1 expression from the WAP-TGF α /*Muc1*^{+/+} tumors indicated a lowered level of Muc1 expression (data not shown).

Evaluation of tumor histotype revealed no significant alteration in the presence or absence of Muc1 expression, with the majority of tumors from both genotypes being either fibroadenomas, myoepitheliomas, or keratoacanthomas.⁴

Overexpression of cyclin D1 in WAP-TGF α correlates with expression of Muc1. Previous studies have shown that *cyclin D1* is up-regulated at the mRNA level in WAP-TGF α transgenic mice (13). Furthermore, increased cyclin D1 expression is a commonly observed downstream event in EGFR-mediated transformation (reviewed in ref. 14). Therefore, we investigated the expression levels of cyclin D1 in hyperplastic glands and tumors derived from WAP-TGF α transgenic mice on *Muc1*^{-/-} and *Muc1*^{+/+} backgrounds. We found that cyclin D1 protein levels were not increased

in hyperplasias respective to their normal glands (data not shown) regardless of *Muc1* background (*Muc1*^{-/-} versus *Muc1*^{+/+}). However, whereas cyclin D1 expression was significantly increased ($P < 0.001$) in tumors derived from WAP-TGF α /*Muc1*^{+/+} glands, there was only a modest increase of cyclin D1 expression in WAP-TGF α /*Muc1*^{-/-} glands (Fig. 3A, top). Note that cyclin D1 separates into two distinct species in the mouse, but only a single species in human (Fig. 3A, ZR-75-1 control). The cyclin D1 antibody specificity of these two bands was verified by using a blocking peptide (data not shown).

To determine the potential mechanism(s) of loss of cyclin D1 expression in WAP-TGF α /*Muc1*^{-/-} versus WAP-TGF α /*Muc1*^{+/+} we evaluated the expression and activation of EGFR on a *Muc1*^{+/+}, *Muc1*^{+/+}, and *Muc1*^{-/-} background. We found that the total levels of EGFR were relatively similar for all Muc1 genotypes (Fig. 3A and B; data not shown for *Muc1*^{+/+}), as shown by densitometry analysis (Fig. 3D). Additionally, we evaluated the activity of EGFR by analyzing the phosphorylation of tyrosine residue 992, which resides in the autophosphorylation domain (Fig. 3B; ref. 28). Again, activation of the receptor, although slightly diminished in the WAP-TGF α /*Muc1*^{-/-} tumors, was not statistically different from WAP-TGF α /*Muc1*^{+/+} tumors upon densitometry analysis (Fig. 3D).

⁴ R. Cardiff (University of California, Davis, Davis, CA), personal communication.

To evaluate potential upstream mediators to cyclin D1 activation, we evaluated the activation of Src, AKT, and p42/44 mitogen-activated protein kinase, and found no differences in WAP-TGF α /Muc1 $^{-/-}$ versus WAP-TGF α /Muc1 $^{+/+}$ (data not shown).

Muc1 expression promotes hyperplastic progression. One of the unique characteristics of the WAP-TGF α model is the development of hyperplastic mammary glands prior to frank tumor development (31). Previous studies show the development of >100 hyperplastic foci per mammary gland in animals undergoing more than five pregnancies (31). We therefore evaluated the effects of Muc1 expression on the formation of hyperplastic foci in the mammary glands of WAP-TGF α transgenic mice.

First, we evaluated contralateral mammary glands from tumor-bearing animals on the WAP-TGF α /Muc1 $^{-/-}$ versus WAP-TGF α /Muc1 $^{+/+}$ backgrounds. Whole-mount analysis showed a dramatic decrease in the amount of hyperplastic foci in WAP-TGF α /Muc1 $^{-/-}$ mammary glands (Fig. 4A and B; Supplementary Fig. S1A and B). Note that whereas hyperplastic foci were so plentiful in the WAP-TGF α /Muc1 $^{+/+}$ glands as to obscure the ducts (Fig. 4A, arrow), hyperplasias were only sparsely observed in the WAP-TGF α /Muc1 $^{-/-}$ glands, and the ductal tree remained clearly visible (Fig. 4B, arrowhead). Whole-mounted mammary glands were also embedded in paraffin, sectioned, and then stained with H&E to evaluate the cellularity of the foci. Microscopic analysis of the lesions verified the presence of multiple hyperplastic foci (Fig. 4C and D; Supplementary Fig. S1C and D).⁴ Analysis of EGFR protein expression in these contralateral hyperplastic glands showed no significant differences (data not shown).

To evaluate the time at which Muc1 expression affects the progression to hyperplasia, we generated animals on the WAP-TGF α /Muc1 $^{-/-}$ versus WAP-TGF α /Muc1 $^{+/+}$ backgrounds, and allowed them to progress through three to five pregnancies each, harvesting the mammary glands >10 days after parturition.

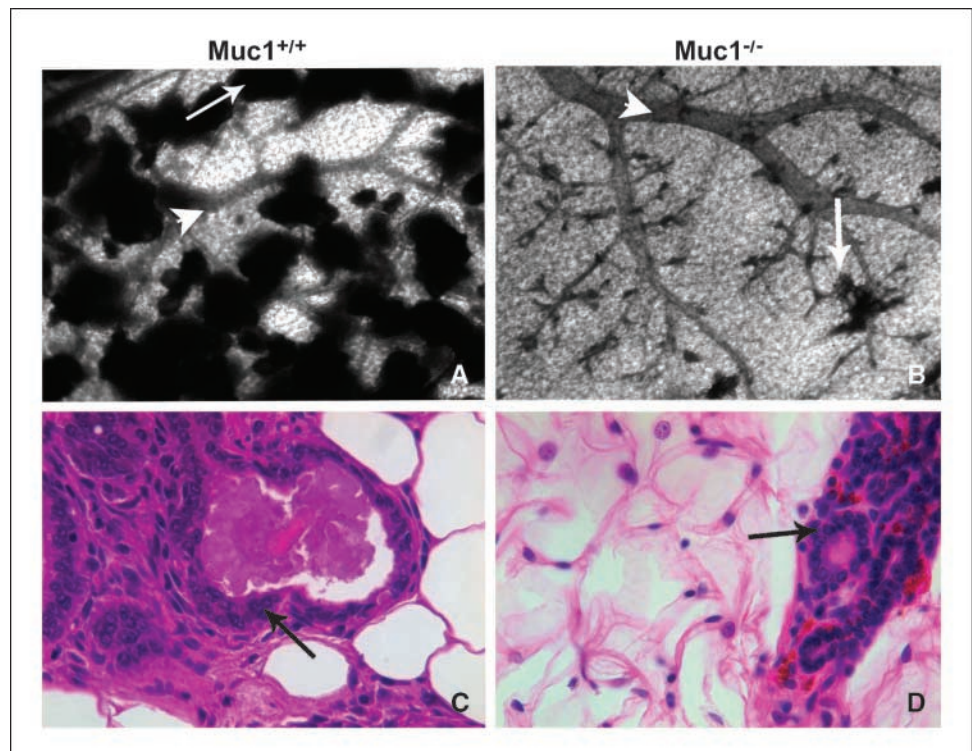
Mammary glands were whole-mounted and the total number of foci \sim 1.0 mm were counted (Fig. 5; Supplementary Fig. S2). This analysis showed that whereas WAP-TGF α /Muc1 $^{+/+}$ mice developed >100 foci with as few as three pregnancies, WAP-TGF α /Muc1 $^{-/-}$ mammary glands never developed more than 10 to 20 foci after as many as five pregnancies (Fig. 5; Supplementary Fig. S2, hyperplastic foci denoted by arrows). These data indicate that Muc1 expression affects early stages of TGF α -dependent breast cancer progression.

Effect of Muc1 expression on pulmonary foci. MUC1 overexpression has been shown to correlate with metastatic progression in a number of studies of breast cancer (24, 32). Therefore, we sought to determine if Muc1 expression induced/alterd pulmonary metastases in the WAP-TGF α mouse model. Although WAP-TGF α mice on a mixed SJL \times C57Bl/6 background show no lung pathology, the WAP-TGF α mice we generated on the inbred C57Bl/6 background displayed pulmonary foci in 28 of 29 lungs. Microscopic examination revealed perivascular lymphocytic infiltrate and perivascular lymphomas (Fig. 6C and D, 'P') of unknown origin in 28 of 29 of the lungs, and a metastatic adenocarcinoma (Fig. 6B, '*') in 1 of 29 lungs.⁴ Interestingly, there was no lung pathology observed in any of the WAP-TGF α /Muc1 $^{-/-}$ animals (neither lymphoma nor adenocarcinoma; Fig. 6A). Note that neither the thymus nor spleen were preserved from these animals, leaving the origin of the lymphomas a subject for future evaluation.

Discussion

TGF α is a potent inducer of transformation and metastatic progression. In the current study, we evaluated the effect of Muc1 expression on TGF α -induced breast neoplasia using the WAP-TGF α transgenic mouse model. Our analyses showed a dramatic effect of Muc1 expression on both the transition from normal

Figure 4. WAP-TGF α /Muc1 $^{-/-}$ mice develop fewer hyperplasias in tumor-contralateral mammary glands than WAP-TGF α /Muc1 $^{+/+}$ mice. Mammary glands contralateral to tumor-forming glands were whole-mounted and analyzed for the formation of hyperplastic foci. Left, WAP-TGF α /Muc1 $^{+/+}$ (A and C); right, WAP-TGF α /Muc1 $^{-/-}$ (B and D). Whole mounts show hyperplastic foci (arrows) embedded within the mammary gland parenchyma of ducts (arrowheads). A and B, magnification, \times 80. H&E staining of mammary gland sections showing cellular hyperplasia (arrows). C and D, magnification, \times 630.



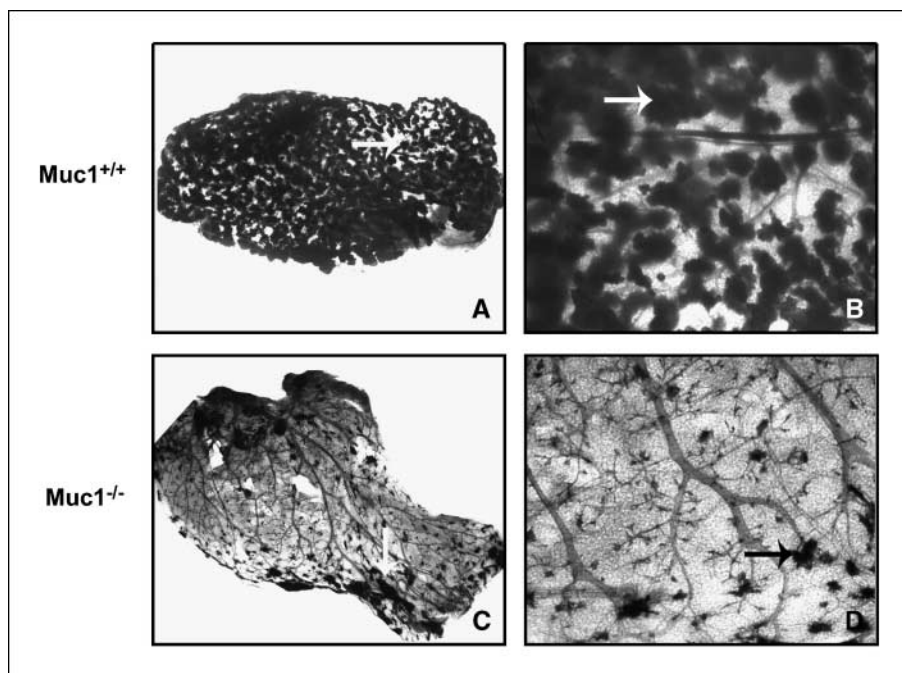


Figure 5. Nonneoplastic WAP-TGF α /*Muc1*^{-/-} mice display reduced hyperplasia in mammary glands compared with WAP-TGF α /*Muc1*^{+/+}. WAP-TGF α mice (either *Muc1*^{+/+}, top; or *Muc1*^{-/-}, bottom) were allowed to progress through five pregnancies, and glands were harvested and whole-mounted. Hyperplastic foci (arrows) were determined to be those foci that had reached >1.0 mm in diameter, and all foci per mammary gland were counted from the inguinal gland of each animal. A and C, magnification, $\times 10$; B and D, magnification, $\times 40$.

glands to hyperplasia and the development of mammary gland tumors. During 1 year of analysis, only 37% of WAP-TGF α animals lacking *Muc1* expression developed breast tumors, whereas 100% of animals expressing *Muc1* progressed to neoplasia. Although EGFR is expressed and activated in both WAP-TGF α /*Muc1*^{+/+} and WAP-TGF α /*Muc1*^{-/-} backgrounds to a similar extent, only tumors expressing *Muc1* induced significant levels of cyclin D1 expression, indicating that cyclin D1 expression can be modulated by *Muc1*. In addition, the loss of *Muc1* in WAP-TGF α mice prevented the formation of perivascular lymphoma in the lungs. Together, these data indicate that TGF α -dependent transformation is dramatically enhanced in the presence of *Muc1*, and that *Muc1* affects multiple stages of neoplasia, including hyperplasia and adenocarcinoma.

The lack of cyclin D1 induction in tumors from the *Muc1*-null background compared with those tumors expressing *Muc1* indicate a function for *Muc1* in the cyclin D1 pathway. Overexpression of cyclin D1 plays an important role in the development of breast cancer, as transgenic expression of cyclin D1 in the mouse mammary gland (MMTV-cyclin D1) results in the development of breast cancer (33). Additionally, *cyclin D1* is amplified and/or overexpressed in >50% of breast cancers (34, 35). In studies of different transgenic mouse models of breast cancer, Yu et al. showed that cyclin D1 expression was absolutely critical for the progression of mammary carcinoma in MMTV-ras and MMTV-neu mouse models, but not in MMTV-Wnt-1 or MMTV-myc mice (36). These studies indicate that although multiple signaling pathways converge on cyclin D1 overexpression, only certain systems of

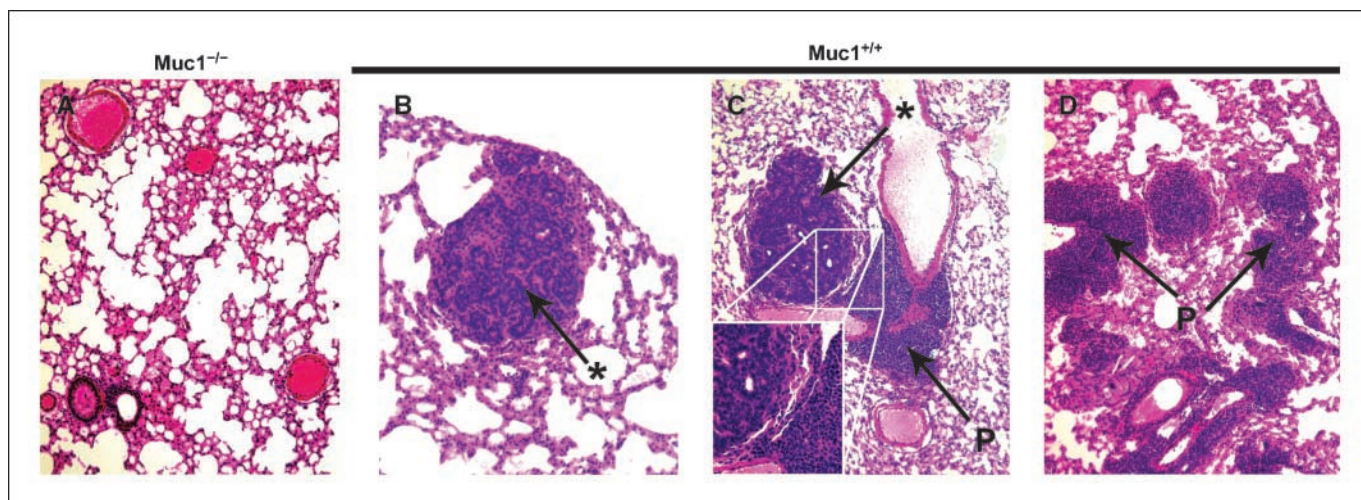


Figure 6. Appearance of lymphoma and metastatic adenocarcinoma in the lung is prevented by loss of *Muc1*. A, lung from a representative WAP-TGF α /*Muc1*^{-/-} mouse was fixed, sectioned, and stained with H&E to show the normal parenchyma. B to D, lungs from WAP-TGF α /*Muc1*^{+/+} mice were fixed, sectioned, and stained with H&E. B, metastatic adenocarcinoma (*). C, perivascular infiltrate (P) adjacent to adenocarcinoma (*). D, perivascular lymphoma (P) in lungs of WAP-TGF α mice. Magnification, $\times 100$ (inset, $\times 630$).

malignant transformation are dependent on cyclin D1 over-expression. It is possible that in the absence of Muc1, cyclin D1 expression cannot be fully induced, and this inhibits tumor formation. In fact, the failure of >60% of WAP-TGF α mice lacking Muc1 expression to develop tumors may be due to the lack of cyclin D1 induction.

WAP-TGF α mice on the *Muc1*^{-/-} background develop significantly fewer hyperplastic foci than their wild-type counterparts. This may be the direct cause for the reduction in tumor formation in the absence of Muc1, although the direct link between the formation of the hyperplastic foci and tumor development has not been firmly established. Recently, our lab has determined that expression of MUC1 modulates the stability of EGF-activated EGFR. This was found to be due to a MUC1-dependent decrease in the ubiquitination of EGFR, resulting in its preferential recycling instead of degradation. In our current studies, we found that there is no significant difference in the total levels of EGFR or activation in the presence of Muc1 in established tumors. The most likely explanation for this is that we were examining those tumors that had alleviated the need for Muc1 expression in stabilizing EGFR expression. It is important to note that the analysis was done on those tumors which were able to form in the absence of Muc1 expression—which may represent a population that has found an alternate route for sustained EGFR activation. In future studies, we plan to fully investigate the stability of EGFR in the developing gland, to determine if Muc1 affects EGFR degradation during mammary gland development and progression to neoplasia.

Our data also point to a potential role for cellular polarization in affecting tumor progression. We found that whereas MUC1 is apically restricted and EGFR is basolaterally restricted in the normal polarized epithelium, subapical colocalization can be found in hyperplastic tissues and abundant cytoplasmic colocalization is observed in tumors (Fig. 1B, arrows). Future investigations will focus on whether loss of polarization is driving the interaction

between MUC1 and EGFR or if the interaction between MUC1 and EGFR is promoting the loss of polarization.

The observation of perivascular lymphomas in the WAP-TGF α model is a novel development, and seems to be the result of expressing the transgene in an inbred C57Bl/6 background. Although the development of the lymphomas is of unknown cause, it is still interesting to observe that a loss of Muc1 expression inhibits the occurrence of these lymphomas in the lung. It has been previously established that Muc1 is expressed in lymphocytes and a variety of hematologic malignancies (21, 37, 38). In a T cell lymphoma cell line (Jurkat), MUC1 was shown to play a critical role in activation and proliferation (39). It is therefore possible that the loss of Muc1 also affected the spontaneous development of lymphomas in this model.

In summary, we have found that the development of hyperplasia and frank breast cancer in the WAP-TGF α transgenic model was highly dependent on the expression of Muc1. Furthermore, the activation of cyclin D1 expression that is commonly linked to EGFR-dependent breast cancer may be dependent on the coexpression of Muc1. We believe that this discovery has a high potential for affecting anti-erbB therapies, as the level of Muc1 expression may be an important corollary to account for in clinical cases.

Acknowledgments

Received 1/8/2007; revised 3/8/2007; accepted 4/26/2007.

Grant support: Funding provided by the NIH (J.A. Schroeder and B.G. Bitler) and the Susan G. Komen Breast Cancer Foundation (M.R. Pochampalli).

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We thank Rachid el Bejjani, Jose Lopez, and Raghu Muthyalampalli for critical reading of the manuscript; David C. Lee (University of North Carolina at Chapel Hill, Chapel Hill, NC) and Sandra J. Gendler (Mayo Clinic, Scottsdale, AZ) for providing us with WAP-TGF α and Muc1 knockout mice, respectively; Robert Cardiff (University of California-Davis, Davis, CA) for histopathological analyses and advice; and the Tissue Acquisition and Cellular Molecular Analysis Shared Service at the Arizona Cancer Center for tissue processing and H&E staining.

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