NORTHWESTERN UNIVERSITY

# The Role of TGF-beta Variants in Breast Cancer

A DISSERTATION

SUBMITTED TO THE GRADUATE SCHOOL
IN PARTIAL FULFILLMENT OF THE REQUIREMENTS

For the degree

DOCTOR OF PHILOSOPHY

Integrated Graduate Program in the Life Sciences

By

Diana S. Rosman

EVANSTON, ILLINOIS

December 2008

# ABSTRACT

The Role of TGF-beta Variants in Breast Cancer

Diana S. Rosman

TGF-β has been named the molecular Jekyll and Hyde of cancer due to its ability to both suppress and promote tumor development. Components of the TGF-β signaling pathway are often mutated in cancer to inhibit the tumor suppressor roles of TGF-β. Our lab studies how a 3-alanine deletion in the signal sequence region of the type 1 TGF-β receptor (TGFBR1) increases cancer risk. This mutation, known as *TGFBR1**6A, confers a 15% increased risk for breast cancer compared to individuals who carry two copies of the wildtype (*9A) receptor. There is a 40% increased risk for homozygosity. My thesis project was to investigate the effects of *TGFBR1**6A on breast cancer development.

TGF-β induces a 30% growth inhibitory response in MCF-7 breast cancer cells. However TGFBR1*6A expression conferred a TGF-β-mediated growth advantage to the cells. In addition, TGFBR1*6A enhanced the ability for MCF-7 cells to migrate and invade through a matrigel barrier independently of TGF-β. Microarray studies identified 2 crucial mediators of migration that are downregulated in MCF-7*6A cells compared to MCF-7*9A cells: *ARHGAP5*, the gene encoding the RhoGTPase activating protein 5, and *FN1*, the gene encoding Fibronectin-1. Downregulation of *ARHGAP5* led to increased activation of RhoA. TGFBR1*6A expression also amplified ERK activation. MCF-7 cells expressing kinase-deficient TGFBR1*6A exhibited TGF-β-mediated growth stimulation and decreased expression of *ARHGAP5* and *FN1*, indicating

that the effects of *6A are mediated through its signal sequence and not through the canonical SMAD signaling. This is the first evidence that TGFBR1*6A can exert functions that are independent of TGF-β and that the effects of TGFBR1*6A are mediated through the signal sequence peptide and not through receptor activation.

Our lab generated a novel *Tgfbr1* haploinsufficient mouse strain and observed that, when crossed with mouse models of colon cancer, there was a significantly increased frequency of colorectal tumors. Additionally, we have recently discovered that germline allele specific expression (ASE) of TGFBR1 increases the risk for human colorectal cancer. I therefore set out to determine if TGFBR1 haploinsufficiency would impact breast cancer development. This *Tgfbr1*$^{+/-}$ mouse model was mated to the commonly used *MMTV-c-Neu* proto-oncogenic mouse model that spontaneously develops mammary tumors. *Neu;Tgfbr1*$^{+/-}$ mice had a significant decrease in tumor latency compared to the *Neu* mice. There is some indication that *Tgfbr1*$^{+/-}$ may enhance lung metastases in the *Neu* mouse model. This is the first evidence that *Tgfbr1*$^{+/-}$ may enhance breast cancer development.

Alterations in the TGF-β signaling pathway are becoming increasingly common in breast cancer. My research has shown how two different variants of TGFBR1, TGFBR1*6A and haploinsufficiency of TGFBR1, have the potential to impact patient's risk for breast cancer and disease progression.

# ABBREVIATIONS

| | |
|---|---|
| *6A | TGFBR1*6A |
| *9A | TGFBR1 or TGFBR1*9A |
| A | Adenine |
| Ala | Alanine |
| ANGPTL4 | Angiopietin like 4 |
| ARHGAP5 | RhoGAP5 activating protein 5 |
| ASE | Allele-specific expression |
| BMI | Body Mass Index |
| BRIP1 | BACH1 |
| BSA | Bovine serum albumin |
| C | Cytosine |
| CCND1 | Cyclin D1 |
| CD | Cowden disease |
| CDK | Cyclin dependent kinase |
| CDKN1A | p21 WAF1 CIP1 |
| CDKN1B | p27 KIP1 |
| CDKN2A | p16 INK4a |
| CDKN2B | p15 INK4b |
| CI | Confidence interval |
| CRC | Colorectal cancer |
| CTGF | Connective tissue growth factor |
| CXCL1 | Also known as GRO1, Melanoma growth stimulating activity, alpha |
| CXCL12 | SDF1, Stromal derived factor-1 |
| DCIS | Ductal carcinoma in-situ |
| DTT | Dithiothreitol |
| ECM | Extracellular matrix |
| EDTA | Ethylene Diamine Triacetic Acid |
| EGFR | Epidermal growth factor receptor |
| EMT | Epithelial to mesenchymal transition |
| ER | Endoplasmic reticulum |
| ERBB1 | EGFR |
| ERBB2 | HER2, HER2/neu, NEU, ErbB2 |
| EREG | Epiregulin |
| FBS | Fetal Bovine Serum |
| FN1 | Fibronectin-1 |

| | |
|---|---|
| G | Guanine |
| GAPDH | Glyceraldehyde -3-phopshate dehydrogenase |
| GDP | Guanosine 5'-Diphosphate |
| GFP | Green Fluorescence protein |
| GST | Glutathione S-Transferase |
| GTP | Guanosine 5'-Triphosphate |
| H&E | Hematoxylin and eosin stain |
| HDAC | Histone deacetylase complex |
| HGF | Hepatocyte growth factor |
| HN | Head and neck |
| hr | Hour |
| HR | Hazard ratio |
| HRAS | H-Ras |
| HRP | Horseradish peroxidase |
| IHC | Immunohistochemistry |
| IL11 | Interleukin 11 |
| ITGAV | Integrin alpha-v |
| ITGB6 | Integrin beta-6 |
| JNK | Jun N-terminal Kinase |
| kb | Kilobases |
| LAP | Latancy associated peptide |
| LFLS | Li-Fraumeni like syndrome |
| LFS | Li-Fraumeni syndrome |
| LMS | Lung metastasis signature |
| LTBP | Latent TGF-β binding protein |
| MAPK | Mitogen activated protein kinase; p44/42, ERK |
| MECP2 | Methyl-CpG-binding protein |
| $MgCl_2$ | Magnesium Chloride |
| MMP1 | Matrix metallopeptidase 1 |
| MMTV | Mouse Mammary Tumor Virus |
| mo | Month |
| Mv1Lu | Mink lung epithelial cells |
| NaCl | Sodium Chloride |
| NCCN | National Comprehensive Cancer Network |
| NCOR1 | Nuclear hormone co-repressor |
| Neo | Neomycin |
| NMuMG | Normal murine mammarg gland cell line |
| OR | Odds ratio |

| | |
|---|---|
| PBS | Phosphate buffered saline |
| PCNA | Proliferating cell nuclear antigen |
| PF4 | CXCL4, Platelet factor 4 (C-X-C motif) ligand 4 |
| PTEN | Phosphatase and tensin homolog |
| PTGS2 | Prostaglandin-endoperoxide sythase 2; also known as COX2 |
| PyVmT | Polyoma virus middle-T antigen |
| RBD | Rho binding domain |
| RR | Relative risk |
| SARA | Smad anchor for receptor activation |
| SBE | Smad binding element |
| SDS | Sodium Dodecyl Sulfate |
| SEER | Surveillance, Epidemiology, and End Results |
| SKIL | SnoN |
| SNP | Single nucleotide polymorphism |
| SRP | Signal recognition particle |
| T | Thymine |
| TBRS | TGF-β response signature |
| TBS | Tris-buffered saline |
| TEB | Terminal end bud |
| TGFBR1 | Type 1 TGF-β receptor; TβRI; Alk5 |
| TGFBR2 | Type 2 TGF-β receptor, TβRII |
| TGF-β | Transforming Growth Factor - beta |
| THBS1 | Thrombospondin-1 |
| WT | Wild-type |

# GLOSSARY

Allele Specific Expression (ASE)- Both alleles of the gene express a transcript, however one allele of the gene has higher transcript expression than the second allele. This is different from monoallelic expression, which is caused by turning off one allele by factors such as genomic imprinting, X-chromosome inactivation, or promoter methylation.

Linkage Disequilibrium- Non-random association of alleles at two or more loci that are not necessarily on the same chromosome. Certain SNP's or genetic markers on multiple allelic locations have a higher propensity for being inherited together

Single Nucleotide Polymorphism (SNP) – A single nucleotide base pair change in DNA that is found in 1% of the population.

Haplotype – Genotype for several SNPs on one allele or chromatid

# NOMENCLATURE

To avoid ambiguity, this manuscript adheres to the established nomenclature guidelines set out by the Human Genome Organization (HUGO) Gene Nomenclature Committee (HGNC). These guidelines were established to avoid confusion due to multiple names for the same gene or protein [Wain et al., 2002]. When used for the first time, alternate names will be shown to identify the gene or protein symbol. The abbreviations section will also act as a reference section to define the newer symbols.

This manuscript discusses both human and mouse genes and proteins, and there are distinct formatting rules for each species. Genes are always italicized and proteins are in normal font. Human genes and proteins are capitalized and mouse genes only capitalize the first character. An example is given below using the gene and protein for the type 1 transforming growth factor beta receptor.

| **Taxonomy** | **Organism** | **Gene** | **Protein** |
|---|---|---|---|
| *Homo sapiens* | Human | *TGFBR1* | TGFBR1 |
| *Mus musculus* | Mouse | *Tgfbr1* | Tgfbr1 |

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# CHAPTER 1

# Introduction

## Breast Cancer Genetics

One in eight women will be diagnosed with breast cancer in her lifetime and it is expected that more than 214,000 new cases will be diagnosed in the U.S. in 2006 [Jemal et al., 2006]. Most cases of breast cancer are sporadic; however, twin studies have shown that heritable factors may cause 20-30% of all breast cancers [Lichtenstein et al., 2000]. While mutations within the *BRCA1* and *BRCA2* genes are common among women with a strong family history of breast cancer, they account for at most 1 to 4% of all breast cancer cases. Mutations in the *TP53* and *PTEN* genes, which cause Li-Fraumeni syndrome and Cowden syndrome respectively, are exceedingly rare, and probably account for less than 0.1% of breast cancers. The large effect of heritability in breast cancer suggests major gaps in our knowledge.

Other candidate genes that may cause breast cancer have been identified in the past few years. Cancer susceptibility genes that are associated with an increased risk of breast cancer include *TGFBR1**6A, *CHEK2**1100delC, and *BRIP1* [Baxter et al., 2002;Meijers-Heijboer et al., 2002;Seal et al., 2006]. Many single nucleotide polymorphisms (SNPs) have also been studied in other genes and a recent study suggests that 5 of them are associated with breast cancer risk: *CASP8* D302H, *IGFB3* -202 C>A, *PGR* V660L, *SOD2* V16A, and TGFB1 *T29C* [Breast Cancer Association Consortium, 2006]. The association between CASP8 D302H and TGFB1 T29C with breast cancer was recently confirmed in a large validation study [Cox et al., 2007]. The respective contribution of these susceptibility genes and candidate SNPs is the focus of several ongoing studies.

These investigations are complicated by the fact that the penetrance of tumor susceptibility genes is highly influenced by other factors such as modifier genes, response to DNA damage, and environmental factors such as exposure to carcinogens, hormonal/ reproductive factors, and weight [King et al., 2003].

Genetic testing is currently used to determine if individuals with a personal and/or family history of breast cancer carry mutations or genomic rearrangements within high penetrance breast cancer susceptibility genes. The results of these tests provide useful guidance in deciding how to follow these high risk individuals in order to prevent the occurrence of breast cancer or permit early cancer detection.

## High Penetrance Genes

### *BRCA1*

BRCA1 was cloned in 1994 [O'Connell et al., 1994]. Since then, researchers have discovered numerous roles for the protein. The exact function for *BRCA1* is unknown as exemplified by the fact that mice that lack one copy of the *Brca1* gene do not exhibit any strong tumor predisposition. However, mice that lack two copies of the *Brca1* gene die *in utero* [Evers and Jonkers, 2006]. These traits have limited *in vivo* analysis of the *Brca1* gene. The BRCA1 protein may not have one specific function, but its interaction with a variety of other proteins is essential for regulating DNA repair, transcription, and cell cycle progression [Greenberg, 2008;Boulton, 2006]. Mutations in BRCA1 that prevent these protein-protein interactions lead to genomic instability and de-regulation of cell-cycle checkpoints [Deng, 2006].

Deleterious mutations within the *BRCA1* gene are a frequent cause of breast cancer among women with a strong family history of breast cancer and are associated with a

significantly increased risk for the disease. A recent analysis of 22 studies involving 8,139 index case patients unselected for family history shows that carrying a deleterious *BRCA1* mutation confers an estimated lifetime risk for developing breast cancer of 65% (95% CI 44% - 78%) [Antoniou et al., 2003]. By the age of 40, carrying a deleterious *BRCA1* mutation confers a 20% chance of developing breast cancer, and the risk increases with age, with the lifetime risk being 82% by age 80 [King et al., 2003]. Mutations in *BRCA1* are strongly associated with ovarian and fallopian tube cancer. The risk for ovarian cancer for a *BRCA1* mutation carrier is 17% by age 40 and increases to 39% by age 70 and 54% by age 80 [Antoniou et al., 2003].

*BRCA2*

The *BRCA2* gene was identified one year after *BRCA1* [Wooster et al., 1995]. The function of *BRCA2* is not as ubiquitous as *BRCA1*. Similarly to what is observed with *Brca1*$^{+/-}$ mice, mice that lack one copy of the *Brca2* gene do not exhibit a strong tumor predisposition [Evers and Jonkers, 2006]. Nonetheless, some functional clues have emerged from *in vitro* studies. After a double strand DNA breaks, BRCA2 induces the translocation of the protein Rad51 into the nucleus and directs Rad51 to the site of the break for homologous recombination-directed repair [Yoshida and Miki, 2004].

A smaller fraction of familial breast cancer cases can be attributed to mutations in *BRCA2* as compared to *BRCA1*. In a combined analysis of 22 studies, *BRCA2* mutation carriers were found to carry a cumulative breast cancer risk by age 70 of 45% (95% CI = 31% - 56%), and for ovarian cancer of 11% (95% CI = 2.4% - 19%) [Antoniou et al., 2003].

*Reliability of Current Genetic Testing for BRCA1 and BRCA2 Deleterious Mutations*

In a study of 300 women who had been diagnosed with invasive breast cancer at any age, had a family history of breast cancer (defined as a family with a minimum of 4 cases of female or male breast cancer, and/or ovarian cancer), and who tested negative for *BRCA1* and *BRCA2* mutations, as assessed by sequencing of the full coding region of each gene, 35 (11.6%) carried genomic rearrangements within the *BRCA1* or *BRCA2* genes. These mutations were more frequent among individuals under 40 years old [Walsh et al., 2006]. These data strongly suggest that genomic rearrangements within the *BRCA1* and *BRCA2* genes should be assessed in young patients with a strong family history of breast cancer, especially if the family history also includes male breast cancer and/or ovarian cancer.

*TP53*

*TP53* encodes the tumor suppressor protein p53, which inhibits cell cycle progression in the presence of radiation-induced DNA breaks. *TP53* mutations are associated with a syndrome named Li-Fraumeni syndrome (LFS) and Li-Fraumeni-like syndrome (LFLS). LFS is defined as a patient diagnosed with a sarcoma prior to age 45 who has both a 1$^{st}$ degree relative also under the age of 45 with any cancer and an additional 1$^{st}$ or 2$^{nd}$ degree relative (under age 45) in the same lineage with any cancer or sarcoma [Li et al., 1988]. LFLS expands on that definition, to include any individual under 45 years old who is diagnosed with sarcomas, brain tumors, adrenocortical carcinomas, or childhood leukemias who has both a 1$^{st}$ or 2$^{nd}$ degree relative in the same lineage with an LFS tumor at any age and another 1$^{st}$ or 2$^{nd}$ degree relative with any cancer prior to age 60 [Birch et al., 1994].

In families with LFS, *TP53* is frequently mutated. Studies have shown that although mutations in *TP53* are extremely rare in the general population, those with the mutation will develop cancer at some point. In a study of 100 women who had breast cancer, 4 women under 31 years old had a mutation in *TP53*, independent of *BRCA*-gene mutation status; 2/37 familial breast cancer cases had features of LFS or LFLS and 2/63 non-familial cases had mutations in *TP53* [Lalloo et al., 2006]. In Walsh's study of 300 women who had a strong family history of breast cancer and had neither mutations nor genomic rearrangements within the *BRCA1* and *BRCA2* genes, three families had LFS and 7 families had LFLS. Two of the 3 families with LFS and 1 in 7 families with LFLS carried mutations in *TP53*. In addition, out of 21 patients with a family history of breast cancer without LFS or LFLS, none carried mutations in *TP53*. In this selected population, about 1% of families with hereditary breast cancer may carry mutations in *TP53* [Walsh et al., 2006]. Another study suggests that that one in 5000 women with breast cancer harbors a *TP53* mutation [Lalloo et al., 2003]. Hence, in the absence of genomic rearrangements within the *BRCA1* and *BRCA2* genes, *TP53* mutations screening should be considered in women with a strong family history of breast cancer and features of LFS or LFLS.

*PTEN*

*PTEN* (phosphatase and tensin homolog) is a tumor suppressor gene that inhibits cell growth during the $G_1$ cell cycle phase by activating the cyclin-dependant kinase inhibitor p27(KIP1) [Li and Sun, 1998]. Mutations in *PTEN* are rare, but are associated with a high penetrance syndrome termed Cowden disease (CD). Individuals with CD have a high risk for developing breast cancer as well as hamartomas and benign tumors in the skin, thyroid, breast,

endometrium, and brain. At least 3 different mutations in *PTEN* have been found in families with CD and early onset breast cancer [Tsou et al., 1997].

*Lifestyle Factors that Affect Breast Cancer Risk*

Many factors influence the penetrance of tumor susceptibility genes, such as environmental factors, carcinogens, hormonal factors, and lifestyle factors. Hormonal factors that influence breast cancer risk include age at menarche, pregnancy, breast-feeding, and contraceptive use. However, these environmental factors may not be strong enough to change the penetrance of the *BRCA* genes. For example, an early age of onset of menstruation increases a woman's risk of breast cancer [Kelsey et al., 1993]. A recent study, though, involving 3947 women showed no correlation between carrying either *BRCA1* or *BRCA2* mutations with age of menarche (p=0.97). However, a matched case-control study with 1311 pairs, showed that for each year that menarche was delayed after age 11 in *BRCA1* carriers, there is a 15% decreased risk of breast cancer ($P_{Trend}$ = 0.0002). For women who experienced menarche at 15 years or older, there is a 54% reduced risk of breast cancer compared to those who experienced it before age 11.

Pregnancy is associated with a protective effect against early onset breast cancer in the general population. Although mutations in *BRCA1* or *BRCA2* are associated with a decreased age of breast cancer onset, the protective effects of pregnancy were the same as in wildtype patients. In the general population, childbirth reduces the risk of breast cancer by 23% (p = 0.009), and among women negative for either *BRCA*-gene mutation, the risk is similarly decreased. *BRCA*-gene mutation carriers seem to have a 29% decreased risk of breast cancer after childbirth, with a similar risk reduction to women who do not have a mutation in either

gene (p = 0.26) [King et al., 2003]. In addition, for women over the age of 40, each additional birth leads to a 14% reduction in the risk for breast cancer in the general population (95% CI = 6% - 22%; $P_{trend} = 0.008$). This trend is seen regardless of *BRCA*-gene mutation status [Andrieu et al., 2006].

It has also been shown that a healthier adolescent lifestyle, measured by adolescent weight within normal limits and physical activity, protects against the risk for early onset breast cancer. Physical activity among teenagers led to a decrease in early onset breast cancer (P = 0.025 in all study participants, and P = 0.034 for women with mutations in the *BRCA* genes) [King et al., 2003]. A study on 11,889 females with breast cancer from Taiwan found that both an increased BMI and hip circumference were associated with an increased risk for breast cancer. Compared to a BMI less than 21.6 $kg/m^2$, having a BMI over 26.2 $kg/m^2$ resulted in a relative risk of 1.9 (95% CI, 1.0-3.4), and compared to a hip circumference of less than 90 cm, one over 100 cm resulted in a RR = 2.9 (95% CI, 1.1-6.7; $P_{Trend} = 0.0485$) [Wu et al., 2006]. For *BRCA* mutation carriers, the OR associated with a 35 pounds weight gain after the age of 18 was found to be 4.64 (95% CI, 1.52-14.12; $P_{Trend} = 0.011$) compared to those who gained less than 12 lbs.

Genetic Testing

Genetic testing is carried out for families with a high risk of breast and ovarian cancer. The criteria for "high risk" is outlined in the National Comprehensive Cancer Network's (NCCN) Clinical Practice Guidelines in Oncology [2006]. The patient must present with a minimum of the one of the following factors: 1) early age at the onset of breast cancer; 2) two primary breast cancers or breast and ovarian cancer in a single patient or 2 primary breast or

breast and ovarian cancers in close relatives from the same side of the family; 3) a clustering of breast cancer with male breast cancer, thyroid cancer, sarcoma, adrenocorticoid cancer, endometrial cancer, pancreatic cancer, brain tumors, dermatologic manifestations, or leukemia/lymphoma on the same side of the family; 4) a member of the family with known mutations in breast cancer susceptibility genes; 5) population at risk (such as the Ashkenazi Jewish population); 6) any male breast cancer; or 7) Ovarian cancer.

Genetic screening for breast cancer consists of screening for mutations in *BRCA1* and *BRCA2*. If the family history is suggestive of either Cowden syndrome or Li-Fraumeni syndrome testing for mutations in *PTEN* or *TP53* may be indicated.

Genetic screening for breast cancer is comprised of screening for specific mutations in *BRCA1* and *BRCA2*. A blood sample is sent to Myriad Genetic Laboratories for their BRACAnalysis® test. The test is comprised of sequencing the 2 genes, as well as looking for 5 specific genomic rearrangements of *BRCA1*: 3.8 kb deletion in exon 13, 510 bp deletion in exon 22, 6 kb deletion in exon 13, 7.1 kb deletion in exon 8 and 9, and 26 kb deletion in exons 14-20 [Petrij-Bosch et al., 1997;Rohlfs et al., 2000;2000]. It has been found that in individuals of Ashkenazi Jewish heritage, 3 specific mutations in BRCA1 and BRCA2 are commonly encountered. Therefore in these individuals screening starts by testing for three founder mutations (185delAG and 5382insC in *BRCA1*, and 6174delT in *BRCA2* [Kadouri et al., 2007]).

A positive result for a deleterious mutation in *BRCA1* or *BRCA2* means that the patient carries a high risk of developing breast and ovarian cancer. A negative test result should be taken with caution. If the individual tests negative for a specific mutation that is present in the affected family members, then it is a true negative result, and the individual's risk of breast cancer is the same as the general populations. However, a negative result in an individual whose

family does not have a known mutation should be viewed with caution since there may be a possibility of missing a mutation with the conventional testing. In this case, the recommendations are individualized and have to do with the person's family and personal history. In some instances a mutation is found which has not been definitively shown to be deleterious, either because it is rare enough or because it does not track with the cancer pattern in families. Finally the lab may report a benign polymorphism which has been shown to not be disease causing.

Although full sequencing of the BRCA genes as well as testing for the five large gene rearrangements is considered the "gold standard" recent studies have shown that breast cancer patients who test negative for BRCA1 and BRCA2 mutations may have up to a 12% incidence of other mutations in the BRCA genes which were missed by conventional testing. Furthermore, it has been shown that there may be a 5% incidence of mutations in *CHEK2* and 1% incidence for a mutation in *TP53* [Walsh et al., 2006]. Other deletions and mutations have also been found [Gad et al., 2002;Mazoyer, 2005]. Therefore, in selected cases it may be beneficial to extend genetic testing to include testing in other regions in BRCA1 and BRCA2 as well as look for other gene mutations.

Low Penetrance Genes

Cancer due to *BRCA1* or *BRCA2* mutations account for only about 1-4 % of all breast cancer cases. Other genes have been identified as breast cancer susceptibility genes, which may account for a proportion of the remainder of heritable breast cancer cases. These genes include *BRIP1, CHEK2**1100delC, and *TGFBR1**6A. They are considered low penetrance breast cancer

susceptibility genes because only a small fraction of individuals who carry these genes will ultimately develop cancer [Stratton and Rahman, 2008].

*CHEK2*

CHEK2 is a cell cycle checkpoint protein that mediates mitotic block in the presence of ionizing radiation-induced DNA damage. Thus inactivating mutations in *CHEK2* would promote cancerous growth in the presence of DNA damage. The *CHEK2**1100delC mutation abolishes kinase activity of the protein, thereby blocking signaling by CHEK2 [Nevanlinna and Bartek, 2006].

The *CHEK2**1100delC variant is present in 1.1% of the population. In contrast, 5.1% of breast cancer patients who are wildtype for the *BRCA* genes carry this mutation. Women carriers of *CHEK2**1100delC have a 2 fold increased risk for breast cancer compared to the normal population [Meijers-Heijboer et al., 2002].

The role of *CHEK2**1100delC in male breast cancer is controversial. Meijers-Heijboer's study in 2002, which included patients from the UK, North America, the Netherlands, and Germany, found the *CHEK2**1100delC mutation in 13.5% of patients from families with male breast cancer. The risk for breast cancer in men who carry *CHEK2**1100delC is increased ten-fold, and 9% of male breast cancer cases are estimated to arise from *CHEK2**1100delC. Other studies have not been able to link *CHEK2**1100delC to male breast cancer cases [Walsh et al., 2006;Syrjakoski et al., 2004].

*BRIP1*

*BRIP1* (also known as *BACH1*) encodes a helicase that functionally interacts with the *BRCA1* gene to contribute to DNA repair [Cantor et al., 2001]. A recent study found that *BRIP1* was mutated in 9 out of 1,212 individuals (0.74%) with breast cancer who had a family history of breast cancer. Within these 9 people, there were 5 different types of truncating mutations. These patients carried wildtype *BRCA* genes. In the control group, which consisted of 2,081 people chosen from a 1958 Birth Cohort Collection in Great Britain, only 2 people (0.1%) had truncating mutations (P=0.0030), conferring an estimated relative risk of breast cancer associated with *BRIP1* truncated mutations to be 2.0 (95% CI, 1.2 – 3.2; P=0.012) [Seal et al., 2006].

HER2 in Breast Cancer

About 20- 30% of breast cancer tumors overexpress the *ERBB2* (*HER2*) oncogene [Slamon et al., 1987]. Patients who are $HER2^+$ have a poorer prognosis than those who are $HER2^-$ [Slamon et al., 1987;Slamon et al., 1989]. ERBB2 is a member of the epidermal growth factor receptor (EGFR) family (also called ERBB). There are 4 EGFR members: ERBB1 (EGFR), ERBB2 (HER2, NEU), ERBB3 (HER3), and ERBB4 (HER4). There are also 10 different ligands, which bind to and activate specific combinations of the receptors. ERBB2 does not have the ability to bind a ligand, and ERBB3 has no autophosphorylation domain. Thus, these 2 receptors rely on heterodimerization for signaling [Olayioye et al., 2000]. Overexpression of ERBB2 also leads to receptor homodimerization to induce signaling [Nagy et al., 2002]. ERBB2 is the preferred heterodimerization partner for all ERBB family members [Tzahar et al., 1996;Graus-Porta et al., 1997]. The intracellular phospho-tyrosines of the ERBB receptors can bind to multiple adapter molecules (such as GRB2, GRB7, SHC, CBL, JAK, in

addition to 10-15 others) to initiate various signaling cascades through out the cell. A variety of signaling events is possible depending on the ligand and dimerization status of the receptors. PI3K preferentially binds to ERBB3 and ERBB4, hence a heterodimer that contains either of those ERBB receptors will have higher PI3K/AKT signaling, which often results in cell survival [Soltoff and Cantley, 1996]. Almost each of the receptor combinations can activate the RAS/RAF/ERK/MAPK pathway [Yarden and Sliwkowski, 2001].

*Neu* is the rat form of *ERBB2.* Mice engineered to express the activated form of the rat *Neu* oncogene driven by the mouse mammary tumor virus (MMTV) will develop adenocarcinomas in the entire epithelium of each mammary gland as early at 78 days, with all mice showing tumors by 95 days. This data suggests that activated *Neu* is a potent enough oncogene that it can induce tumor formation with few, if any, other genetic alterations [Muller et al., 1988]. Mice harboring the *MMTV-c-Neu* proto-oncogene, the form which has been correlated with human breast cancer [Slamon et al., 1989], begin to develop focal mammary tumors by 4 months of age, with 50% of females developing mammary tumors by 205 days. 73% of mice harboring the *Neu* protooncogene also exhibited lung metastasis [Guy et al., 1992].

In addition to the above meantioned genes and proteins, common variants in the TGF-β signaling pathway that may modify breast cancer risk have been identified. The TGF-β pathway and implications for the role of TGF-β in cancer is discussed in the following sections.

## TGF-β Signaling

The TGF-β signaling pathway is conserved across species, from worms and flies to mammals [Shi and Massague, 2003;Schmierer and Hill, 2007]. The signaling molecules can be broken down into three different areas: ligands, receptors, and signaling molecules.

### Ligands

The TGF-β superfamily of proteins consists of a diverse set of proteins that share at least 25% homology between members. The family is characterized by the presence of a "cystein knot," a structural motif in the mature protein formed by three intramolecular disulfide bonds between six strictly conservative cystein residues [Sun and Davies, 1995]. The superfamily consists of two subfamilies, the TGF-β/Activin/Nodal subfamily, and the bone morphogenic (BMP), growth and differentiation factor (GDF), and Muellerian inhibiting substance (MIS) subfamily, which are classified by the specific signaling pathways that are activated in the cell [Shi and Massague, 2003].

TGF-β has three distinct isoforms which are encoded on different chromosomes. TGF-β1 is encoded by *TGFB1* on chromosome 19q13.1 [Fujii et al., 1986]; TGF-β2 is encoded by *TGFB2* on chromosome 1q41 [Fujii et al., 1986]; TGF-β3 is encoded by *TGFB3* on chromosome 14q23-q24 [Ten et al., 1988]. Each isoform is expressed in a tissue specific manner: TGF-β1 is expressed in endothelial, hematopoietic, and connective tissue cells; TGF-β2 is expressed in epithelial and neuronal cells; TGF-β3 is expressed in mesenchymal cells [Bian et al., 2003].

TGF-β is synthesized as a precursor molecule with a propeptide region located at its N-terminus, which is known as the latency associated peptide (LAP). TGF-β forms homodimers while in the cell and the LAP remains attached in the small latent complex. Both the TGF-β and

LAP homodimers are held together by disulfide bonds [Gentry and Nash, 1990;Gentry et al., 1988]. When the small latent complex is secreted, LAP is cleaved from the mature TGF-β by furin-like endoproteinase[Dubois et al., 1995;Dubois et al., 2001], however LAP stays non-covalently attached to TGF-β [Saharinen et al., 1999]. The LAP shields the receptor binding sites on the TGF-β homodimers keeping the complex inactive. When the secreted TGF-β complex was purified from platelets, a larger structure was identified, cloned, and named Latent TGF-β Binding Protein (LTBP). LTBP is a large molecular weight protein (125-160 kD) that binds the LAP by disulfide bonds [Miyazono et al., 1988;Wakefield et al., 1988]. LTBP enhances the secretion of the large TGF-β complex out of the cell and targets the complex to the extracellular matrix (ECM), thereby keeping TGF-β inactive until needed [Saharinen et al., 1999].

LTBP can be cleaved from the TGF-β complex by several different mechanisms. *In vitro*, acidic or basic pH releases LTBP from TGF-β [Lawrence et al., 1985;Brown et al., 1990]. TGF-β is activated in physiological conditions by acidic pH [Jullien et al., 1989], thrombospondin-1 (THBS1) [Schultz-Cherry and Murphy-Ullrich, 1993;Crawford et al., 1998], and ITGAV/ITGB6 ($\alpha_v\beta_6$ integrin) [Munger et al., 1999]. Proteases target the LAP propeptide for degredation, thereby releasing active TGF-β. In co-culture experiments with endothelial cells and smooth muscle cells, TGF-β is primarily activated by the protease plasmin, whereas when each of these cells are cultured alone, only latent TGF-β is present [Sato and Rifkin, 1989;Sato et al., 1990;Antonelli-Orlidge et al., 1989]. It has also been shown that fibronectin (FN1) is required for ITGAV/ITGB6 -induced ligand activation. ITGAV/ITGB6 binds to the LAP propeptide and FN1 binds to LTBP; it is hypothesized that the role for FN1 in activating TGF-β is to bring the latent complex in close proximity to ITGAV/ITGB6 to bind the LAP [Fontana et al., 2005].

Receptors

The type 1 and type 2 TGF-β receptors (TGFBR1 and TGFBR2, respectively) are part of the serine/threonine family of kinase receptors. The receptors have an N-terminal extracellular domain for binding the ligand, a transmembrane domain, and C-terminal ser/thr kinase domain. TGF-β only can recognize and bind TGFBR2. TGFBR2 is a constitutively active kinase, however, it is not until TGFBR2 is brought into close proximity to TGFBR1, that it is capable of having any signaling capabilities [Wrana et al., 1992]. Thus, a TGF-β dimer binds two TGFBR2 receptors, which then incorporate 2 TGFBR1 receptors, leading to the formation of a tetrameric complex. TGFBR1 contains a characteristic SGSGSG sequence (termed GS domain) directly following the kinase domain. The GS site is phosphorylated by TGFBR2 which then acts as a docking platform for pSMAD2, a key TGF-β signaling molecule [Huse et al., 2001]. FKBP12, an inhibitor of TGF-β signaling, binds to the unphosphorylated GS region of TGFBR1 to block TGFBR2-induced phosphorylation of this GS region [Okadome et al., 1996;Chen et al., 1997]. Therefore, the GS site is a key regulatory domain in TGFBR1 that is either bound by FKBP12 to keep the kinase activity repressed or phosphorylated by TGFBR2 to initiate signaling via the SMADs.

SMAD Signaling

The SMAD pathway is the primary signaling mechanism initiated by TGF-β. The SMAD protein was first identified and cloned from *Drosophila* (named MAD) and orthologs were discovered shortly thereafter in worms and vertebrates (named SMAD) [Sekelsky et al., 1995;Derynck et al., 1996]. SARA, the SMAD anchor for receptor activation, binds to and transports the R-SMADs (SMAD2 and SMAD3 complex) to the receptors. SARA contains a

phospholipid binding domain which recruits R-SMAD to the cell membrane [Tsukazaki et al., 1998]. The R-SMADs are targeted to early endosomes, which is in close proximity to the receptors when signaling is initiated [Di Guglielmo et al., 2003]. Active (phosphorylated) TGFBR1 recruits R-SMAD to the receptor where they are in turn phosphorylated. This phosphorylation causes SARA to dissociate from the SMAD complex, so SMAD2 and SMAD3 can then bind to SMAD4. The SMAD2/3/4 complex then translocates to the nucleus where it can initiate transcription of target genes. SMAD4 can also associate with SMAD2/3 complex once in the nucleus. SMAD4 has the ability to shuttle in and out of the nucleus independently of TGF-β signaling [Pierreux et al., 2000;Watanabe et al., 2000]. Once inside the nucleus, the SMAD complex binds to different co-activators (CBP, p300, ARC105, SMIF) and transcription factors [MIZ1, E2F4/5, DP1) to initiate transcription [Shi and Massague, 2003].

SMAD signaling can be disrupted by SKI and SKIL (also known as SnoN), both part of the SKI family of protooncogenes. The SKI family induces SMAD4 to dissociate from the activated R-SMADs to inhibit signaling. SKI/SKIL also recruits transcriptional co-repressors including the nuclear hormone co-repressor NCOR1, the histone deacetylase SIN3A, and the methyl-CpG-binding protein MECP2 to inhibit transcription [Shi and Massague, 2003;Luo, 2004]. TGIF is another inhibitor of TGF-β-dependent gene activation which recruits HDACs to the SMAD complex to inhibit transcription [Wotton et al., 1999].

SMAD7, an inhibitory SMAD protein, is upregulated in response to EGFR, interferon-γ via STATs, tumor necrosis factor α via NF-κB, and by autocrine TGF-β signaling [Kaklamani and Pasche, 2005]. SMAD7 blocks TGF-β signaling by binding to phosphorylated TGFBR1 and recruiting SMURF1 or SMURF2, E3 ubiquitin ligases, to target the receptor for degradation [Park, 2005]. Thus TGF-β signaling can be regulated at several different steps.

Non-Canonical TGF-β Signaling

TGF-β can also activate signaling independently of the SMADS. In fact, many pro-oncogenic effects of TGF-β are through the non-canonical pathways. TGF-β has been shown to activate ERK, p38, and JNK in a cell line and context specific manner [Derynck et al., 2001]. Specific examples of TGF-β activating MAPK signaling will be discussed further in the following section.

TGF-β signaling can be diverted from the SMAD pathway to these alternate pathways by a few mechanisms. Limiting the availability of SMADS shifts the balance into the non-canonical signaling pathway. SMAD4 is degraded by the ubiquitin-proteosome pathway after upregulation of RAS [Saha et al., 2001]. The loss of SMAD4 then results in hyperactivation of RAS/ERK, which can lead to further pro-oncogenic effects [Iglesias et al., 2000]. In addition, the co-repressor, TGIF, is stabilized to the SMAD complex in response to EGF signaling through RAS/MEK, thereby limiting the SMADs ability to signal and thus diverting TGF-β signaling to the MAPK signaling pathways [Lo et al., 2001].

## Role of TGF-β in Cancer Suppression and Promotion

TGF-β plays a pivotal, although somewhat contradictory, role in cancer development. Although TGF-β inhibits tumor formation by inducing growth arrest in normal cells, TGF-β can also signal to tumor cells to induce late stage progression and metastasis.

TGF-β as a Tumor Suppressor

The primary role of TGF-β is to induce cell senescence in most cell types, however it can also promote cellular differentiation and apoptosis [Bian et al., 2003;Siegel and Massague,

2003;Rahimi and Leof, 2007]. TGF-β mediates $G_1$ cell cycle arrest by inducing expression of cyclin dependant kinase inhibitors CDKN1A ($p21^{CIP1}$), CDKN1B ($p27^{KIP1}$), CDKN2A ($p16^{INK4A}$), and CDKN2B ($p15^{INK4B}$), as well as CDK4 and CDC25A [Feng et al., 2000;Datto et al., 1995;Iavarone and Massague, 1997;Bian et al., 2003]. TGF-β also inhibits cell proliferation by downregulating the oncogene MYC, a transcription factor that directly transcribes genes involved in cell cycle progression, apoptosis, and cell transformation [Wilkins and Sansom, 2008]. The SMAD complex binds the TGF-β-inhibitory element on MYC promoter, thereby inhibiting transcription of the gene [Chen et al., 2001;Pietenpol et al., 1990].

Decreases in TGF-β signaling result in an increased risk of cancer due to a lack of inhibitory growth signals. Mutations in the different components of the signaling pathway, including TGFBR1, TGFBR2, TGFB1, and the SMADs have all been implicated in different types of cancer. Mutations in TGFBR1 has been well documented in ovarian, breast, kidney, and bladder cancers [Wang et al., 2000;Chen et al., 1998;Chen et al., 2004]. Hypermethylation of the TGFBR1 promoter has been shown to be involved in MSI sporadic gastric tumors [Pinto et al., 2003]. Mutations in TGFBR2 are found in colon, head and neck (HN), and gastric cancers [Markowitz et al., 1995;Garrigue-Antar et al., 1995;Pinto et al., 2003]. Mutations in SMAD4 have been reported in ovarian and cervical cancers [Wang et al., 2000;Baldus et al., 2005].

Our lab is particularly interested in the role of TGFBR1-mediated TGF-β signaling. To address this, we developed a novel *Tgfbr1*$^{+/-}$ mouse model, which has been fully backcrossed into three strains of mice that are commonly used to study cancer development: C57BL/6, 129SvIm, and FVB/N. *Tgfbr1*$^{+/-}$ mice had decreased TGF-β signaling in various tissues (Pasche Lab, unpublished data). The C57BL/6 *Tgfbr1*$^{+/-}$ mice were mated to C57BL/6 *Apc*$^{Min/+}$ mice, a model commonly used to study colon cancer [Moser et al., 1990;Su et al., 1992], and we found a

two-fold increase in the number of intestinal tumors in $Apc^{Min/+};Tgfbr1^{+/-}$ mice compared to the $Apc^{Min/+}$ mice (Pasche Lab, unpublished data). Similar results were obtained when $Tgfbr1^{+/-}$ mice were treated with azoxymethane. This dramatic effect of *Tgfbr1* haploinsufficiency in the $Apc^{Min/+}$ and azoxymethane models, led us to ask whether decreased Tgfbr1 expression is also found in humans. Our lab, in collaboration with Albert de la Chapelle, discovered that allele-specific expression (ASE) of *TGFBR1* occurs in 1-2% of the general population. Moveover, 10-20% of sporadic colorectal cancer (CRC) patients have *TGFBR1* ASE. ASE in *TGFBR1* confers an increased risk for CRC (OR 8.7; 95% CI 2.6-29.1) [Valle et al., 2008]. This is another example demonstrating the role of TGF-β signaling as a tumor suppressor.

TGF-β as a Tumor Promoter

TGF-β was first described as a transforming growth factor because of its ability to transform mouse 3T3 fibroblasts. Transformation was measured by loss of density-dependent growth inhibition and gain of anchorage-independent growth [De Larco and Todaro, 1978].

Tumor cells often secrete excess TGF-β [Derynck et al., 1987] and TGF-β has been implicated in tumor cell invasion and changes in the microenvironment. An important step in initiating invasion is the ability for the tumor cells to undergo EMT, whereby cells loose their adhesive properties and gain the ability to migrate and invade the stroma [Thiery, 2003]. TGF-β mediates EMT in a variety of cell lines including: mouse epithelial mammary cells (NMuMG), human normal and transformed breast epithelial cells, mink lung cells (Mv1Lu), pancreatic tumor cells (BxPc3), and primary mouse keratinocytes [Gotzmann et al., 2004;Derynck et al., 2001]. The mechanism by which TGF-β induces EMT is specific to the cell model [Zavadil and Bottinger, 2005]. For example, in the NMuMG cell line, TGF-β induces N-cadherin expression,

disrupts E-cadherin expression, and induces actin stress fiber assembly through RhoA and ROCK and are independent of the Smad signaling [Bhowmick et al., 2001]. EMT has been shown to be enhanced in breast tumor cells through RAS/RAF, P13K/AKT, and the RhoA pathways [Oft et al., 1996;Oft et al., 1998;Bakin et al., 2000;Bhowmick et al., 2001]. The ERK signaling pathway has also been implemented in EMT in keratinocytes [Zavadil et al., 2001].

TGF-β also enhances cell migration through various MAPK signaling pathways. For instance, migration of human lung fibroblasts cells has been associated with signaling through both p38 and ERK [Pechkovsky et al., 2008;Caraci et al., 2008]. TGF-β induces migration in MDA-MB-231 breast cancer cells through AKT, ERK, and NF-κB [Wei et al., 2008]. Secker et al. found that TGF-β induces the migration of human corneal epithelial cells after wounding through signaling through the RAS/MEK/ERK MAPK pathway [Secker et al., 2008]. Invasion of kidney epithelial cells was enhanced by RAF signaling [Lehmann et al., 2000].

TGF-β also induces tumor growth by activating angiogenesis. Angiogenesis is a crucial process in tumor progression, as the formation of new blood vessels allow oxygen to reach the inner portion of tumor cells to survive. Increased vasculature in the tumor also provides easier access for the tumor cells to invade and metastasize [Hanahan and Weinberg, 2000]. TGF-β has been shown to be a potent inducer of angiogenesis [Roberts et al., 1986;Yang and Moses, 1990;Gajdusek et al., 1993]. Prostate tumors derived from Chinese hamster ovary (CHO) cells that overexpress TGF-β1 showed increased angiogenesis compared to non-overexpressing TGF-β cells [Ueki et al., 1992]. In addition, a positive correlation was shown between TGF-β2 or the combination of TGF-β2/TGFBR1/TGFBR2 and increased microvessel density in invasive breast tumors [de Jong et al., 1998].

**TGF-β Polymorphisms in Breast Cancer**

Germline mutations in both the *TGFB1* ligand and the TGF-β receptors have been linked to breast cancer risk [Kaklamani et al., 2005;Kaklamani and Pasche, 2005]. Although no specific mutations in *TGFBR2* have been linked to breast cancer, mutations and decreased TGFBR2 expression have been observed. Lucke and colleages screened 17 primary breast tumors and 17 recurrent breast tumor samples for mutations in *TGFBR2*, and although they were unable to find any mutations in the primary tumor samples, they identified 4 novel mutations in the kinase domain in the recurrent tumor samples [Lucke et al., 2001]. Decreased TGFBR2 expression has also been correlated with an increase in aggressiveness of tumor development. One study found that women who had breast epithelial hyperplastic lesions lacking atypia (EHLA) with low TGFBR2 staining, had a higher risk for developing invasive breast cancer[Gobbi et al., 1999]. The same group later showed that a decrease in TGFBR2 expression may contribute to breast cancer progression. Decreased expression of TGFBR2 was found to be associated with high grade ductal carcinoma *in situ* (DCIS) and invasive mammary carcinomas [Gobbi et al., 2000].

*TGFBR1 (Int7G24A)*

A polymorphism resulting from a G→A transition in intron 7 of *TGFBR1* (*Int7G24A*) was first identified by a screen of structural alterations that may lead to cervical cancer, and was subsequently linked to an increased risk of bladder, kidney, and lung cancers [Chen et al., 1999;Chen et al., 2004;Zhang et al., 2003]. Moreover, *Int7G24A* was found in 48% of invasive/metastatic breast cancers, compared to 26% of the controls. Carrying at least one allele of *Int7G24A* results in a 161% increased risk for invasive breast cancer (OR, 2.61; 95% CI, 1.65-

4.11, p<0.0001) and may represent a marker for breast cancer progression [Chen et al., 2006]. Although a recent report failed to verify this association with *Int7G24A* to breast cancer risk in Swedish familial or sporadic breast sample population[Song et al., 2007], more studies are needed to determine if the risk is significant. The functional role of *Int7G24A* has yet to be determined.

*TGFBR1**6A

Our lab has previously identified a common polymorphism of *TGFBR1*, named *TGFBR1**6A (or *6A for short), which is a low penetrant, tumor susceptibility allele [Pasche et al., 1998;Pasche et al., 1999]. 14.1% of the population carries *TGFBR1**6A. *6A confers a 22% increased risk for cancer [Zhang et al., 2005] and is associated specifically with colon, ovarian, cervical, and breast cancer [Kaklamani et al., 2003;Pasche et al., 2004;Baxter et al., 2002;Chen et al., 1999;Bian et al., 2005]. In particular, homozygosity for *6A confers a 107% and 200% increased risk for ovarian and prostate cancer, respectively, compared to individuals homozygous for *TGFBR1**9A [Zhang et al., 2005]. The risk of colon cancer increase 20% for *6A carriers and 102% for homozygotes [Pasche et al., 2004].

The most recent combined analysis on the association of *TGFBR1**6A with breast cancer risk includes all published reports as of February 2008 as well as recent data our lab derived using breast cancer cases and their sibling controls from the NCI-sponsored Breast Cancer Family Registry (BCFR). The results from this combined analysis that now includes a total of 6,694 breast cancer cases and 8,579 controls reveal that *TGFBR1**6A is associated with breast cancer risk (O.R. 1.15, 95% CI 1.04-1.28) (Table 1.1). The results establish the presence of an allelic dosing effect as the risk incurred by *TGFBR1**6A homozygotes (O.R. 1.40, 95% CI 1.04-

1.88) is higher than the risk incurred by *TGFBR1**6A heterozygotes (O.R. 1.12, 95% CI 1.00-1.25) (Table 1.2). *TGFBR1**6A has recently been found to be associated with stage 3 breast cancer (O.R. 2.3, 95% CI 1.0-5.1; P=0.05) in sporadic breast cancer study from Sweden [Song et al., 2007].

**Table 1.1:**
**Combined analysis of all published breast cancer case control BCFR studies:**
**The risk of breast cancer from carrying *6A**

| Number | Study | Effect | OR (95%CI) | P value | Cases | Controls |
|---|---|---|---|---|---|---|
| 1 | Pasche [Pasche et al., 1999] | Any6A | 1.530(0.951,2.460) | 0.080 | 24/152 | 78/735 |
| 2 | Baxer [Baxter et al., 2002] | Any6A | 1.549(1.057,2.270) | 0.025 | 87/355 | 41/248 |
| 3 | Reiss [Pasche et al., 2004] | Any6A | 0.714(0.315,1.615) | 0.418 | 11/98 | 14/91 |
| 4 | Caldes [Pasche et al., 2004] | Any6A | 1.533(1.012,2.322) | 0.044 | 57/275 | 42/294 |
| 5 | Offit [Pasche et al., 2004] | Any6A | 1.366(0.918,2.033) | 0.124 | 71/463 | 39/330 |
| 6 | Northwestern [Pasche et al., 2004] | Any6A | 0.885(0.289,2.708) | 0.830 | 4/29 | 18/123 |
| 7 | Jin [Jin et al., 2004] | Any6A | 0.996(0.727,1.364) | 0.979 | 74/391 | 89/437 |
| 8 | Kaklamani [Kaklamani et al., 2005] | Any6A | 1.468(1.081,1.992) | 0.014 | 96/611 | 78/690 |
| 9 | Pasche Italy [Pasche et al., 1999] | Any6A | 1.048(0.446,2.461) | 0.915 | 11/48 | 12/50 |
| 10 | Chen [Chen et al., 2006] | Any6A | 1.479(0.789,2.776) | 0.222 | 23/104 | 19/129 |
| 11 | Feigelson [Feigelson et al., 2006] | Any6A | 1.000(0.733,1.364) | 1.000 | 72/363 | 100/484 |
| 12 | Cox [Cox et al., 2007] | Any6A | 0.942(0.788,1.126) | 0.511 | 219/968 | 321/1352 |
| 13 | Song [Song et al., 2007] | Any6A | 1.122(0.901,1.398) | 0.304 | 165/764 | 170/856 |
| 14 | BCFR | Any6A | 1.096(0.949,1.266) | 0.212 | 316/2073 | 387/2760 |
| Fixed | | | 1.124(1.038,1.216) | 0.004 | 1230/6694 | 1408/8579 |
| **Random** | | | **1.152(1.039,1.276)** | **0.007** | **1230/6694** | **1408/8579** |

**Heterogeneity p =0.18**

**Table 1.2:**
**Combined analysis of all published breast cancer case control BCFR studies:**
***6A Heterozygosity vs Homozygosity: An allelic dosage effect**

| Number | Study | Effect | OR (95%CI) | P value | Cases | Controls |
|---|---|---|---|---|---|---|
| 1 | Pasche [Pasche et al., 1999] | 9A/6A | 1.530(0.951,2.460) | 0.080 | 24/152 | 78/735 |
| 2 | Baxer [Baxter et al., 2002] | 9A/6A | 1.551(1.041,2.312) | 0.031 | 83/355 | 39/248 |
| 3 | Reiss [Pasche et al., 2004] | 9A/6A | 0.714(0.315,1.615) | 0.418 | 11/98 | 14/91 |
| 4 | Caldes [Pasche et al., 2004] | 9A/6A | 1.474(0.970,2.239) | 0.069 | 56/275 | 42/294 |
| 5 | Offit [Pasche et al., 2004] | 9A/6A | 1.277(0.846,1.926) | 0.244 | 67/463 | 38/330 |
| 6 | Northwestern [Pasche et al., 2004] | 9A/6A | 0.998(0.323,3.085) | 0.997 | 4/29 | 17/123 |
| 7 | Jin [Jin et al., 2004] | 9A/6A | 0.845(0.603,1.182) | 0.325 | 66/391 | 86/437 |
| 8 | Kaklamani [Kaklamani et al., 2005] | 9A/6A | 1.378(1.007,1.884) | 0.045 | 92/611 | 77/690 |
| 9 | Pasche Italy [Pasche et al., 1999] | 9A/6A | 0.853(0.350,2.077) | 0.726 | 10/48 | 12/50 |
| 10 | Chen [Chen et al., 2006] | 9A/6A | 1.658(0.869,3.162) | 0.125 | 23/104 | 18/129 |
| 11 | Feigelson [Feigelson et al., 2006] | 9A/6A | 0.908(0.653,1.263) | 0.567 | 66/363 | 96/484 |
| 12 | Cox [Cox et al., 2007] | 9A/6A | 0.952(0.790,1.148) | 0.609 | 207/968 | 302/1352 |
| 13 | Song [Song et al., 2007] | 9A/6A | 1.072(0.848,1.353) | 0.562 | 152/764 | 160/856 |
| 14 | BCFR | 9A/6A | 1.092(0.924,1.291) | 0.301 | 266/2073 | 326/2760 |
| Fixed | | | 1.096(1.007,1.193) | 0.035 | 1127/6694 | 1305/8579 |
| **Random** | | | **1.118(1.000,1.249)** | **0.050** | **1127/6694** | **1305/8579** |

**Heterogeneity p =0.16**

| Number | Study | Effect | OR (95%CI) | P value | Cases | Controls |
|---|---|---|---|---|---|---|
| 1 | Pasche [Pasche et al., 1999] | 6A/6A | / | / | 0/152 | 0/735 |
| 2 | Baxer [Baxter et al., 2002] | 6A/6A | 1.402(0.420,4.681) | 0.583 | 4/355 | 2/248 |
| 3 | Reiss [Pasche et al., 2004] | 6A/6A | / | / | 0/98 | 0/91 |
| 4 | Caldes [Pasche et al., 2004] | 6A/6A | 5.365(0.257,111.99) | 0.279 | 1/275 | 0/294 |
| 5 | Offit [Pasche et al., 2004] | 6A/6A | 2.867(0.607,13.545) | 0.184 | 4/463 | 1/330 |
| 6 | Northwestern [Pasche et al., 2004] | 6A/6A | 0.836(0.040,17.646) | 0.908 | 0/29 | 1/123 |
| 7 | Jin [Jin et al., 2004] | 6A/6A | 3.022(1.177,7.761) | 0.022 | 8/391 | 3/437 |
| 8 | Kaklamani [Kaklamani et al., 2005] | 6A/6A | 4.540(0.962,21.422) | 0.056 | 4/611 | 1/690 |
| 9 | Pasche Italy [Pasche et al., 1999] | 6A/6A | 5.317(0.252,112.20) | 0.283 | 1/48 | 0/50 |
| 10 | Chen [Chen et al., 2006] | 6A/6A | 0.246(0.012,5.153) | 0.366 | 0/104 | 1/129 |
| 11 | Feigelson [Feigelson et al., 2006] | 6A/6A | 2.017(0.820,4.960) | 0.127 | 6/363 | 4/484 |
| 12 | Cox [Cox et al., 2007] | 6A/6A | 0.881(0.527,1.473) | 0.628 | 12/968 | 19/1352 |
| 13 | Song [Pasche et al., 2005] | 6A/6A | 1.464(0.814,2.634) | 0.203 | 13/764 | 10/856 |
| 14 | BCFR | 6A/6A | 1.021(0.706,1.476) | 0.913 | 50/2073 | 61/2760 |
| Fixed | | | 1.245(1.019,1.521) | 0.032 | 103/6694 | 103/8579 |
| **Random** | | | **1.400(1.042,1.882)** | **0.026** | **103/6694** | **103/8579** |

**Heterogeneity p =0.22**

*TGFB1** T29C

A polymorphisms in the *TGFB1* gene, *TGFB1* T29C results in a leucine to proline substitution at residue 10 (also named *TGFB1* L10P). This substitution causes a 2.8 fold increase in the amount of circulating TGF-β1 in HeLa cells compared to cells expressing the normal T29 (leucine) gene [Dunning et al., 2003]. In addition, individuals with *TGFB1*CC* had lower serum levels of TGF-β1 compared to individuals with either *TGFB1**TC or *TGFB1**TT [Yokota et al., 2000]. In a study of 3,075 postmenopausal Caucasian women with a median age of 70, *TGFB1**T carrier status, was significantly associated with a 64% increased incidence of breast cancer when compared with *TGFB1**CC homozygous carrier status signifying that increased TGF-β secretion protects against breast cancer [Ziv et al., 2001]. However, opposite results were found in a pooled analysis of three European case-control studies that included 3,987 cases and 3,867 controls with a median age of 50. In that study, there was a 21% decreased risk of breast cancer in carriers of the *TGFB1**T allele [Dunning et al., 2003] when compared with homozygous carriers of the *TGFB1**C allele, suggesting that age and menopausal status might modify the association between *TGFB1* T29C variant and breast cancer risk. In a Japanese hospital-based study of 232 cases and 172 controls with a median age of 55, there was no overall association between the *TGFB1**T allele and breast cancer [Hishida et al., 2003]. However, for premenopausal women, homozygous carriers of the *TGFB1**T allele had a 55% reduced risk of breast cancer in comparison with the *TGFB1**CC genotype. A German study of 500 cases and 500 controls with a median age of 57 did not find any statistically significant association between either the *TGFB1**T allele and breast cancer [Krippl et al., 2003]. Similarly, a large multiethnic case-control study of 1123 breast cancer cases and 2314 controls with a

median age of 63 from Los Angeles and Hawaii also did not find any association between the *TGFB1**T allele and breast cancer risk [Marchand et al., 2004]. Our recent study of 658 breast cancer cases and 841 controls did not show any significant association between the *TGFB1**T allele and breast cancer risk but there was a trend towards an association between the *TGFB1**C allele and decreased breast cancer risk [Kaklamani et al., 2005]. A combined analysis of these studies conducted by the Breast Cancer Association Consortium has shown that the *TGFB1* T29C SNP is associated with breast cancer risk [Breast Cancer Association Consortium, 2006]. Of note, only five of the sixteen candidate SNPs studied were found to be associated with breast cancer risk and the association of *TGFB1* T29C with breast cancer risk was the strongest of all SNPs studied ($p = 0.0088$). These results were recently validated in another large replication study conducted by the Breast Cancer Association Consortium, which confirmed the association of the *TGFB1* T29C with breast cancer (O.R. 1.07, 95% CI 1.02-1,13) and homozygotes (O.R. 1.16, 95% CI 1.08-1.25) [Cox et al., 2007]. Higher levels of circulating TGF-β1 have recently been found to be associated with worse overall survival in the Shanghai breast cancer study [Grau et al., 2007]. These findings strongly suggest that the naturally-occurring variants of the TGF-β ligands are associated with both breast cancer risk and outcome.

Combined assessment of *TGFB1**T29C and *TGFBR1**6A

Our lab has recently assessed the combined effects of *TGFB1* T29C and *TGFBR1**6A SNP's on breast cancer risk. Individuals were grouped according to TGF-β signaling status and risk evaluated. Individuals who were homozygous for both *TGFB1**CC and *TGFBR1**6A were classified as high signalers (activated ligand, and wild-type receptor). Because *TGFBR1**6A has hypomorphic signaling compared to the wildtype receptor [Pasche et al., 1999], all *6A carriers

were classified as low signalers, with the exception of *6A carriers with the genotype *TGFB1**CC, which were classified as intermediate signalers. Intermediate signalers also included those homozygous for *9A that carried at least one *TGFB1**T allele. Compared to high signalers, the low signalers had a significantly higher risk for breast cancer (O.R. 1.69, 95% CI 1.08-2.66), with the biggest effect seen on women over 50 years who were classified as low signalers (O.R. 2.05, 95% CI 1.01-4.16) [Kaklamani et al., 2005].

TGF-β and Transgenic Breast Cancer Mouse Models

A common approach to studying protein expression specifically in the mammary gland is to express the protein under the control of the mouse mammary tumor promoter virus (MMTV). *MMTV-Tgfb1* mice or *MMTV-Tgfb1*$^{S223/225}$ (constitutively active TGF-β) mice fail to develop spontaneous mammary tumors [Pierce, Jr. et al., 1993;Pierce, Jr. et al., 1995].

When *MMTV-c-Neu* mice were crossed with *MMTV-Tgfb1*$^{S223/225}$ mice, tumors from the *Neu;Tgfb1*$^{S223/225}$ mice were smaller in volume, more poorly differentiated, and exhibited local invasion, even though the latency did not change. Moreover, these double transgenic mice had 100% occurrence for metastasis to the lung 100 days after tumor formation, as compared to 60% of the *MMTV-Neu* mice. *Neu;Tgfb1*$^{S223/225}$ tumors were highly vascularized as well [Muraoka et al., 2003]. Another *MMTV-c-Neu* model expressing constitutively active Tgfbr1 (*MMTV-Alk5*$^{TD}$) showed that 78% of *Neu;Alk5*$^{TD}$ mice had lung metastasis compared to 31% of *Neu* controls, while the tumor latency and tumor burden remained unchanged [Muraoka-Cook et al., 2006].

Mice engineered to express *MMTV-Tgfbr1(AAD)*, a different constitutively active Tgfbr1 model, were bred with mice containing the activated *MMTV-Neu* in which Neu is mutated to

allow signaling only through the Grb or Shc adapter proteins. Both the *Neu-Grb;Tgfbr1(AAD)* and *Neu-Shc;Tgfbr1(AAD)*mice had an increased tumor latency compared to the *Neu-Grb* or *Neu-Shc* mice, respectively, indicating that increased TGF-β signaling has a tumor suppressive effect. However *Neu-Shc;Tgfbr1(AAD)* mice had a 3-fold increased formation of extravascular lung metastasis compared to mice only able to signal through Grb [Siegel et al., 2003]. In addition, *MMTV-Tgfbr2(ΔCyt)* (dominant negative Tgfbr2) decreased tumor latency and decreased the formation of extravascular lung metastasis 2-fold in the *Neu-Grb* model [Siegel et al., 2003].

Although the effect of TGF-β signaling on tumor latency and tumor burden are highly dependant on the mouse model being used, there is a clear indication that increased TGF-β signaling leads to more breast cancer metastases.

**TGFBR1*6A**

*TGFBR1**6A has 3 –CGC- repeats (coding for 3 alanines) deleted from a 9 polyalanine sequence located in the signal sequence of *TGFBR1* [Pasche et al., 1998]. In mink lung epithelial cells lacking TGFBR1, TGFBR1*6A expression results in lowered TGF-β-mediated growth inhibition compared to cells where *9A was re-expressed. SBE4 luciferase activity, which measures SMAD binding to the SMAD promoter element, was also reduced by *6A expression in the presence of TGF-β [Pasche et al., 1999;Chen et al., 1999].

The signal sequence of a protein is responsible for directing a secreted or membrane-bound protein to the endoplasmic reticulum (ER) for further processing. The nascent polypeptide chain emerging from the ribosome is bound by a signal recognition particle (SRP) on its signal sequence. The SRP then binds to two-GTPs on the SRP receptor located in the ER

membrane. The GTP is hydrolyzed causing the release of SRP and allowing translation to continue through the SEC61A1 (Sec61α) protein. At some point during the translocation process, the signal sequence is cleaved from the protein by signal peptidases, and the polypeptide chain is released into the ER lumen for further processing [Rapoport et al., 1996;Enns, 2001].

To determine if the deletion in the polyalanine tract would affect the primary function of the signal sequence to direct the protein to the ER and subsequently the membrane, the membrane portion of HEK293 cells expressing TGFBR1 or TGFBR1*6A was isolated and the receptor was taken for automated Edman degredation and peptide sequencing by ion trap tandem mass spectrometry. The results from both techniques showed that the deletion does not affect the signal sequence cleavage site, however a minor secondary cleave site was detected in the WT form that was not detected in the *6A form (Figure 1.1) [Pasche et al., 2005]. *In-vitro* translation assay using rough dog pancreas microsomes also demonstrated that the *6A mature receptor is directed to the membranes and processed by glycosylation as efficiently as the WT mature receptor [Pasche et al., 2005]. The glycosylation occurs at unique Asnyy-X-X sites in the extracellular domain of the protein (which would be the luminal side in the *in-vitro* translation assay). The gel shift in figure 1.2 demonstrates that glycosylation occurs. (TGFBR1*10A in the figure is another polymorphism that was identified). Further processing of the *in-vitro* translated receptor with Proteinase K, indicated that the protease-sensitive cleavage sites on the cytoplasmic domain of the receptor remained intact, as indicated by the 30 kD band present in figure 1.2. In addition, we also showed that membrane localization of TGFBR1 in MCF-7 breast cancer cells was identical for both WT and *6A by using a GFP-tagged TGFBR1 and *6A construct (Pasche Lab unpublished data).

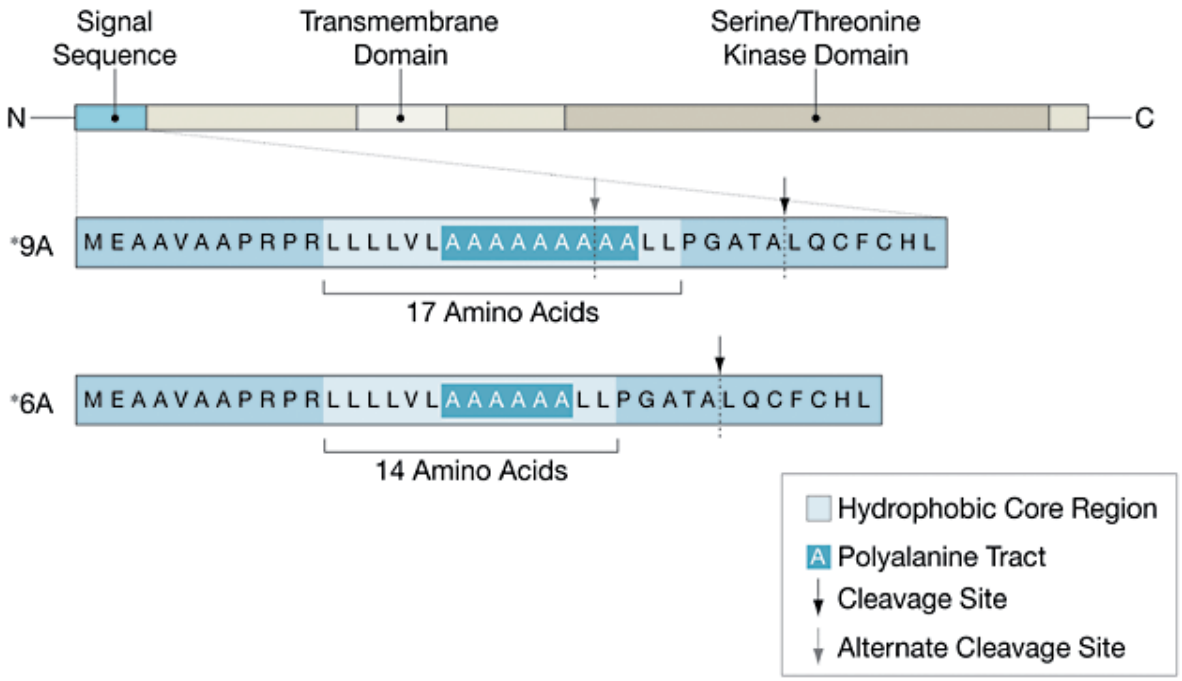


**Figure 1.1 The signal sequence cleavage site is identical in TGFBR1*6A and TGFBR1*9A**

Amino terminus sequencing of *6A and *9A reveals that the predicted signal sequence cleavage site remains intact in TGFBR1*6A. The signal sequence is cleaved between Ala30 and Leu31 in *6A and between Ala33 and Leu34 in *9A.

An alternate cleavage site was located in the polyalanine tract of the *9A receptor that was not present in *6A.

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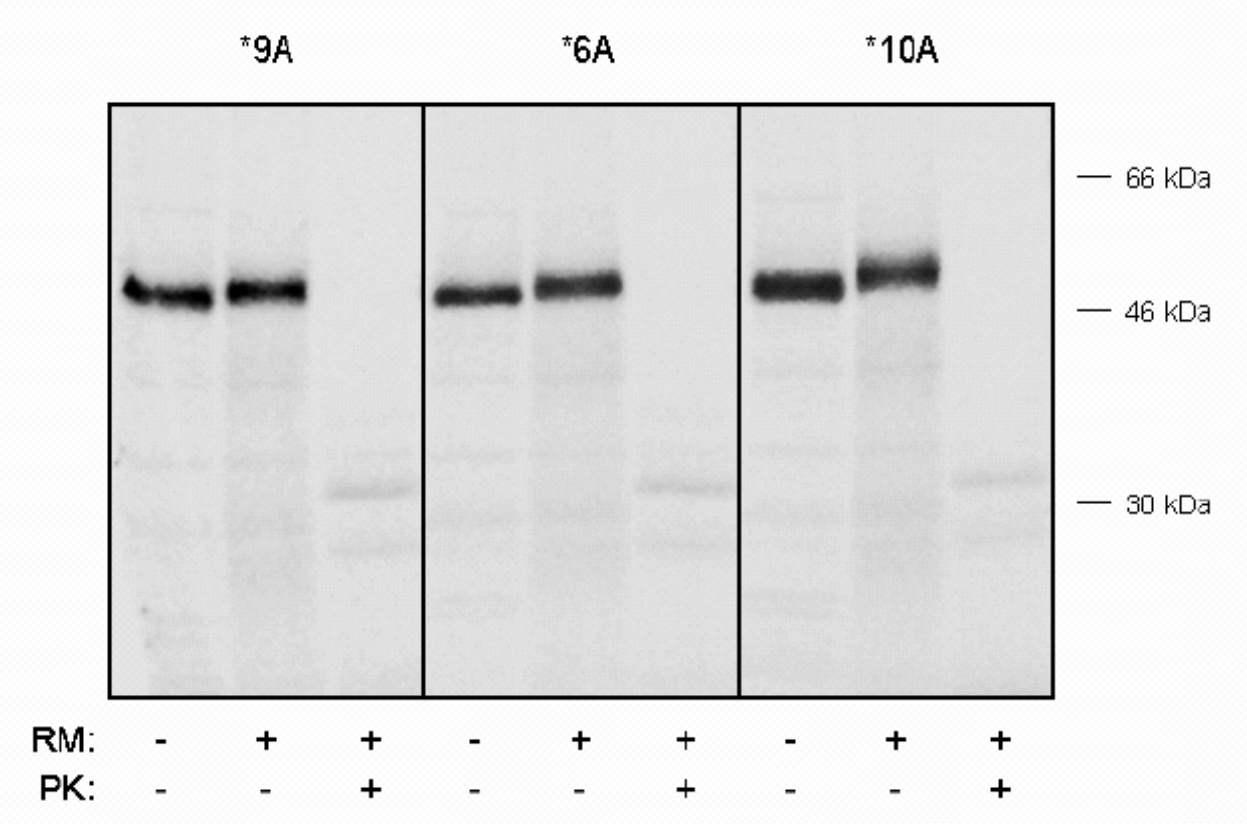


**Figure 1.2 TGFBR1*6A does not affect membrane insertion or protease sensitivity *in-vitro***

TGFBR1*9A, TGFBR1*6A, and TGFBR1*10 (an alternate form) were translated *in vitro* in the presence (+RM) or absence (-RM) of dog pancreas rough microsomes. The minor band shift seen in the +RM samples indicate glycosylation at a unique Asnyy-X-X site in the luminal side (extracellular domain) of the protein. Glycosylation was identical in all 3 samples. Proteinase K (PK) was then added to the system. PK cleaves the protein on the cytoplasmic side of the membrane. The presence of the 30 kD band in each sample indicates that protease-sensitive sites in the cytoplasmic domain remain intact in each receptor form.

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Genotyping studies of several types of cancers indicate that *TGFBR1**6A is somatically acquired in head and neck (HN) and colorectal cancers (Figure 1.3). In a study of 226 HN cancers, 24 tumors were *9A/*6A. Four out of these 24 patients had *9A/*9A in their germline indicating that 16.7% of these HN tumors acquired 6A. Likewise, 30 out of 157 colorectal tumors were *9A/*6A, and 13% of these tumors had acquired *6A. Furthermore, out of 44 liver metastases derived from colorectal cancer 50% of the metastatic tumors (22/44) contained the *9A/*6A genotype. Germline DNA was available for 15 of those 22 *9A/*6A liver metastases, and genotyping showed that 13 (87%) were homozygous for *9A in the germline. Out of those 13 patients who had the *9A/*9A in their germline, only 2 of them had acquired *6A at the site of the primary tumor indicating that the high *6A frequency seen in liver metastases from colorectal cancer is due to somatic acquisition either at the primary site of the tumor or, more commonly, in the processes of metastasis. Conversely, breast tumors did not show any somatic acquisition of *6A [Pasche et al., 2005].

*TGFBR1*6A* is associated with cancer risk. The fact that *TGFBR1*6A* carriers have a 15% increased risk in developing breast cancer demonstrates that this gene may play a significant role in breast cancer development. Although we have strong epidemiological evidence of an association of this gene with cancer risk, we do not yet know the mechanism of action that this mutation plays in the development of breast cancer. We do know that TGFBR1*6A is acquired at high frequency in liver metastasis derived from colon cancer.

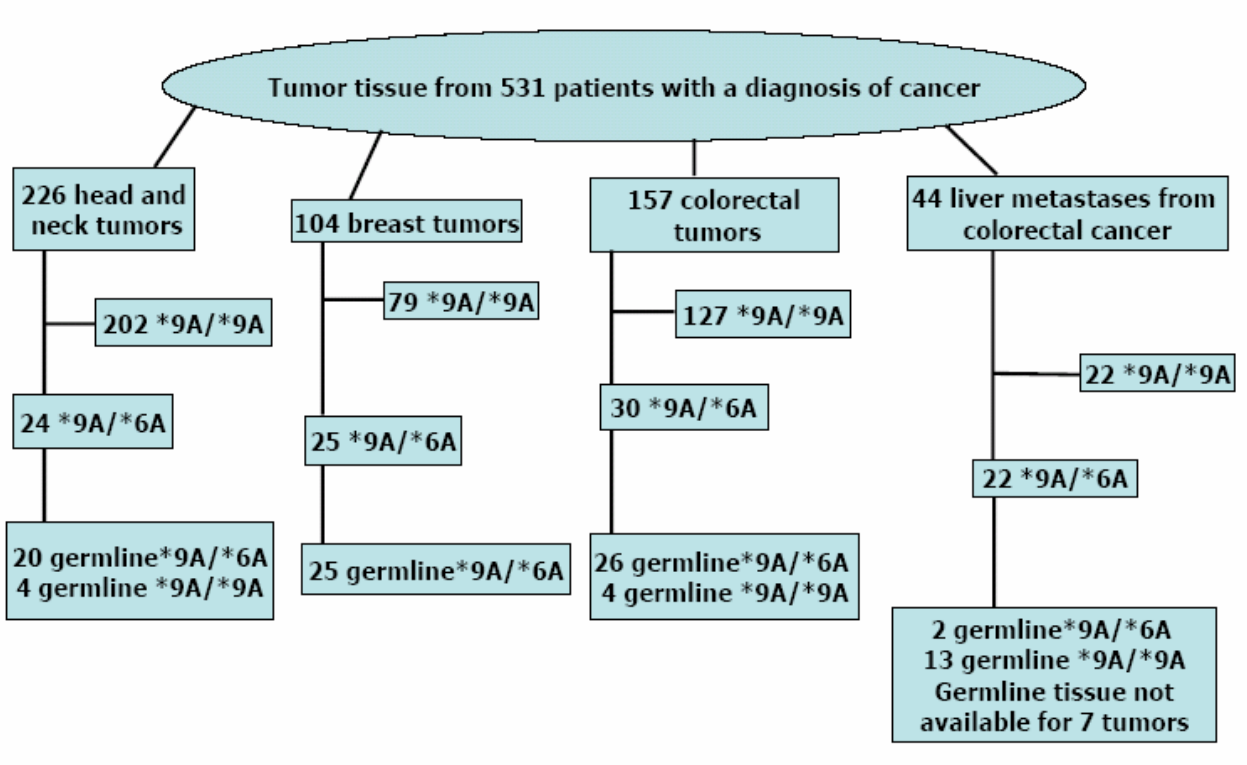


**Figure 1.3 *TGFBR1**6A is somatically acquired in colorectal tumors**
*TGFBR1**6A is somatically acquired in 16.7% of head and neck tumors, 13% of colorectal tumors, and 50% of liver metastasis derived from colorectal cancer. 13 out of 44 (29.5%) of liver metastases had acquired *6A either at the site of the primary tumor or during the process of metastases. There was no evidence of *6A somatic acquisition in breast tumors.
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My thesis project looks at the role that two different TGF-β variations (*TGFBR1**6A and TGFBR1 haploinsufficiency) play in the development of breast cancer. The first part of my project determines the effects of TGFBR1*6A on growth, migration, and invasion of MCF-7 breast cancer cells. I find that not only does TGFBR1*6A switch TGF-β-mediated growth inhibition signals into growth stimulatory signals in MCF-7 cells, but I also discover that TGFBR1*6A enhances migration and invasion of MCF-7 cells, independent of TGF-β signaling. Through a gene array, we learned that RhoA activation is higher in *6A expressing cells, and this is the mechanism most likely causing the increased migratory activity of the cells. This is the first evidence that TGFBR1*6A has a TGF-β-independent phenotype, and we also show evidence for the actions being signal sequence mediated.

To study the role of TGFBR1-mediated TGF-β signaling in breast cancer, we crossed our FVB *Tgfbr1*$^{+/-}$ mice to the *MMTV-c-Neu* proto-oncogene mouse model. We find a significant decrease in tumor latency in *Neu* mice harboring *Tgfbr1*$^{+/-}$, and we also see evidence that *Tgfbr1*$^{+/-}$ may enhance lung metastasis in the *Neu* background. This is the first evidence that *Tgfbr1* haploinsufficiency may enhance breast cancer tumor formation in mice.

# CHAPTER 2

## *TGFBR1**6A enhances the migration and invasion of MCF-7 breast cancer cells through RHOA activation

### INTRODUCTION

TGF-β plays dual roles during cancer development and progression. TGF-β acts as a tumor suppressor by inhibiting the growth of most cell types including epithelial, endothelial, hematopoietic, and neuronal cells. However, once tumors form, most cells become resistant to TGF-β growth inhibition and TGF-β becomes pro-oncogenic [Elliott and Blobe, 2005].

As a tumor suppressor, TGF-β inhibits cell growth predominantly by signaling via the SMAD pathway. Secreted TGF-β is normally kept inactive by a latency-associated peptide (LAP) and latent TGF-β binding protein (LTBP) [McMahon et al., 1996;Bottinger et al., 1996;Miyazono et al., 1988]. Thrombospondin-1 (THBS1) and ITGAV/ITGB6 ($\alpha_v\beta_6$ integrin) bind LTBP thereby activating TGF-β [Crawford et al., 1998;Munger et al., 1999]. Upon TGF-β binding to TGFBR2, TGFBR1 is recruited to the complex and becomes phosphorylated, thus making TGFBR1 active. Active TGFBR1 induces phosphorylation of SMAD2/3, thereby allowing SMAD2/3 to bind to SMAD4. Although SMAD4 is not required for translocation into the nucleus, it is required for the SMAD complex to act as a transcription factor [Liu et al., 1997].

TGF-β signaling is both enhanced by, and runs in parallel to, the MAPK signaling. TGF-β induces migration by activating AKT and ERK1/2 [Dumont et al., 2003;Ao et al., 2006]. The role of SMADs in migration is controversial. There is evidence that SMAD4 is required for

TGF-β-induced migration in human immortalized keratinocytes (HaCaT) and in pancreatic tumor cells (Colo-357) [Levy and Hill, 2005]. The fact that the metastatic ability of RAS-transformed MCF10At1k cells signaling through TGFBR1 requires the SMAD2/3 binding domain [Tian et al., 2004] is in agreement with these findings. Other groups have suggested that TGF-β-induced migration requires TGFBR1, but is independent of SMADs [Dumont et al., 2003] . In a SCID mouse model, invasion and metastasis of MDA-MB-231 cells is mediated by MEK-ERK signaling, which results in the activation of MMP-9, a SMAD4-independent event [Safina et al., 2006]. ERK, JNK, and RhoA regulate TGF-β induced migration in MCF-7 cells as well as in the SMAD4-deficient breast cancer cell line, MDA-MB-468. TGF-β induced activation of ERK has also been shown to be independent of TGFBR1 [Imamichi et al., 2005].

A common variant of *TGFBR1*, *TGFBR1**6A, is a low penetrance, tumor susceptibility allele that is found in 14.2% of the general population. Carriers of this mutation have a 15% increased risk of cancer. Breast cancer risk appears to be increased by 12% for heterozygotes and 40% for homozygotes [most recent, unpublished data]. *TGFBR1**6A results from a 9 bp in-frame deletion, which truncates 3 alanines from a 9 alanine tract located within TGFBR1 signal sequence. The wild type allele is referred to as *TGFBR1**9A or *9A. Previous studies have shown that transient and stable transfection of mink lung epithelial cells with *6A results in a small but significant decrease in TGF-β-induced growth inhibition [Chen et al., 1999;Pasche et al., 1999]. We have also shown that the TGFBR1*6A and TGFBR1 share the same signal sequence cleavage site and that TGFBR1*6A mature receptor is identical to and processed similarly to its wild-type counterpart [Pasche et al., 2005]. This suggests that TGFBR1*6A biological effects are mediated by its signal sequence, not its mature receptor. This chapter will look at the effects of *TGFBR1**6A in MCF-7 breast cancer cells. The effect of *TGFBR1**6A on

TGF-β-mediated cell growth, migration, and invasion are assessed, as well as the potential for *TGFBR1**6A to act as an oncogene.

## RESULTS

### TGFBR1*6A expression results in TGF-β mediated growth stimulation in MCF-7 breast cancer cells

MCF-7 cells were stably transfected with pIRES vector, pIRES-*TGFBR1**6A-HA-FLAG or pIRES-*TGFBR1*-HA-FLAG. Clones were chosen based on similar transgene expression levels and designated as: *9A-low and *6A-low, *6A-int (intermediate expressing clone), and *9A-high. We did not obtain any *6A clones that expressed equal levels of protein as the *9A-high, so we chose the highest *6A expressing clone we obtained (figure 2.1). MCF-7 cells have two wild-type copies of TGFBR1, so transfecting in *6A essentially makes them heterozygous for the allele.

TGF-β-mediated growth inhibition was assessed by thymidine ($^3$H) incorporation after 18 hr of TGF-β treatment. Vector control and *9A transfected MCF-7 cells were on average 28.5% growth inhibited after TGF-β treatment (Figure 2.2). A striking phenotype was seen after TGF-β treatment in the *6A expressing cells. Not only did TGF-β decrease the growth inhibitory signals in these cells, but it reversed the growth inhibition into 26.3% growth stimulation.

To determine whether the TGF-β mediated growth stimulation by *6A depends on receptor activation, a kinase deficient TGFBR1*6A construct was created and transfected into MCF-7 cells. The lysine residue at position 232 was mutated to an arginine (K232R), which results in knocking out the kinase domain thereby blocking TGFBR1-mediated signaling (Figure 2.1). Blocking receptor signaling also resulted in an 30.3% growth stimulation after TGF-β treatment (Figure 2.2) indicating that the growth stimulatory effects of *6A are independent of receptor signaling and result from the activity of the signal sequence.

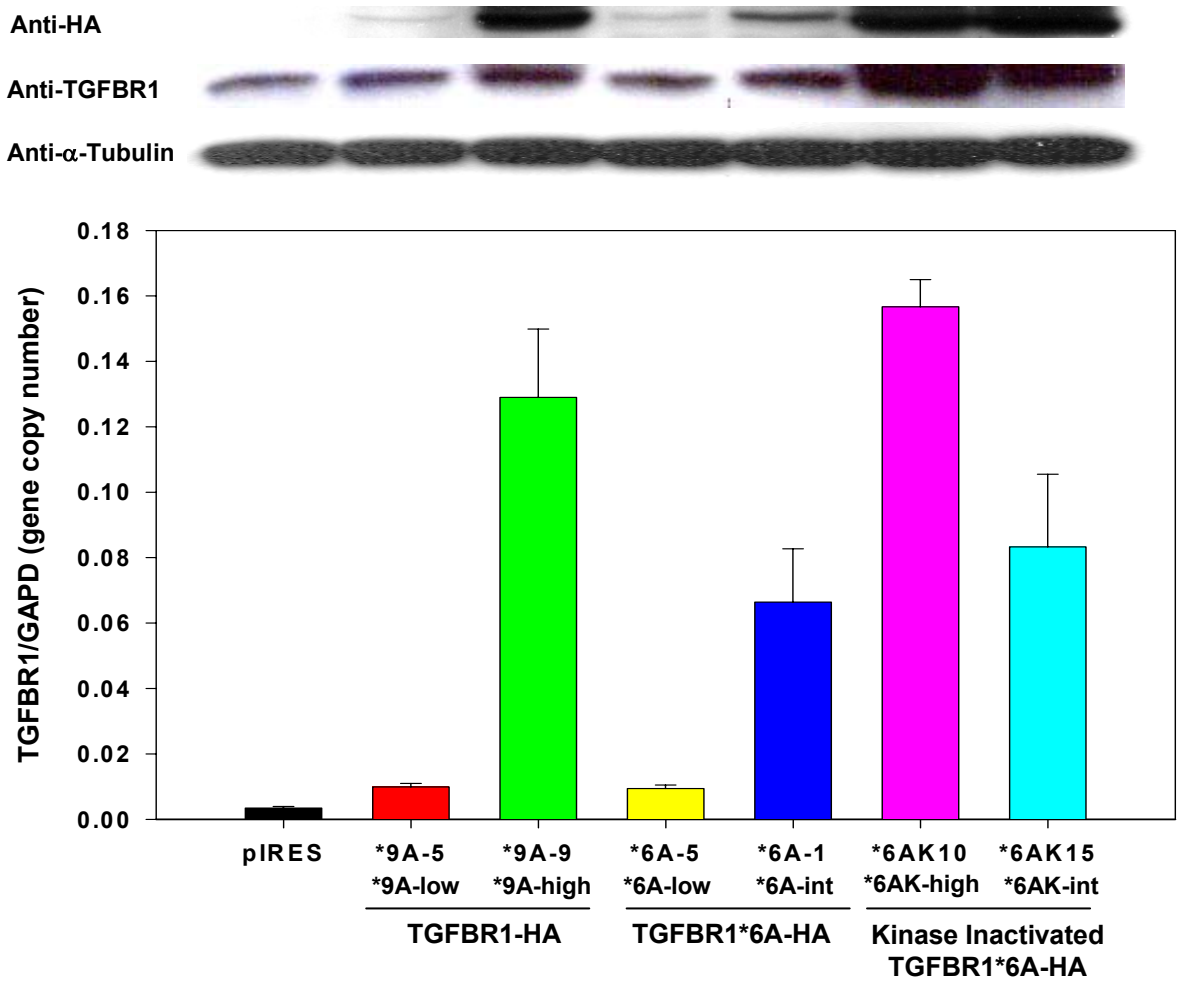


**Fig 2.1 TGFBR1 expression levels of stably transfected MCF-7 clones**

A. Lysates were collected from MCF-7 clones stably transfected with TGFBR1*9A-HA-FLAG, TGFBR1*6A-HA-FLAG, or a kinase inactivated TGFBR1*6A construct (*6AK) and separated using SDS-PAGE. Membranes were probed with anti-HA to detect transgene expression, anti-TGFBR1, and anti-α-tubulin as a loading control. MCF-7 cells are homozygous for *9A, so *6A expression would result in a heterozygous genotype.

B. Receptor expression was also assessed at the mRNA level by real-time PCR. All samples were normalized to GAPD and the results are represented as ratio of TGFBR1 mRNA level over GAPD. Error bars represent 95% confidence intervals.

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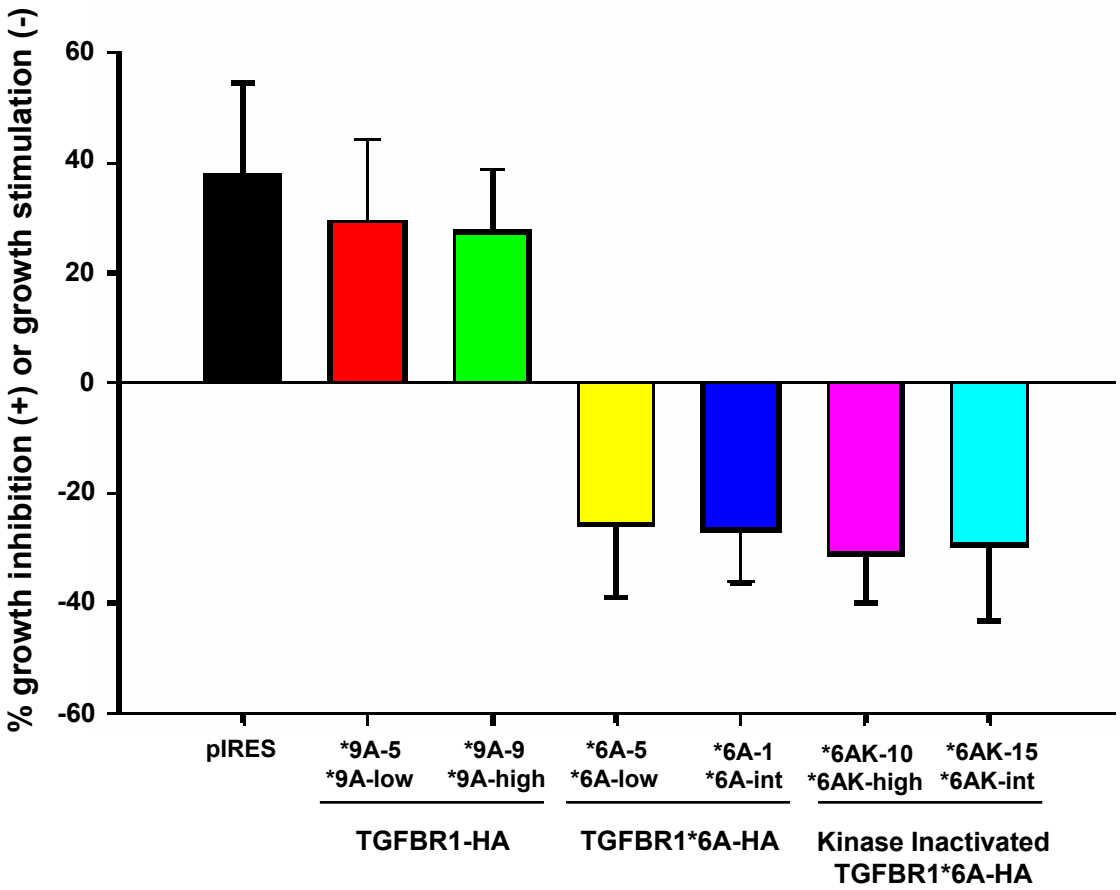


**Fig 2.2 TGFBR1*6A is a dominant allele, and *6A expression switches TGF-β mediated growth inhibition to growth stimulation in MCF-7 cells**

TGF-β mediated growth inhibition was assessed by $^{3}H$ incorporation. The average growth inhibition for the vector controls and the *9A clones was 28.5%. *6A cells were 26.3% growth stimulated after TGF-β treatment. The kinase deficient clones were also 30.3% growth inhibited, indicating that growth stimulation is a result of the *6A signal sequence, as the kinase deficient receptor would result in no TGF-β signaling capability. Error bars represent the SD.

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**TGFBR1*6A is not an oncogene**

Because the epidemiological data linking TGFBR1*6A to cancer risk is so strong, and because TGFBR1*6A expression confers a growth advantage in MCF-7 breast cancer cells, we hypothesized that *TGFBR1**6A may act as an oncogene [Pasche et al., 2005]. To test this hypothesis, I stably transfected NIH-3T3 cells with *6A, *9A, or the empty vector (Figure 2.3.A). Transfected cells were plated and foci formation was assessed after 21 days (Figure 2.3.B). Although the *6A-high expressing cells appears to have some darker staining patterns, this is due to the slightly higher concentration of cells in those areas, however there was no detectable foci. Neither the *9A or *6A expressing plates exhibited foci indicating that *6A alone cannot act as an oncogene.

HRAS is a bone fide oncogene, which transforms NIH-3T3 cells via the PI3K pathway and leads to uncontrollable cell proliferation. A constitutively active mutant HRAS known as HRAS-$V^{12}$ maintains the gene in its activated GTP state [Li et al., 2004]. The high expressing *9A clone cooperates with HRAS-$V^{12}$ to increase transformation (Figure 2.3.C). This is not surprising given the fact that TGF-β was first identified as a fibroblast transforming growth factor [De Larco and Todaro, 1978]. In contrast, *6A has no effect on HRAS-$V^{12}$ induced transformation, thus indicating that *6A's known decrease in TGF-β-mediated signaling results in decreased oncogenesis when compared with its wild-type counterpart (Figure 2.3.C).

In the colony formation assay in soft agar, neither *9A nor *6A alone was able to transform NIH-3T3 cells. *9A and *6A were also unable to synergize with HRAS-$V^{12}$ to induce more colony growth than just the HRAS-$V^{12}$ alone (data not shown).

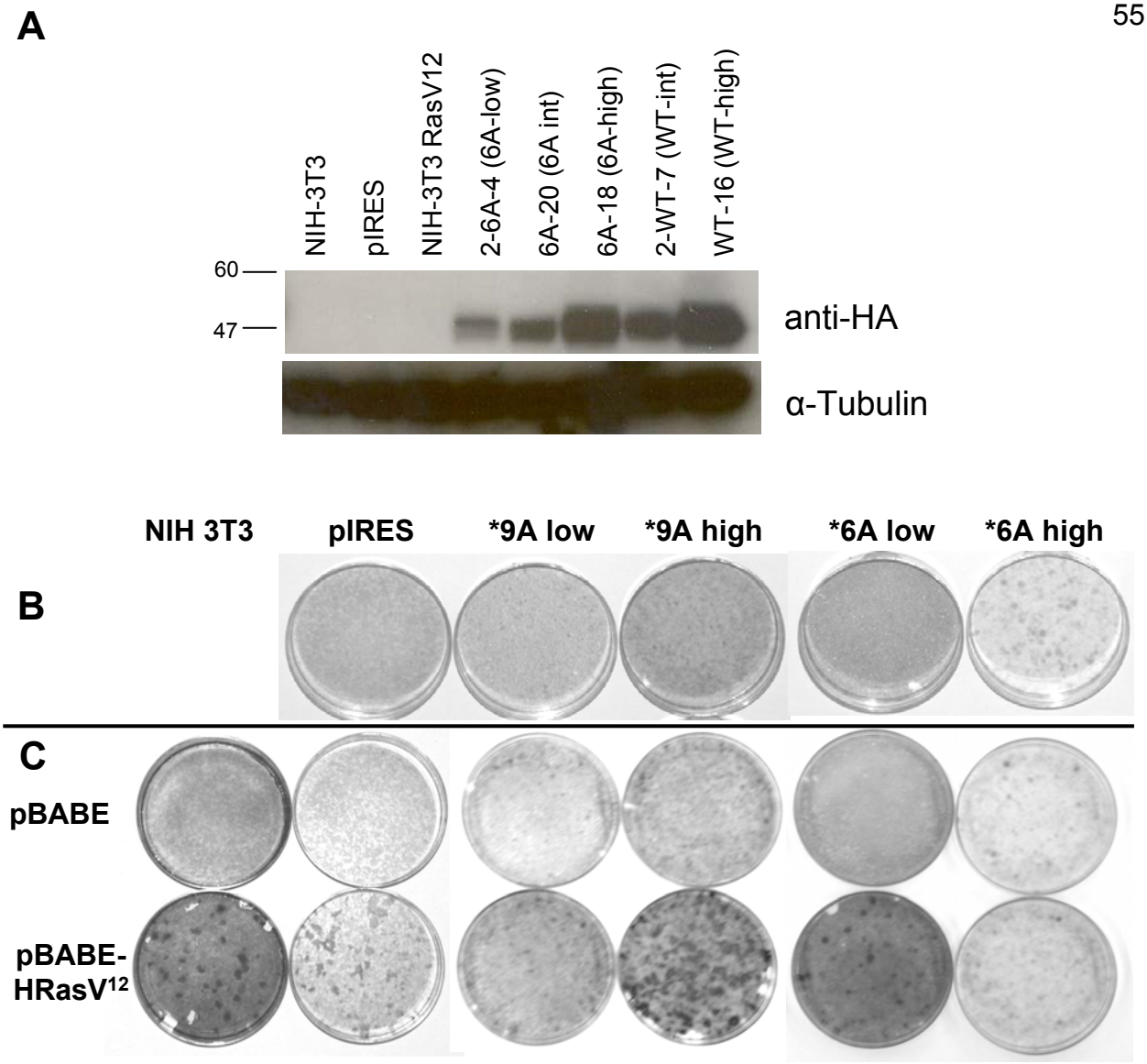


**Figure 2.3 TGFBR1*6A does not induce foci formation either alone or with HRasV$^{12}$**

A. NIH-3T3 cells were stably transfected with pIRES, pIRES-TGFBR1-HA-FLAG, TGFBR1*6A-HA-FLAG, or pBABE-HRasV$^{12}$. Western showing transgene expression (anti-HA) and loading control (α-tubulin).

B. After 21 days of culture, there was no evidence of foci formation in any of the cell lines generated.

C. NIH-3T3 cells stably transfected with pIRES-*6A or *9A were transiently transfected with pBABE or pBABE-HRasV12 to check for foci formation. The high expressing *9A clone enhanced HRasV12 transformation. Few to no foci appeared in either *6A expressing plate.

There was no evidence of a difference between TGFBR1*6A-transfected and TGFBR1*9A-transfected NIH-3T3 cells with respect to TGF-β mediated growth inhibition (Figure 2.4). Various *6A clones are growth inhibited upon TGF-β treatment. Hence, transfection of NIH-3T3 cells with the *6A allele does not significantly alter TGF-β mediated growth inhibition.

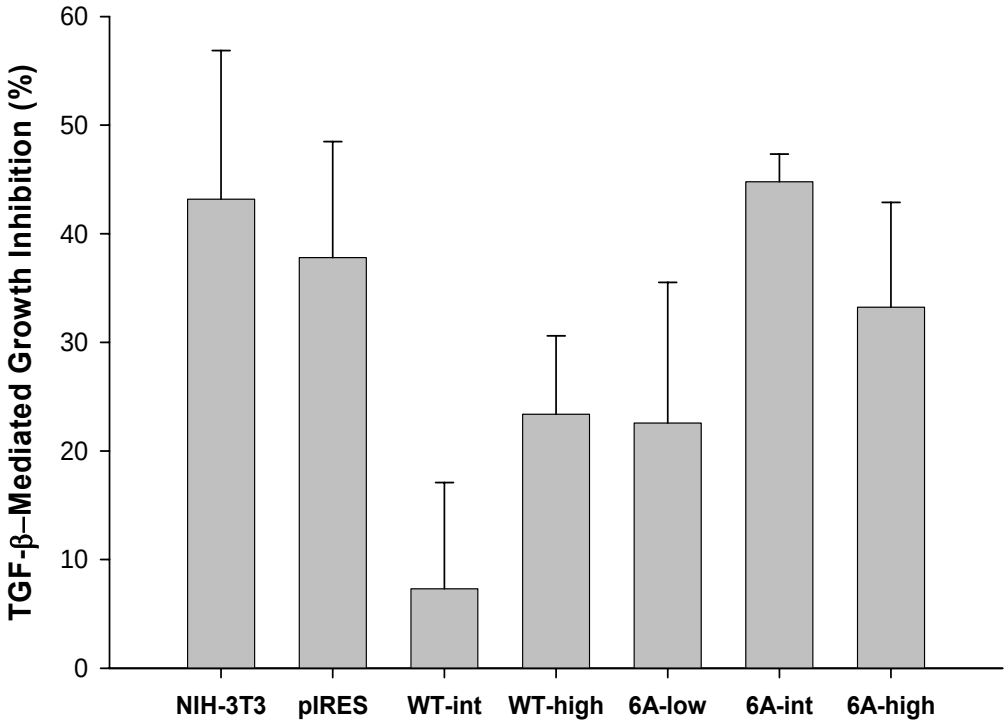


**Figure 2.4 *TGFBR1*6A does not confer growth stimulation in NIH-3T3 fibroblasts**
Growth inhibition response to TGF-β was assessed by H3-thymidine incorporation after 18 hr of TGF-β treatment. Results are the average of 3 trials and error bars are standard deviation of 3 trials. Expression of *6A does not decrease growth inhibition compared to the vector transfected cells.

In addition to NIH-3T3 cells, we tested the ability for TGFBR1*6A to transform the normal breast epithelial cell line, MCF-10A, as judged by the ability to confer growth stimulation after TGF-β treatment. MCF-10A cells were transfected with pBABE-TGFBR1*9A or pBABE-TGFBR1*6A and stable pools were collected (Figure 2.5.A). The growth inhibitions of MCF-10A, vector controls, *9A, and *6A cells were 69%, 71%, 75%, and 77%, respectively, indicating that *6A did not confer a growth advantage to normal breast epithelial cells (Figure 2.5.B).

**Canonical TGF-β signaling is identical in cells expressing *9A and *6A**

To assess TGF-β signaling, MCF-7 *9A-low and *6A-low cells were transiently transfected with the luciferase reporter constructs SBE4-lux or 3TP-lux. The SBE4 construct measures the ability for SMAD2 and SMAD3 to induce transcription of the reporter construct [Zawel et al., 1998]. The 3TP-Lux expresses luciferase under the control of three TPA response elements and a segment of the plasminogen inhibitor promoter [Carcamo et al., 1995]. After TGF-β treatment, the amount of induction in the SBE4-lux reporter is the same in pIRES cells and *9A cells: 1.3 and 1.2 fold induction, respectively. SMAD signaling in *6A cells is decreased after TGF-β treatment, however, the difference is not statistically significant ($p<0.067$) (Figure 2.6.A).

TGF-β signaling downstream of SMADs was assessed by using the 3TP-lux reporter assay [Carcamo et al., 1995]. TGF-β treatment resulted in 1.7, 1.3 and 1.1 fold induction of reporter gene expression for pIRES, *9A and *6A cells, respectively. The differences between the different cell lines are again not statistically significant ($p<0.175$) (Figure 2.6.B). Thus, transfection of MCF-7 cells with *6A results in a minor but not statistically significant decrease in TGF-β signaling when compared with *9A.

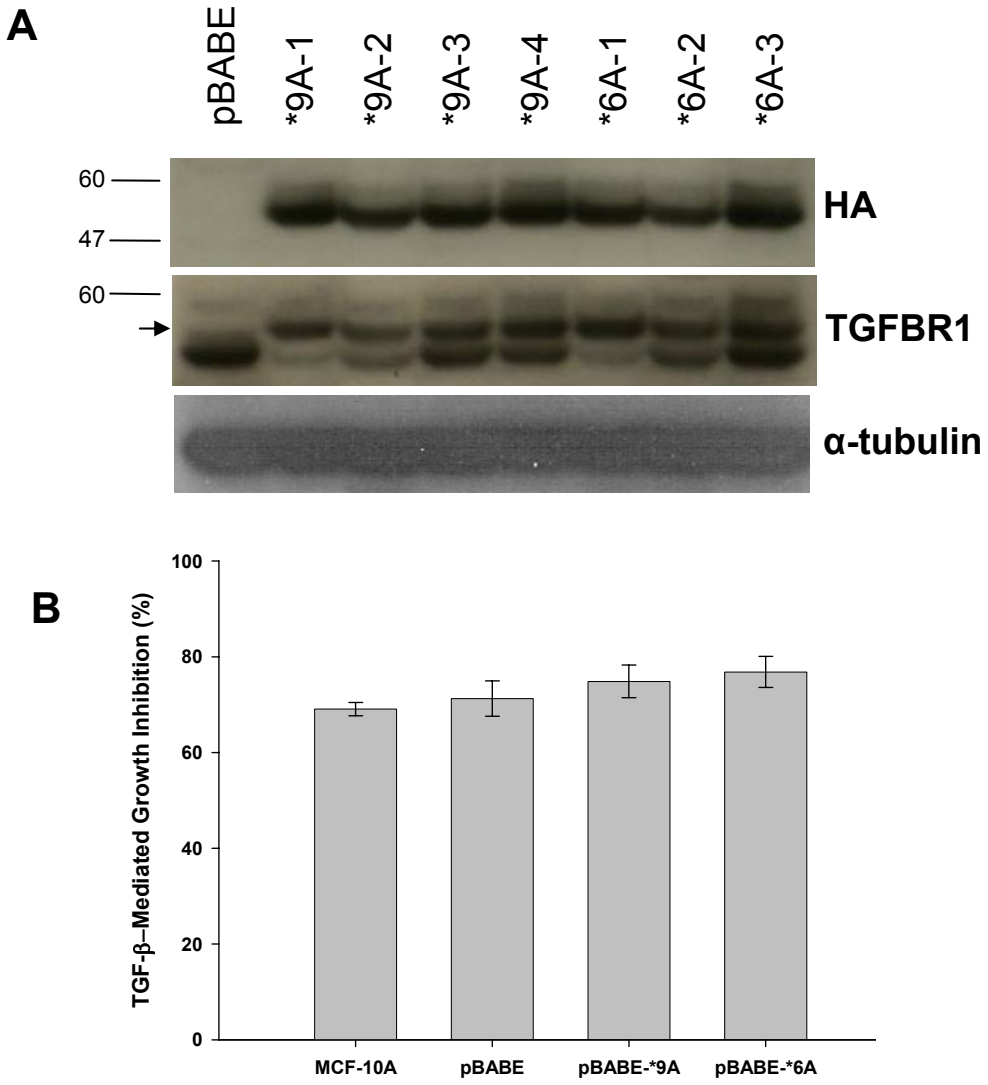


**Figure 2.5 *TGFBR1**6A does not confer growth stimulation in normal breast epithelial cells**

A. MCF-10A normal breast epithelial cells were transfected with pBABE, pBABE-*TGFBR1*-HA-FLAG or pBABE-*TGFBR1**6A-HA-FLAG. Western blot showing transfection efficiency (HA-tagged), TGFBR1 expression levels, and α-tubulin, loading control. Each lane represents a different mixed population of transfected cells. WT-1 and 6A-1 were used for experiments.

B. MCF-10A Growth inhibition was assessed by H3-thymidine incorporation after 18 hr of TGF-β treatment. Results are the average of 3 trials and error bars are standard deviation of 3 trials. Expression of *6A does not affect growth inhibition compared to the vector transfected cells.

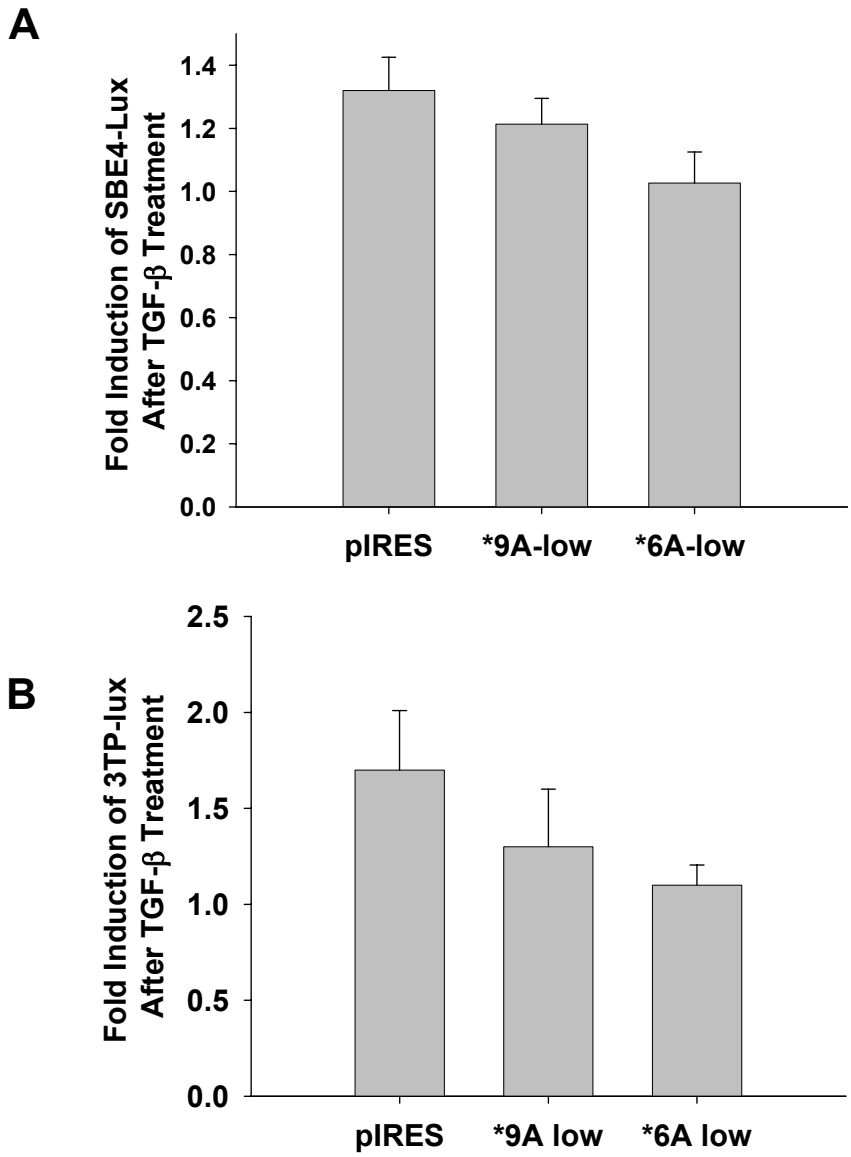


**Figure 2.6 There is no difference in TGF-β signaling between *9A and *6A**

Transcriptional regulation was assessed using luciferase assays with the SBE4-luciferase (A) and 3TP-lux (B) reporter systems. SBE-4 activity represents the ability for Smad4 to activate transcription, and 3TP-lux activity represents overall downstream TGF-β signaling. In both cases, there was no difference between *9A and *6A expressing cells. Error bars indicate the standard deviation for 4 experiments. p-value <0.067 for SBE4, and p<0.175 for 3TP-lux.

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Lastly, we assessed SMAD-mediated TGF-β signaling by measuring the levels of phosphorylated SMAD2 (pSMAD2) and SMAD3 (pSMAD3). Exposure to TGF-β leads to similar levels of pSMAD2 for MCF-7*6A cells and MCF-7*9A cells (both for low and high expressers) (Figure 2.7). MCF-7 cells secrete endogenous TGF-β even after serum starvation [Fenig et al., 2001;Arnold et al., 1999]. Other groups have shown that pSMAD2 is present in non-stimulated MCF-7 cells as well [Buck et al., 2004;Fanayan et al., 2002]. This autocrine secretion may be the cause of baseline levels of pSMAD2 after serum deprivation [Fanayan et al., 2002]. Upregulation of pSMAD2 after TGF-β treatment is minimal and consistent with the minimal increase we saw in the SMAD luciferase assays. There is no difference in the amount of SMAD3 phosphorylation between *6A and *9A cells. Likewise, *6A and *9A cells have the same degree of upregulation of pSMAD3 after TGF-β treatment. These experiments demonstrate that the effect of *6A is not mediated by differences in SMAD signaling, which was anticipated from our earlier evidence showing kinase deficient *6A cells behave the same as *6A cells in regards to TGF-β-mediated cell growth (see Figure 2.2) [Pasche et al., 2005].

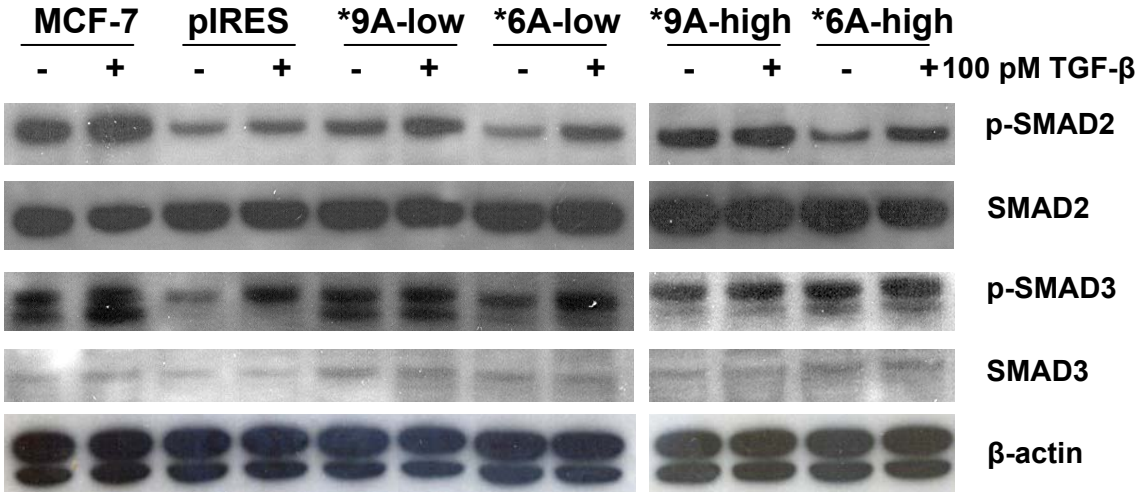


**Figure 2.7 TGFBR1*6A does not alter the TGF-β canonical SMAD signaling pathway**
Cells were serum starved overnight before addition of 100 pM TGF-β for 18 hr. Western blotting for pSmad2 after TGF-β treatment shows that upregulation of pSMAD2 is the same for both the *9A and *6A cells. Minimal pSMAD3 upregulation is seen in each cell line.

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**TGFBR1*6A enhances migration and invasion of MCF-7 cells independently of TGF-β**

To comprehensively explore possible roles for *6A in tumor development and progression, we investigated its ability to modify cell migration and invasion of MCF-7 cells. Transwell chambers were used to assess the migratory potential of MCF-7 cells. The upper chambers contained the cells seeded in starvation media, and the bottom wells held media containing complete media with 5 ng/ml TGF-β. In the presence of TGF-β we observed migration of an average of 20 MCF-7 parental cells and pIRES vector control cells (Figure 2.8.A). The *9A and *6A low cells migrated 1.3 times and 1.9 times more than the parental cells, respectively, with the difference between the *9A and *6A cell number being highly significant ($p<0.005$). Higher TGFBR1 expression levels resulted in an overall increase in migration in the presence of TGF-β, with a ratio of 1.8 and 2.2 migrating cells for *9A-high and *6A-int cells, respectively, compared to the parental MCF-7 cells. This 1.2 fold difference in the amount of migrated *6A-int cells compared to the *9A-high cells was also significant ($p<0.05$). These results demonstrate that *6A enhances the migration of MCF-7 cells.

To further characterize the differences between *6A and *9A cells, we selected the low expressing *6A and *9A clones because they are more likely to reflect *in vivo* conditions. First, we assessed migration in the absence of exogenously added TGF-β. Parental MCF-7 and pIRES control cells had an average of 37 and 33 migrating cells, respectively. While the migration of *9A cells was essentially identical to that of the control cells, the number of migrated *6A cells was 3.2 fold higher than the *9A cells ($p<0.005$) (Figure 2.8.B).

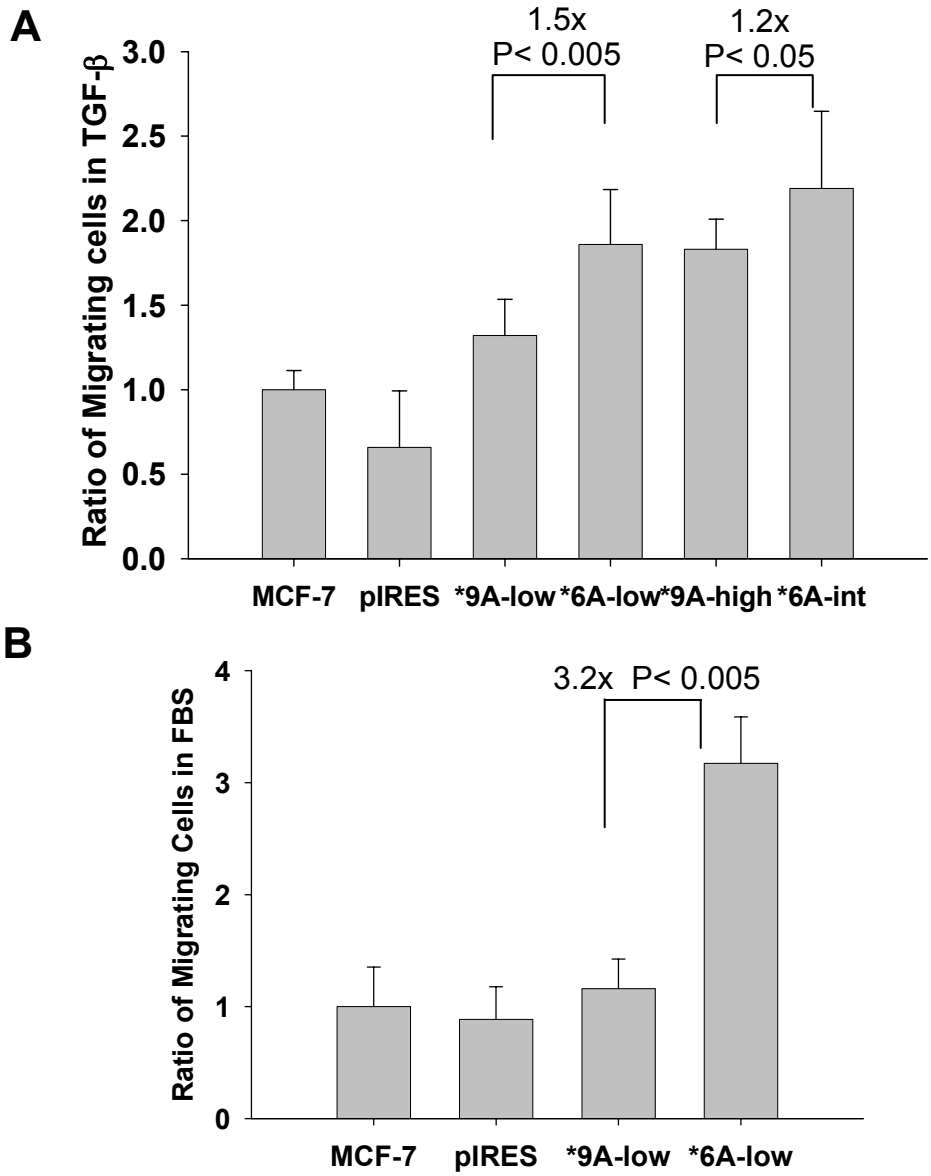


**Figure 2.8 TGFBR1*6A enhances the migration of MCF-7 cells**

A and B: MCF-7 cells stably transfected with the empty vector (pIRES), *9A or *6A were first plated in transwell chambers in starvation media overnight. Then either complete media containing 5ng/ml TGF-β (A) or complete media (B) was added to the bottom wells. After 24 hrs cells were scraped off the top of the insert and the cells on the bottom of the insert were counted. The experiment was performed 5 times in TGF-β and 7 times in FBS and error bars represent the standard error. Migration of *6A clones was significantly higher than that of *9A clones (A). In the absence of exogenously added TGF-β the difference was greater (B).

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Migration in MCF-7 cells was further assessed with two clones each from cells transfected with *TGFBR1**6A or *TGFBR1**9A inserted into the pBABE vector. Compared to vector only controls, low expressing *9A clones migrated 2.6 fold more in the presence of FBS ($p<0.001$) (Figure 2.9). Low expressing *6A cells on the other hand migrated 3.5 fold more than pBABE cells in FBS ($p<0.001$). This 1.3 fold difference between the *9A-low and *6A-low cells is also highly significant ($p<0.001$). TGF-β does not increase the amount of migration in pBABE, *9A-low, or *6A-low cells ($p<0.1$ for each cell line). Although the high expressing *9A clones migrated 0.4 fold less than vector cells, the high expressing *6A cells migrated 1.8 times more than the vector cells in FBS ($p<0.001$). Again, addition of TGF-β did not alter the amount of migrating cells in the *6A-high cells ($p<0.1$) demonstrating that *6A expression results in an increase in migration, that is not enhanced by TGF-β.

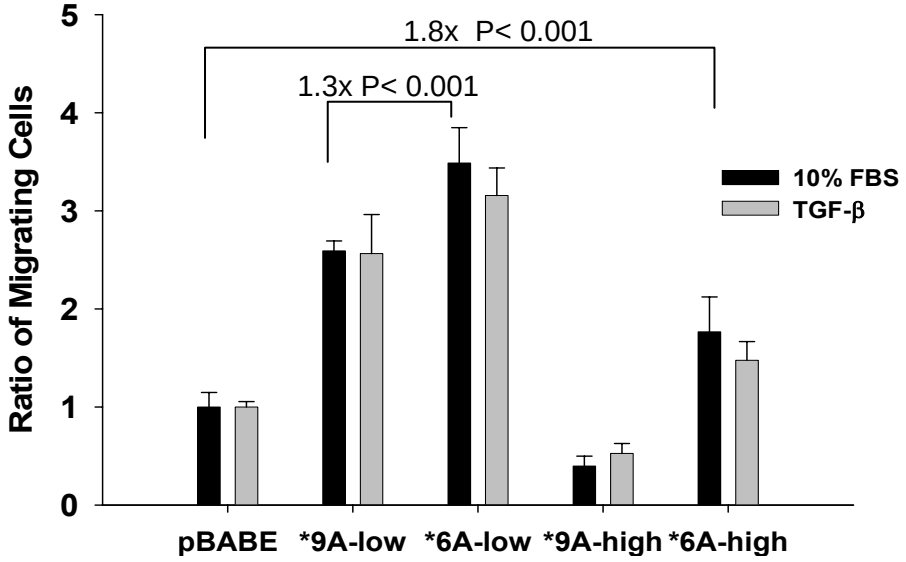


**Figure 2.9 TGFBR1*6A enhances the migration of MCF-7 cells**
MCF-7 cells stably transfected with the empty vector (pBABE), *9A or *6A were first plated in transwell chambers in starvation media overnight. Then either complete media alone or complete media containing 5ng/ml TGF-β was added to the bottom wells. After 24 hrs cells were scraped off the top of the insert and the cells on the bottom of the insert were counted. The experiment was performed 3 times and error bars represent the standard error. Migration of *6A clones was significantly higher than that of *9A clones.

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Migration of MCF-7 cells was also determined by performing a scratch wound assay (Figure 2.10) At 24 hr in the presence of 10% FBS, *6A-low cells closed the entire wound, while *9A-low cells only closed 66%. The *6A-int cells closed 85% while the *9A-high cells closed 60% after 24 hr. These results mirror the results from the transwell assays. These data indicate that *6A enhances migration in MCF-7 cells, both in the presence and in the absence of exogenously added TGF-β.

To examine the possible role played by traces of TGF-β in FBS, the differential effect of *6A and *9A on cell migration was further characterized by the addition of a pan-TGF-β neutralizing antibody to the media. As seen in figure 2.10, TGF-β blockade resulted in slightly less wound closure when compared to closure in FBS alone. The percent closure for *9A-low, *6A-low, *9A-high, and *6A-int was 47%, 89%, 80%, and 93%, respectively, after TGF-β neutralization. This further demonstrates that the increase in migration that *6A cells have over *9A cells is independent of TGF-β signaling. The high percent of wound closure seen after blocking TGF-β also shows that TGF-β is not required for migration or wound closure, and although migration is higher in complete media (with possible traces of TGF-β present in serum), TGF-β is not the driving force for cell migration.

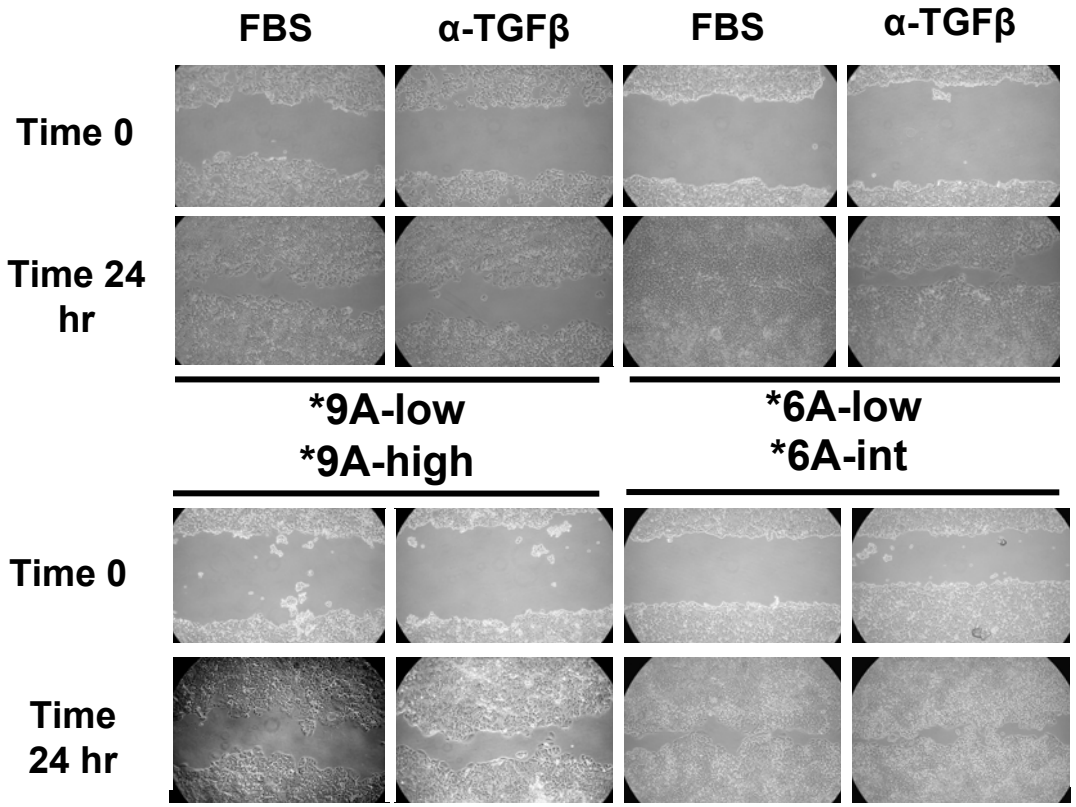


**Figure 2.10 TGFBR1*6A enhances the migration of MCF-7 cells independently of TGF-β**

A confluent monolayer of MCF-7-*9A and MCF-7-*6A cells (in the pIRES vector) was scratched with a pipet tip and the gap was measured after 24 hr. *6A-low cells closed the wound completely in FBS while *9A-low cells closed the wound 66%. Addition of a TGF-β neutralizing antibody resulted in 89% and 47% wound closure in *6A-low and *9A-low cells, respectively. *6A-int cells closed the wound 85% and 93% in FBS and TGF-β, respectively, while *9A-high cells closed the wound 60% and 80% in FBS and TGF-β, respectively. The pictures are representative of three assays.

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Scratch assays were also done in MCF-10A normal breast epithelial cells transfected with either pBABE-TGFBR1*6A or pBABE-TGFBR1*9A (Figure 2.11). TGF-β1 exerts the same effects in MCF-10A *9A and *6A cells. After 8 hr of TGF-β treatment, MCF-10A*9A cells close 25% of the wound while MCF-10A*6A cells close 18%. Cells in normal media (5% serum) or treated with anti-TGFβ only close at a maximum of 4% at 8 hr. These results indicate that TGF-β1 is required for cells to initiate wound closure.

By 15 hr, vector control cells treated with TGF-β1 or anti-TGFβ close about 80% while the cells in complete media close about 60% (Figure 2.11). *9A cells close 100% in complete media, 90% in TGF-β1, and 80% after anti-TGFβ treatment. *6A cells close 70% in complete media, 100% in TGF-β1, and 85% closure with anti-TGFβ. This indicates that although TGF-β1 is required to induce wound closure at the earlier stages (8 hr), TGF-β1 alone is not enough to finish the closure, and other growth factors are involved in closing the gap. These data indicate that *6A does not modify migration in non-cancerous breast cells such as the MCF-10A cells.

Having demonstrated that *6A enhances the migration of the MCF-7 breast cancer cell line, we sought to assess its impact on invasion. Cell invasion of MCF-7 cells was determined by the cells' ability to invade through a matrigel barrier. Cells were plated in starvation media on top of the matrigel coated inserts, while the bottom wells contained either complete media with 10% FBS or complete media with 10% FBS and 5 ng/ml TGF-β. As with cell migration, we observed that *6A enhances cell invasion independently of TGF-β. In the presence of FBS alone, MCF-7 parental cells, pIRES control cells, and *9A cells have the same number of invading cells (Figure 2.12.A). However, *6A cells demonstrate a 1.8 fold greater amount of invasion than the *9A cells ($p < 0.005$).

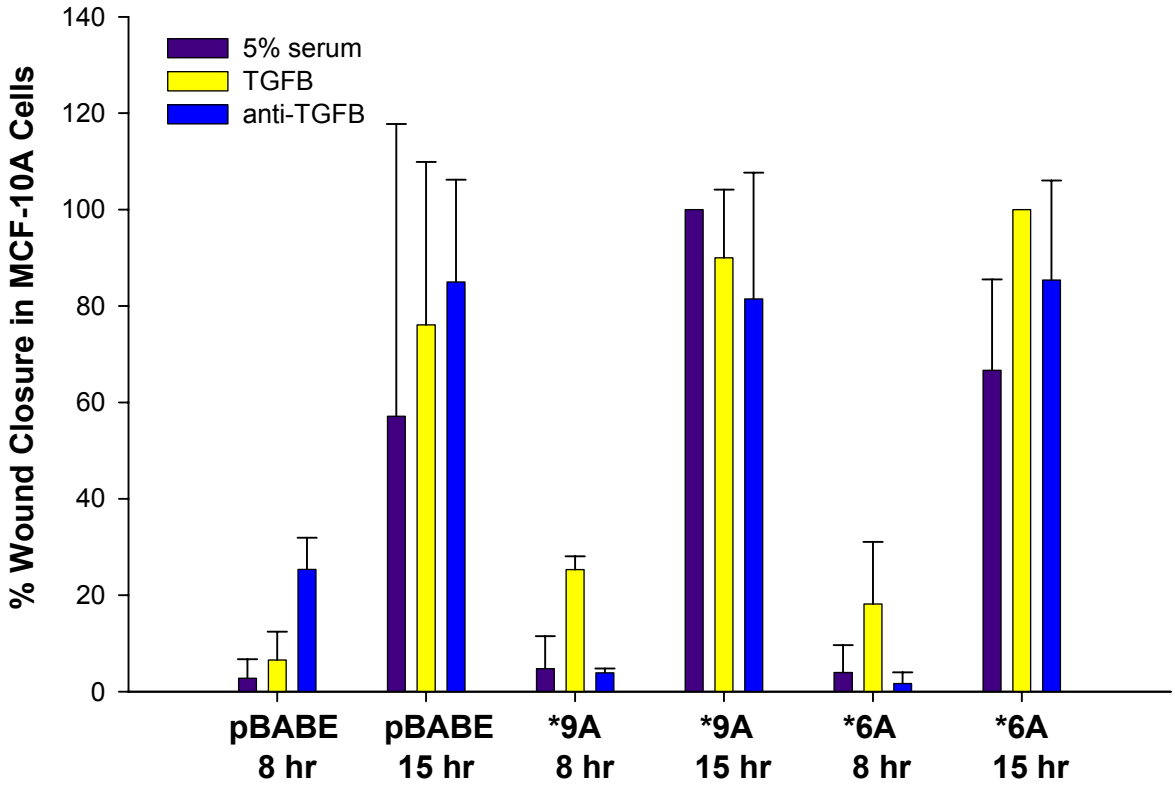


**Figure 2.11 Wound closure in MCF-10A cells is not altered by TGFBR1*6A**

A confluent monolayer of MCF-10A vector only, MCF-10A*9A, and MCF-10A*6A cells were scratched with a pipet tip and wound closure was evaluated at 8 and 15 hrs. In the *9A and *6A cells, TGF-β seems to be required to initiate the migration of cells at 8 hr, however by 15 hr the wound closure is the same for pBABE, *9A, and *6A cells in the anti-TGFβ treated wells indicating that other growth factors are involved in migration and TGF-β is only required at initial stages of migration.

Addition of TGF-β results in similar proportions of invading cells in each of the cell lines tested. The pIRES and *9A cells have a slight decrease in invading cells compared to the MCF-7 cells, however, *6A cells invade the same as the parental cells, indicating that *6A has no effect on TGF-β induced cell invasion (Figure 2.12.A).

Invasion was also tested in the MCF-7 pBABE clones. The *9A-low cells invade 1.3 times more than the vector controls ($p<0.001$), while the *6A-low cells invade 2.2 times more than the vector cells ($p<0.001$) (Figure 2.12.B). This 1.7 fold increase in *6A-low cell invasion over *9A-low cells is also significant ($p<0.001$). Similar to what was seen with the pIRES clones, TGF-β does not alter the amount of invasion, as compared with the basal invasion, ($p<0.1$ for *9A-low and $p<0.1$ for *6A-low cells). *9A-high clone invasion was decreased compared to pBABE controls, however the *6A high clones invaded 1.9 fold more than the pBABE cells ($p<0.001$), demonstrating that *6A increases invasion. TGF-β caused a 2.1 fold induction in invasion in the *6A-high cells compared to pBABE cells ($p<0.001$), however TGF-β did not change the amount of invading cells compared to non-treated wells ($p<0.2$).

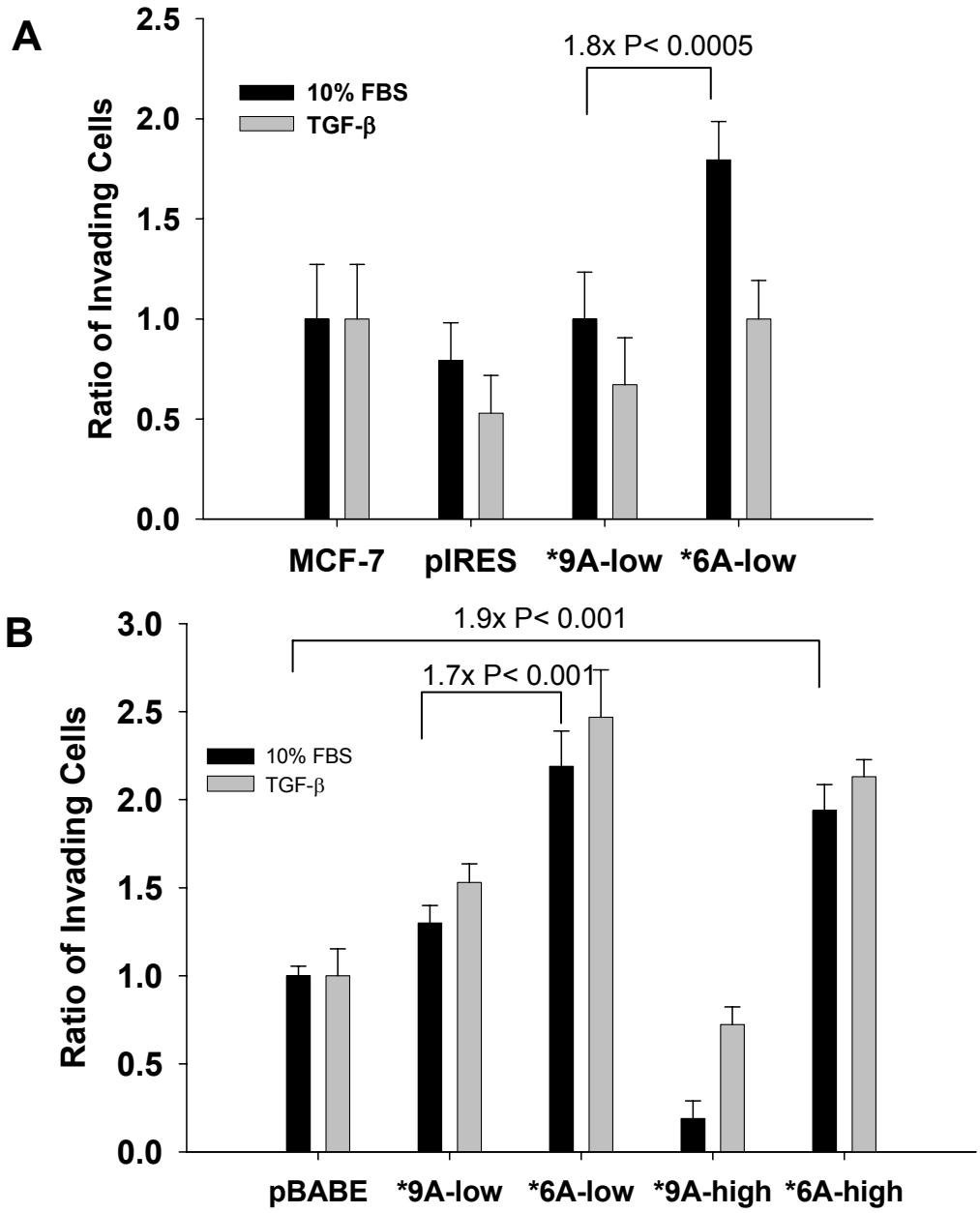


**Figure 2.12 TGFBR1*6A enhances MCF-7 cell invasion**

A and B, MCF-7 cells stably transfected with the empty vector (pIRES), *9A or *6A (A) or pBABE empty vector, or with pBABE*9A or pBABE*6A (B) were seeded on top of matrigel coated transwell chambers in starvation media overnight. Cells invaded towards complete media (black bars) or complete media with 5 ng/ml TGF-β (grey bars) for 72 hr. Vertical bars represent the average of seven experiments (A) or 3 experiments (B). Error bars represent standard error. The number of *6A cells invading the matrigel was significantly higher than that of *9A cells ($p < 0.005$).

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To determine whether the increase in cell migration, wound closure, and invasion seen in *6A cells is partially or entirely due to increased cell growth compared to *9A cells, basal growth rate was assessed by thymidine incorporation. As seen in figure 2.13, there was no difference between the growth rate of *6A compared to *9A or to parental MCF-7 or vector control cells. These data strongly suggest that the differences in actual cell migration are not due to differential cell growth.

These results establish the fact that both migration and invasion of MCF-7 cells are significantly enhanced by *6A in a TGF-β independent manner.

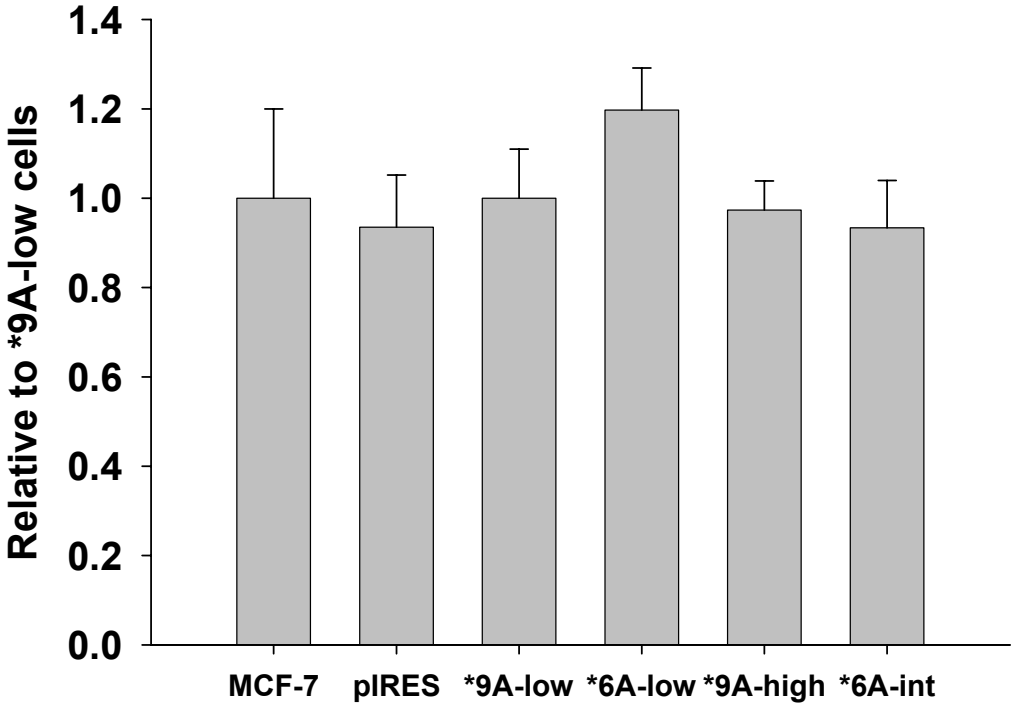


**Figure 2.13 TGFBR1*6A does not alter basal level of growth in MCF-7 cells**
Basal growth rate of MCF-7 cells was determined by thymidine ($^3$H) incorporation over 4 hrs following 18 hr of normal cell growth in culture. For *9A-low and *6A-low, n =10 assays done in triplicate. MCF-7, pIRES, *9A-high, and *6A-int, n=3 assays done in triplicate

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**TGFBR1*6A expression leads to downregulation of *ARHGAP5* and *FN1***

To dissect the molecular mechanisms underlying the differences in migration and invasion of *6A and *9A cells, we analyzed the differential gene expression of the two low expressing cell lines. The Affymetrix GeneChip® Human Genome U133 Plus 2.0 Array was used to identify differentially-expressed genes in MCF-7 pIRES*6A cells compared to MCF-7 pIRES*9A cells. The low expressing *6A and *9A cells were used for the array. The results were analyzed using Ingenuity Pathway Analysis focusing on genes involved in cell migration and invasion. Using a cut-off of 1.5 fold difference in gene regulation and a p-value of <0.01, two genes involved in cell migration were identified that were down-regulated in *6A cells compared to *9A cells: *ARHGAP5* and *FN1*.

*ARHGAP5* encodes the Rho GTPase activating protein 5 (ARHGAP5). Affymetrix gene array analysis shows that ARHGAP5 expression is 3.4 fold lower in *6A cells than in *9A cells in the presence of normal growth media (p=0.00017). Real-time PCR confirmed these findings and showed a 3.8 fold reduction in the expression of ARHGAP5 in *6A cells compared to *9A cells (Figure 2.14.A), as well as a 4.3 fold reduced expression in the *6A-int cells compared to the *9A-high cells, indicating that this response is not due to clonal variation. Furthermore, we observed a slight 1.2 and 1.5 fold reduction in ARHGAP5 in a TGFBR1*6A kinase-inactivated MCF-7 cell line compared to the low expressing *9A cells (Figure 2.14.A), suggesting that ARHGAP5 downregulation is independent of TGFBR1 kinase signaling. Western blotting for ARHGAP5 also shows that ARHGAP5 is downregulated in *6A compared to *9A for cells transfected with both the pIRES and pBABE vectors (Figure 2.14.B).

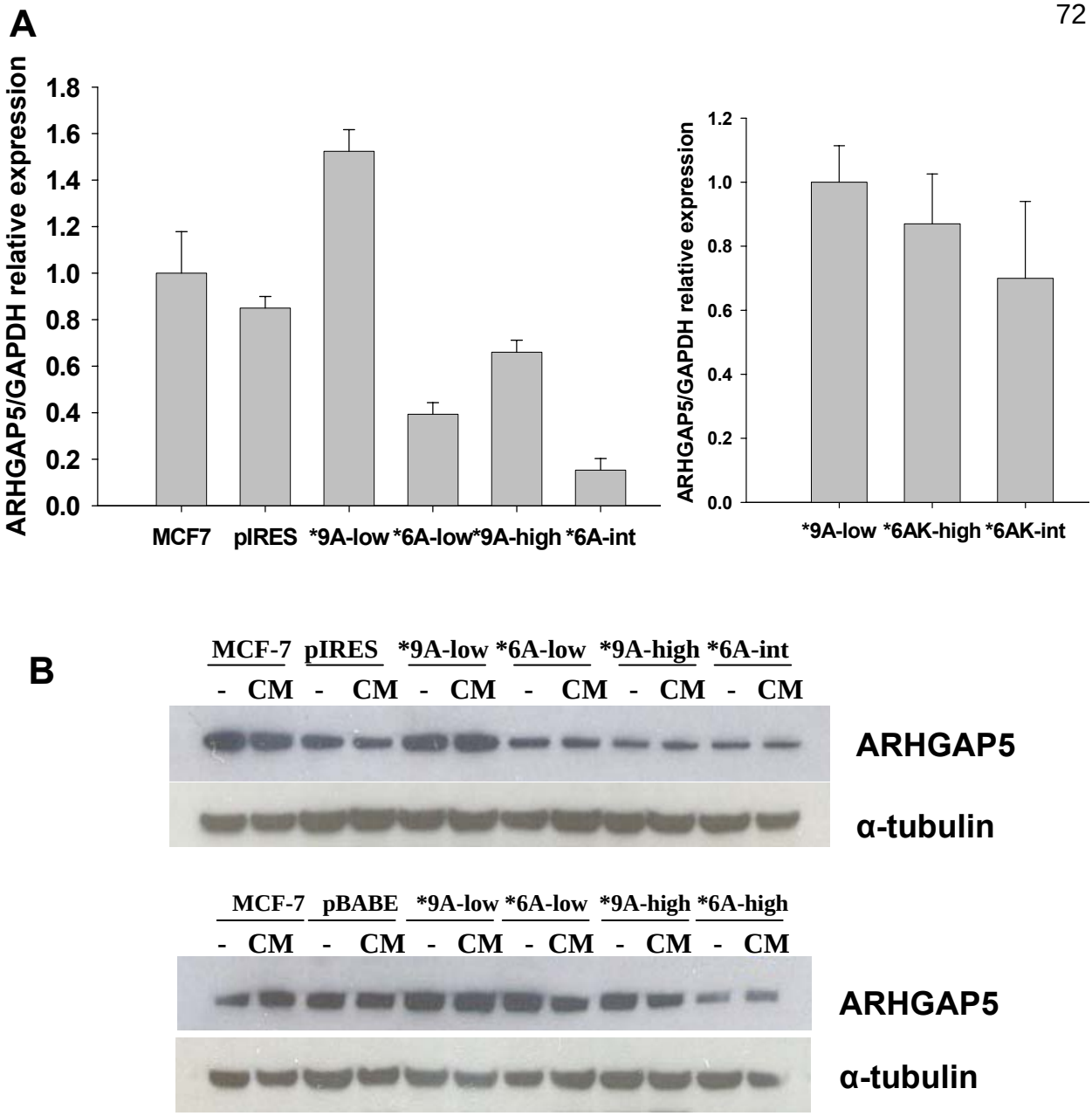


**Fig 2.14 ARHGAP5 is downregulated in TGFBR1*6A expressing cells**

A. Real-time PCR was performed on both the low and high expressing clones to determine expression of ARHGAP5 (A). Compared to the the respective *9A cells, ARHGAP5 is downregulated 3.8 fold in the *6A-low cells, and 4.3 fold in the *6A-int cells. In the *6A kinase deficient clones, ARHGAP5 is downregulated 1.2 and 1.4 fold compared to *9A cells. Results represent one experiment done in triplicate. Error bars represent the standard deviation.

B. Western blot of MCF-7 pIRES and pBABE clones confirms downregulation of ARHGAP5 in all *6A clones compared to *9A clones.

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RHO GAPs down-regulate GTP-bound RHOA by hydrolyzing GTP into GDP. By downregulating ARHGAP5, RHOA remains bound to GTP and remain active. We utilized two different methods to determine whether the differential regulation of ARHGAP5 alters ARHGAP5 activity: a commercially available G-LISA™ RHOA Activation Assay kit and the pull-down method described by Ren *et. al*. [Ren et al., 1999] It has been shown that the serum component LPA rapidly induces RHOA activation before declining [Ren et al., 1999]. As shown in figure 2.15.A, RhoA activation was consistently higher in *6A cells compared to *9A cells. The greatest differences were observed at 10 and 20 min and 60 min post serum induction.

Because GTP-bound RhoA is rapidly hydrolyzed into the GDP form, a pull down assay was used that utilizes the ability of the Rhotekin protein to specifically bind GTP-bound RhoA [Reid et al., 1996]. At 10 min post FBS induction, we were able to confirm our finding that GTP-RHOA is higher in *6A-low cells than in *9A-low cells, indicating that RhoGAP activity is decreased in *6A cells (Figure 2.15.B).

*FN1* encodes fibronectin (FN1). FN1 was downregulated 2.5 fold in *6A cells when compared with *9A cells (p=0.0046). Real-time PCR confirmed this finding and showed a 6.4 fold reduction of FN1 in the low expressing *6A cells compared to *9A. The difference among high expressing clones was also significant with a 3.1 fold lower level in the *6A cells compared to *9A cells (Figure 2.16.A). FN1 is downregulated 11 fold and 2 fold in the kinase-dead *6A MCF-7 cells compared to the *9A-low cells (Figure 2.16.B), indicating that *6A-mediated downregulation of FN1 is independent of TGFBR1 kinase signaling. Western blotting analysis further confirmed FN1 downregulation, as seen by the almost complete disappearance of FN1 in all *6A clones tested compared to its *9A control (Figure 2.17).

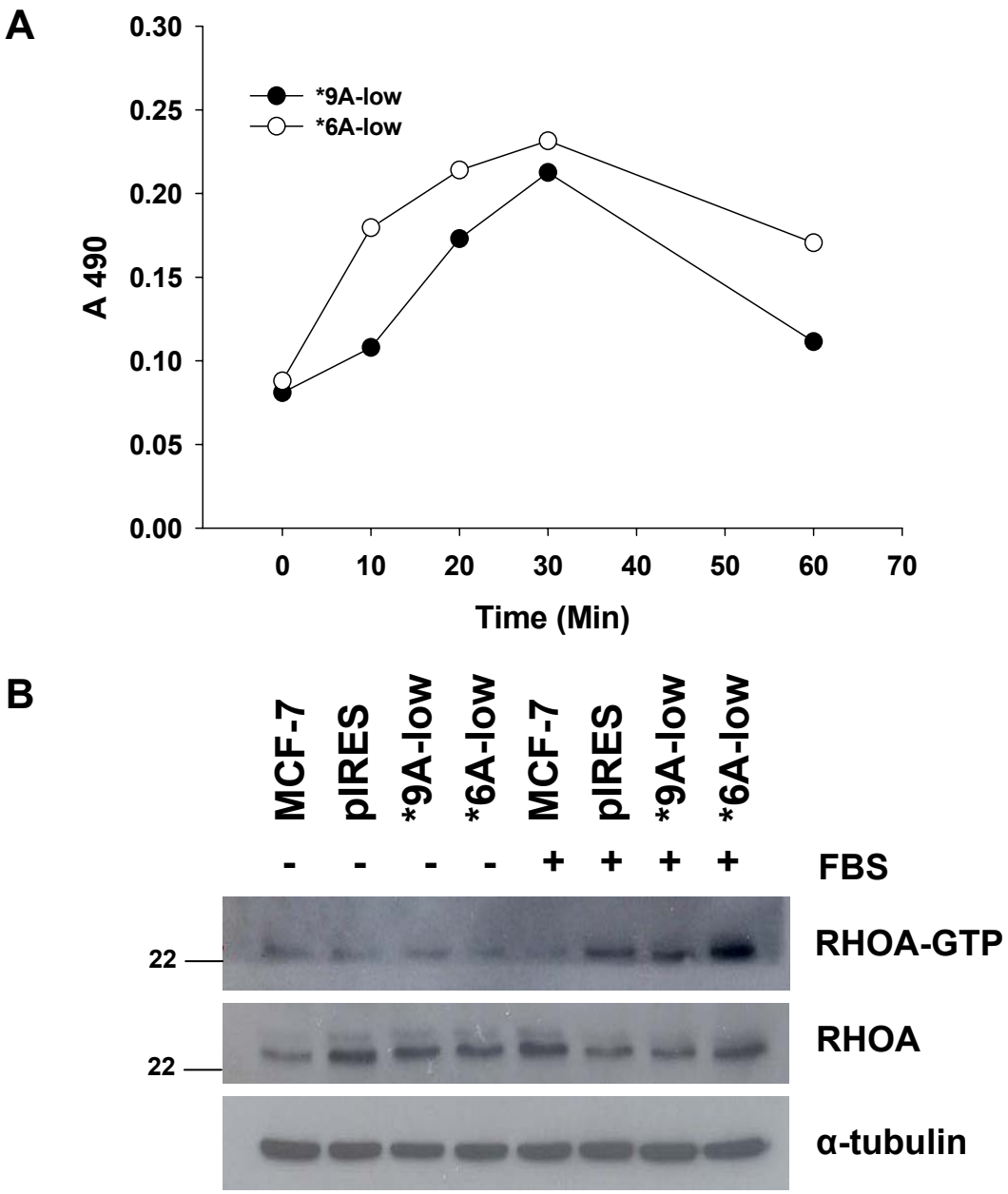


**Figure 2.15 Downregulation of ARHGAP5 leads to RHOA activation**

A. A G-LISA assay was performed to measure GTP-bound RHOA. MCF-7-*6A cells have higher amounts of RHOA activation than MCF-7-*9A cells at each time point. The data represents one assay done in triplicate.

B. After overnight starvation, cells were stimulated with FBS for 10 min prior to collecting lysates. Lysates were immunoprecipitated using the Rhotekin Rho Binding Domain to detect GTP-bound RHOA, and blotted with anti-RHOA. MCF-7-*6A cells have an increased amount of GTP-bound RHOA than MCF-7-*9A cells.

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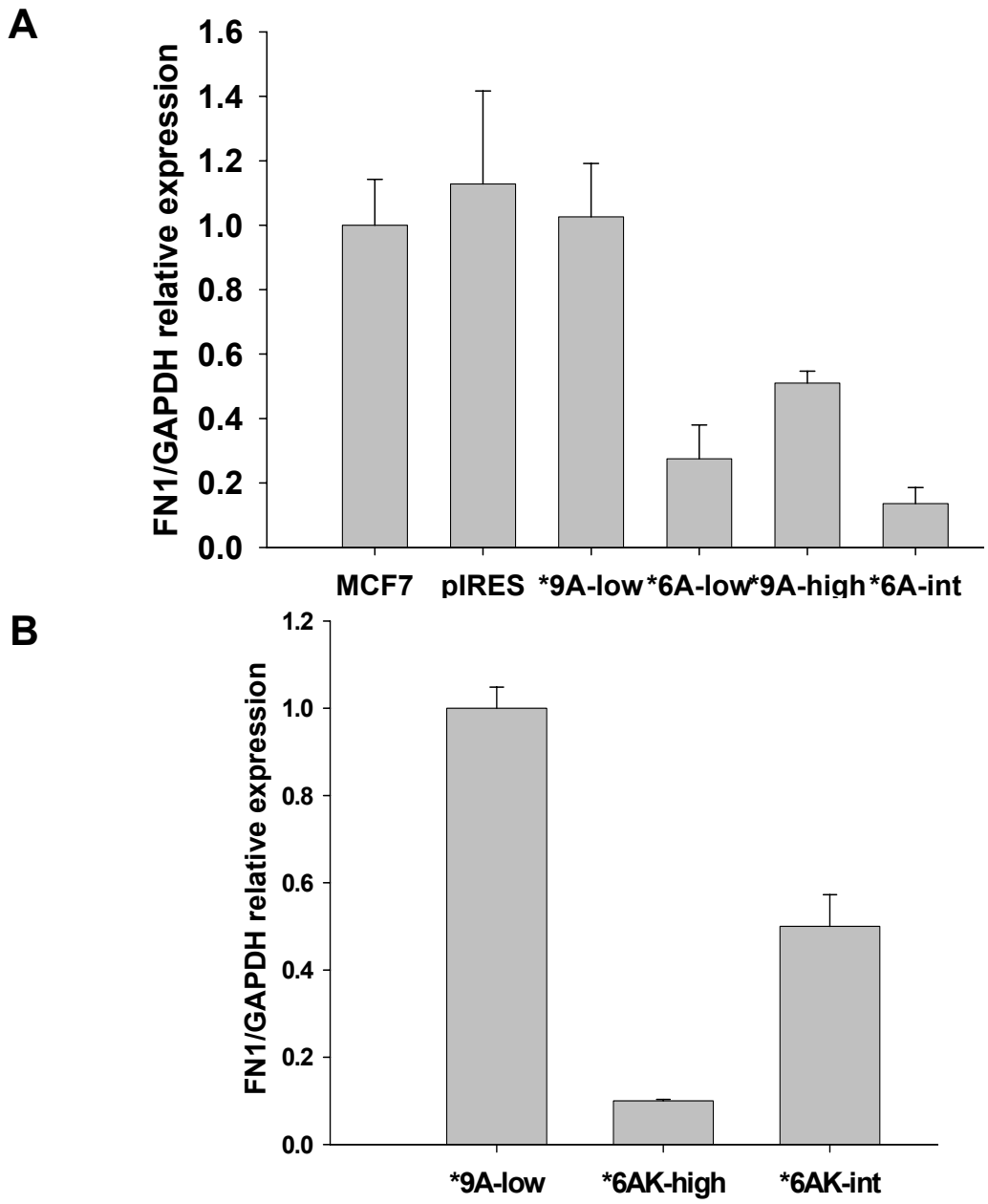


**Fig 2.16 FN1 is downregulated in TGFBR1*6A expressing cells**

A and B: Real-time PCR was performed on both the low and high expressing clones to determine expression of FN1. (A) FN1 is downregulated 6.4 fold in the *6A-low cells, and 3.1 fold in the *6A-int cells compared the *9A-low and *9A-high cells, respectively. (B) FN1 expression in the kinase deficient *6A clones are 11 fold and 2 fold reduced compared to *9A cells. Results represent one experiment done in triplicate. Error bars represent the standard deviation.

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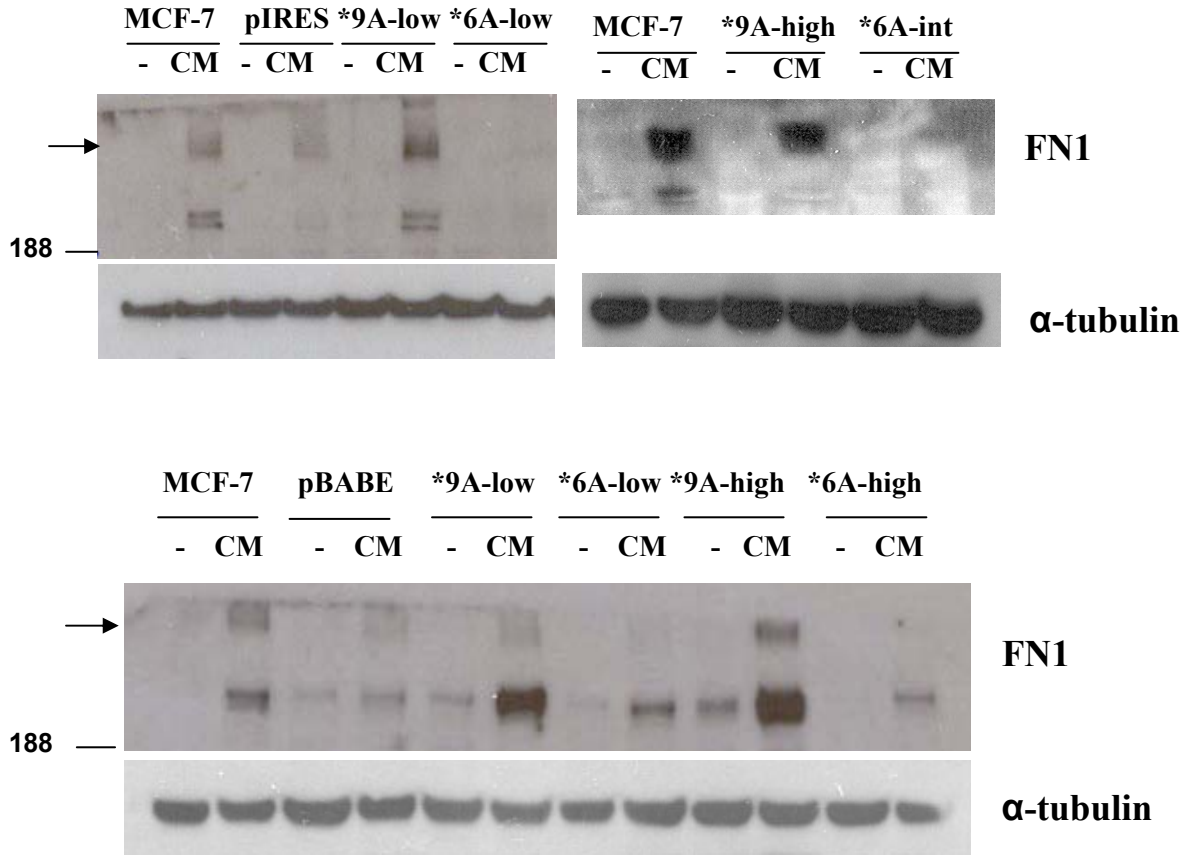


**Fig 2.17 FN1 is downregulated in TGFBR1*6A expressing cells**

Western immunoblotting of FN1 confirms downregulation of FN1 in *6A cells compared to *9A cells for both the low and high expressing clones in both the pIRES (upper panel) and pBABE (lower panel) clones.

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**TGFBR1*6A expression increases ERK activation**

Signaling through the MAPK pathway contributes to migration and invasion [Huang et al., 2004]. To determine whether *TGFBR1**6A increases migration in our model via activation of the MAPK pathway, we examined the various components involved in MAPK signaling. After growth in complete growth media (10% FBS) for 24 hrs following overnight serum-starvation, we observed 44% higher levels of ERK1/2 phosphorylation in the *6A-low over the *9A-low clones, and 43% higher levels of ERK1/2 phosphorylation in the *6A-int over the *9A-high clones (Figure 2.18). Considering that the *6A-int clones express TGFBR1 to a lesser extent than the *9A-high cells (Figure 2.1), the enhancing effect of *6A on ERK1/2 phosphorylation may be underestimated. Assessing earlier time points for ERK activation may also reveal more insight into the time of *6A-induced ERK activation.

There was no consistent difference in signaling between p38 and JNK activation in *9A cells compared to *6A cells grown in complete media, however looking at earlier time points after TGF-β treatment may provide more clues to p38 and JNK regulation.

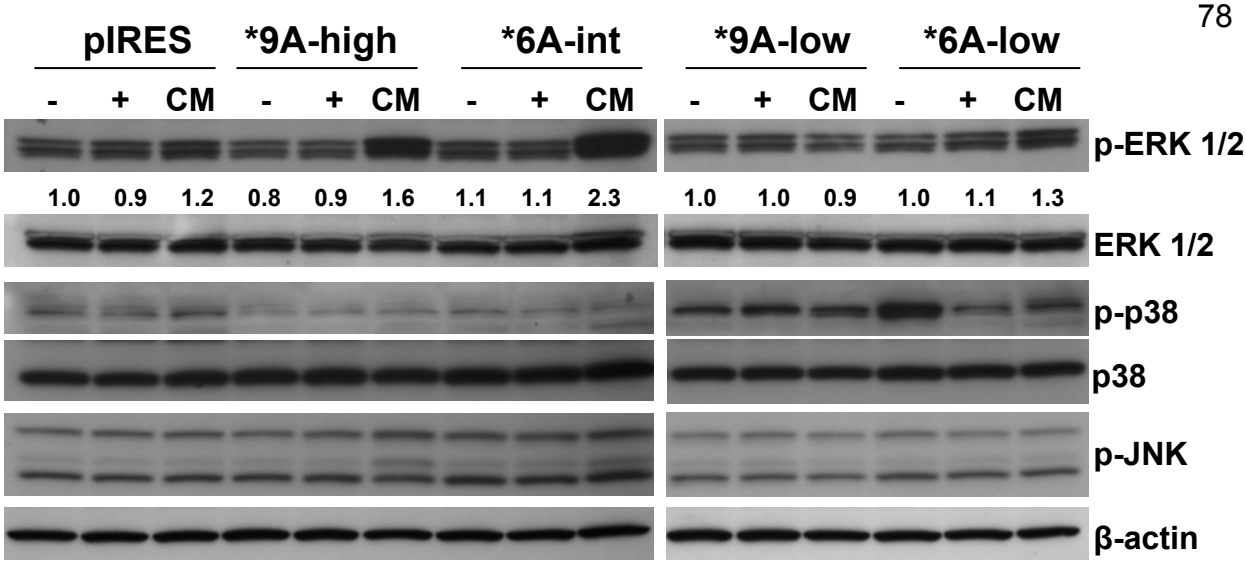


**Fig 2.18 ERK activation is higher in TGFBR1*6A cells**

Western blots of phosphorylated ERK, phosphorylated p38, and phosphorylated JNK. CM – complete media; - starvation media; + 100 pM TGF-β treatment for 18 hr after overnight serum starvation. In complete media (CM), both the *6A-low expresser and *6A-int cells show higher phosphorylated ERK compared to their respective *9A controls. ERK signaling is not affected by TGF-β. Neither p38 nor JNK are involved in *6A-mediated signaling in the presence of complete media. However, p-p38 levels were increased in *6A-low cells in starvation media. Numbers under p-ERK represent ratio of expression normalized after densitometry analysis.

**DISCUSSION**

One the most essential homeostatic functions of TGF-β is inhibition of cell proliferation, thereby functioning as a tumor suppressor gene. TGF-β signaling components are often mutated during tumor formation.

In this chapter, we demonstrate that transfection of *TGFBR1**6A into MCF-7 breast cancer cells results in a significant increase in both cellular migration and invasion. The difference in migration and invasion were observed with multiple clones both in the presence and in the absence of exogenously added TGF-β. However, the difference between *6A cells and *9A cells was more pronounced in the absence of TGF-β, which indicates that this phenomenon is independent of TGF-β signaling and that TGF-β may blunt the effect of other serum components.

Gene expression profiling analysis identified two differentially expressed genes involved in migration, *ARHGAP5* and *FN1*. Differential expression of these genes was confirmed in high and low expressing *6A and *9A clones. Furthermore, our functional assays established that downregulation of ARHGAP5 is associated with increased RhoA activation. RhoGTPases (RHO, RAC, and CDC42) regulate cell migration by mediating distinct cytoskeletal changes. RAC induces lamellipodia extensions and membrane ruffling, CDC42 regulates filopodia formation, and RHO induces stress fiber formation [Ridley, 2001]. *ARHGAP5* encodes the RhoA GTPase activating protein 5 (ARHGAP5), which causes the inactivation of GTP-bound RhoA. We found that down-regulation of ARHGAP5 is associated with increased levels of activated RhoA as early as 10 min post induction. Although mutations in RhoA are rare, RhoA is often overexpressed or functionally hyperactive in breast cancer tissue, and overexpression correlates with more advanced breast disease [Fritz et al., 2002;Fritz et al., 1999]. Furthermore, knockdown

of RhoA using siRNA inhibited migration and invasion of aggressive MDA-MD-231 and Hs578T breast cancer cell lines [Zuo et al., 2006;Pille et al., 2005]. Expression of constitutively activated RhoA in MCF-7 breast cancer cells resulted in an increase in serum-mediated motility which was directly blocked by ROCK inhibition. In addition, RhoA activation signaling via ROCK led to a direct increase in MMP-9 activity and ERK1/2 activation, leading to the increase in motility [Caceres et al., 2005]. These findings lead us to hypothesize that the observed increase in ERK activation is a direct effect of RhoA hyperactivation induced by *TGFBR1**6A expression, resulting in the observed increased migratory phenotype of MCF-7*6A cells.

While IHC staining on patient breast tumor samples indicates that the tumor cells do not express ECM-associated (fibrillar) FN1, the same studies show that FN1 is concentrated in the stromal compartment of breast tumors and is often condensed in areas surrounding proliferating "tumor islands" [Christensen et al., 1988]. In accordance with this observation, a study looking at FN1 production in a sample of tumor cells obtained from primary or metastatic tumors , found that FN1 was absent in many of the metastatic tumor cell lines, while FN1 was present in cell lines derived from either nonmalignant tissues or primary carcinomas [Smith et al., 1979;Urtreger et al., 2006]. Additionally, metastatic tumor cells did not synthesize FN1 [Smith et al., 1979]. Urtreger et al. determined that tumor cells derived from a metastatic murine tumor do not express either plasma (soluble) FN1 or ECM-associated FN1. Re-expression of plasma FN1 resulted in both increased levels of plasma and secreted FN1. However, the secreted FN1 was not incorporated into the ECM [Urtreger et al., 1998]. FN1 re-expression decreased the migration of these tumor cells. Furthermore, a mutated FN1 that could not form fibrils also was sufficient to inhibit cell migration [Urtreger et al., 1998]. These results further provide strong support for the notion that loss of FN in tumor cells results in enhanced migration.

Horii and colleagues showed that FN1 production in MCF-7 cells is inhibited by 17β-estrodiol, which is a stringent growth stimulator for ER+ MCF-7 cells. Furthermore, treatment of MCF-7 cells with tamoxifen, an anti-estrogen, inhibits cell growth and restores FN1 expression, which is partially mediated by TGF-β induction [Horii et al., 2006]. The restoration of FN1 by tamoxifen treatment may also inhibit further tumor progression by inhibiting the ability for the tumor cells to migrate or invade. These results indicate that FN1 secreted by breast tumors is implicated in migration and invasion.

Western blotting of FN1 showed that FN1 protein expression was downregulated to the same extent as the FN1 mRNA levels for MCF-7*6A cells compared to *9A cells. The FN1 antibody we used recognizes ECM associated FN1 and not soluble FN1[Leu et al., 2003]. The levels of soluble FN1 needs to be further investigated, however because overall FN1 was decreased, soluble and fibrillar FN1 may be downregulated to the same extent. Decreased FN1 expression in MCF-7*6A cells most likely results in loss of adhesion, thus promoting early events that lead to migration. The next step would be to reintroduce FN1 into the MCF-7*6A cells to see if migration or invasion decreases to the level that the *9A cells migrate or invade.

FN1 in the stroma functions as an anchor for tumor cells and suppresses cell migration [Kaspar et al., 2006]. Stromal FN is often lost in breast cancer tumors and is associated with a poorer prognosis, lower relapse-free survival, and higher rate of metastasis compared to patients with FN present in the stroma [Takei et al., 1998;Christensen et al., 1988]. Women with invasive breast carcinoma (IBC) who died without metastases (27 out of 31 patients, 87%) had a higher frequency of FN1-positive stromal cells compared to women who died with disseminated IBC (3 out of 26 patients, 12%; $p<0.0005$) [Christensen et al., 1988]. Loss of stromal FN1 promotes metastasis by causing the cell to loose adhesiveness to the stroma thus allowing the

tumor cell to leave the tumor and invade. Because *6A association with breast cancer is due to germline mutation status and not somatic acquisition by the tumor, it would be interesting to look at FN1 expression patterns in breast cancer samples from patients who harbor *6A and to see if FN1 expression correlated with metastatic occurrences in humans.

Because the mature *9A and *6A receptors are identical [Pasche et al., 2005], it is expected that TGFBR1 kinase-mediated TGF-β signaling is identical in *9A and *6A cells. Indeed, MCF-7 cells expressing either *9A or *6A do not show a difference in TGF-β signaling as assessed by levels of pSMAD2 or pSMAD3. Similarly to our findings with respect to TGF-β-mediated growth inhibition the observed effects are likely due to secondary signaling events triggered by *6A signal sequence. This explanation is supported by the findings that MCF-7 cells transfected with a kinase-deficient *TGFBR1**6A construct also show decreased expression of ARHGAP5 and FN1 when compared to cells transfected with *9A.

*TGFBR1**6A causes TGF-β mediated growth stimulation in both MCF-7 breast cancer cells and DLD-1 colon cancer cells and transduces TGF-β growth-inhibitory signals less effectively than TGFBR1 in mink lung epithelial cells [Pasche et al., 2005;Pasche et al., 1999;Chen et al., 1999]. These previous findings led us to test the hypothesis that *TGFBR1**6A may act as an oncogene. Using foci and colony formation assays in NIH-3T3 cells, we were unable to uncover any oncogenic properties for *6A, either alone, or collectively with HRAS-$V^{12}$. Actually, overexpression of the wild-type receptor increased HRAS-$V^{12}$ oncogenic transformation in NIH-3T3 cells to a greater degree than overexpression of the *6A receptor. Furthermore, transfection of *6A did not provide NIH-3T3 cells with a growth advantage after TGF-β treatment. The inability for *TGFBR1**6A to transform normal cells was also validated in the MCF-10A normal breast epithelial cell line. The inability for *6A to confer both a growth

advantage to normal cells is significant in the fact that *6A is a low penetrant tumor susceptibility allele. If *6A had the advantage of transforming normal cells, then more people who carried *6A would develop breast cancer. Additionally, *6A could not enhance migration in the normal breast epithelial cells, possibly indicating that *6A-mediated migratory effects may only occur to tumor cells.

In summary, we show that *6A enhances MCF-7 cell migration and invasion and results in the activation of the RhoA and ERK pathways as well as the downregulation of two genes that play a central role in the inhibition of cell motility (Figure 2.19). These effects were observed in the absence of exogenously added TGF-β. This is the first report of a *TGFBR1**6A phenotype that is independent of TGF-β signaling. Given the fact that approximately 16% of patients with breast cancer harbor the *6A allele [Kaklamani et al., 2005] our findings may have important implications for the relatively large proportion of patients suffering from this disease.

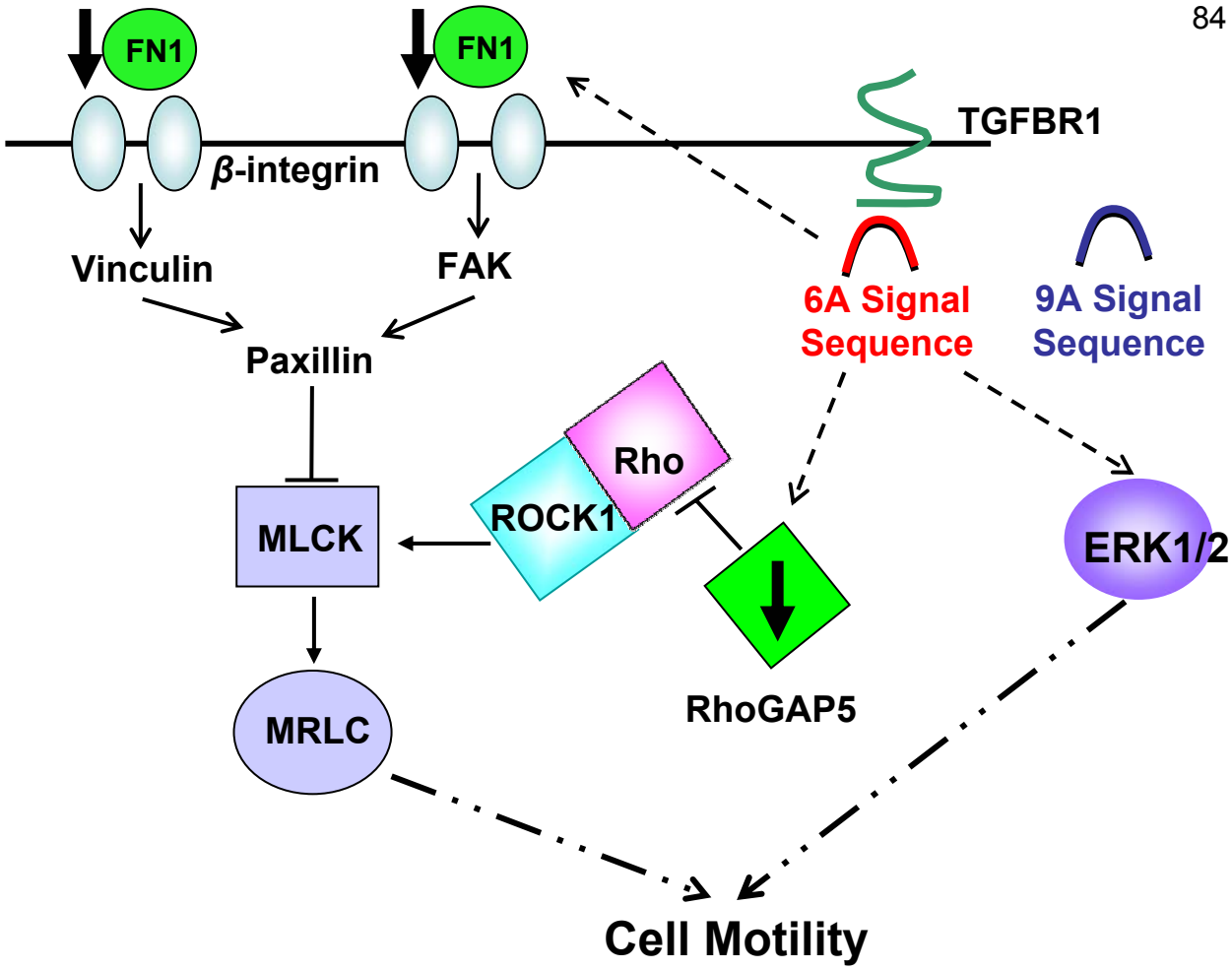


**Figure 2.19 *TGFBR1**6A, ERK signaling and cell motility**

*TGFBR1* and *TGFBR1**6A encode for identical mature receptors but the signal sequence of TGFBR1*6A is 3 amino acids shorter than the signal sequence of TGFBR1. Transfection of MCF-7 cells with TGFBR1*6A results in the downregulation of *FN1* and *ARHGAP5*, and enhances RhoA and ERK activation. RhoA activates ROCK, which activates MLCK (myosin light chain kinase), then MRLC (myosin regulatory light chain 2), causing myosin II to induce stress fiber formation and enhancing motility.

# CHAPTER 3

# *Tgfbr1* haploinsufficiency accelerates mammary tumor formation in *MMTV-Neu* mice

## INTRODUCTION

TGF-β is a pleiotropic cytokine involved in regulating cell growth, differentiation, apoptosis, adhesion, migration, angiogenesis, immune surveillance, and matrix remodeling [Massague, 2008]. TGF-β plays an important role in mammary gland development as well. The mammary gland is a unique organ that undergoes the majority of its development postnataly. At birth, a rudimentary mammary gland is present, and ducts continue to grow slowly until puberty where hormones influence the development of a mature epithelial ductal tree that extends through the entire mammary fat pad. The terminal end bud (TEB) is the structural unit that grows and branches to form the ductal tree. TEBs are a dual-layer structure, with an inner layer of luminal cells surrounding a hollow lumen, and an outer layer of myoepithelial cells that borders the basement membrane of the gland [Lanigan et al., 2007].

In mammary gland development, TGF-β mostly plays a growth inhibitory role. In mouse mammary epithelial tubules forming in a three-dimensional culture, TGF-β concentration was lowest in the stromal areas around branching points in the mammary ducts, where cell growth needs to occur [Nelson et al., 2006]. In mice there is a high concentration of TGF-β in the periductal stroma where lateral budding is suppressed, and the extracellular matrix (ECM) associated TGF-β is selectively lost in areas where the lateral buds are forming [Silberstein et al.,

1992]. Mice engineered to express constitutively active TGF-β in the mammary glands have transiently delayed ductal epithelium progression at 12 weeks that is overcome by 40 weeks [Muraoka et al., 2003].

Several mouse models exist that are used to study breast cancer development. A common technique used to determine a specific gene's action in the mammary gland is to express it under the mouse mammary tumor virus (MMTV) promoter. Mice engineered to express the activated form of the rat *Neu* oncogene driven by the MMTV promoter will develop adenocarcinomas in the entire epithelium of each mammary gland as early at 78 days, with all mice showing tumors by 95 days. These data suggest that activated *Neu* is potent oncogene that can induce tumor formation with few, if any, other genetic alterations [Muller et al., 1988]. Mice harboring the *MMTV-c-Neu* proto-oncogene, the form which is found in 20-30% of human breast cancer [Slamon et al., 1989], begin to develop focal mammary tumors by 4 months of age, with 50% of females developing mammary tumors by 205 days. Seventy three percent of mice harboring the *Neu* proto-oncogene also exhibited lung metastasis [Guy et al., 1992].

TGF-β plays a pivotal, although paradoxical, role in cancer development. Although TGF-β inhibits tumor formation by inducing growth arrest in normal cells, TGF-β can also signal to tumor cells to induce late stage progression and metastasis. When the *MMTV-c-Neu* mice were crossed with mice expressing constitutively activate TGF-β1 in the mammary gland (*MMTV-Tgfb1*$^{S223/225}$), tumor volume was smaller, poorly differentiated, and exhibited local invasion, even though the latency did not change. Moreover, these double transgenic mice had 100% occurrence for metastasis to the lung at 100 days after initial tumor formation, as compared to 60% of the *MMTV-Neu* mice. The breast tumors were highly vascularized as well in the *Neu;Tgfb1*$^{S223/225}$ mice [Muraoka et al., 2003]. Likewise, 78% of bigenic mice expressing

*MMTV*-N*eu* and *MMTV-Alk5*$^{TD}$ (activated Tgfbr1) had lung metastasis present at 68 days after initial tumor formation, compared to 31% of *Neu* controls, while the tumor latency and tumor burden remained unchanged [Muraoka-Cook et al., 2006].

In another model, mice engineered to express *MMTV-Tgfbr1(AAD)*, constitutively active Tgfbr1, were bred with mice containing the activated *MMTV-Neu* in which Neu is mutated to allow signaling only through the Grb or Shc adapter proteins [Siegel et al., 2003]. Both the *Neu-Grb;Tgfbr1(AAD)* and *Neu-Shc;Tgfbr1(AAD)*mice had an increased tumor latency compared to the *Neu-Grb* or *Neu-Shc* mice, respectively, indicating that increased TGF-β signaling has a tumor suppressive effect. However *Neu;Tgfbr1(AAD)* mice that retained the ability to signal only through Shc had a 3-fold increased formation of extravascular lung metastasis compared to mice only able to signal through Grb. In addition, *MMTV-Tgfbr2(ΔCyt)* (dominant negative Tgfbr2) decreased tumor latency and decreased the amount of extravascular lung metastasis in the *Neu-Grb* model [Siegel et al., 2003]. Although the effects of TGF-β signaling on tumor latency and tumor burden are highly dependent on the mouse model being used, there is a clear indication that increased TGF-β signaling leads to more breast cancer metastases.

The *TGFB1*T29C* SNP results in increased TGF-β secretion and activation [Dunning et al., 2003]. In a combined assessment with *TGFBR1**6A and *TGFB1**T29C, patients were classified according to their predicted TGF-β signaling response, and it was found that patients classified as low TGF-β signalers had a significantly higher risk for breast cancer (O.R. 1.69, 95% CI 1.08-2.66) then those classified as high signalers [Kaklamani et al., 2005].

Our lab created a *Tgfbr1* haploinsufficient mouse model that disrupts *Tgfbr1* in exon 1 where the signal sequence is located. When *Tgfbr1*$^{+/-}$ mice are crossed with *Apc*$^{Min/+}$ mice (mouse model for intestinal cancers [Moser et al., 1990]), *Apc*$^{Min/+}$*;Tgfbr1*$^{+/-}$mice develop twice

as many intestinal tumors than the *Apc*$^{Min/+}$*;Tgfbr1*$^{+/+}$ mice (Pasche Lab, unpublished data). Similar results are observed when *Tgfbr1*$^{+/-}$ mice are treated with azoxymethane. About 1-2% of the population have what is known as allele specific expression (ASE) of *TGFBR1*, where one allele produces more transcripts than the second allele. *TGFBR1* ASE results in decreased TGF-β signaling. ASE in *TGFBR1* confers an increased risk for CRC (OR 8.7; 95% CI 2.6-29.1) and occurs in 10-20% of sporadic colorectal cancer (CRC) patients [Valle et al., 2008].

With the combined assessment between *TGFBR1*6A* and the *TGFB* T29C SNP demonstrating that patients with decreased TGF-β signaling have a higher risk for breast cancer, and the data that decreased TGF-β signaling causes an increase in CRC in both mouse and human, we decided to test the effect of decreased TGFBR1-mediated TGF-β signaling in a breast cancer model.

To investigate the effects of decreased TGF-β signaling at the receptor level on breast cancer development, *Tgfbr1*$^{+/-}$ mice were crossed to the *MMTV-c-Neu* proto-oncogene mouse model. We found a striking decrease in tumor latency in the *Neu;Tgfbr1*$^{+/-}$ compared to the *Neu* mice, however, tumor burden was not significantly altered. We also see evidence that metastasis may be increased in the *Neu;Tgfbr1*$^{+/-}$ mice as well, but these studies are still in progress.

## RESULTS

### Generation of *Neu;Tgfbr1*$^{+/-}$ mice

A classical knockout vector was generated by inserting a Neomycin resistance cassette (Neo) into a Not I site located immediately after the start codon in exon 1 and removing 1.1kb of mouse genomic sequence immediately upstream of this Not I site. Following transfection and selection of 129SvIm embryonic stem (ES) cells, KO clones were karyotyped and injected into C57BL/6 blastocysts. Germline transmission from the resulting chimeras was obtained and a colony established. F3 *Tgfbr1*$^{+/-}$ mice were backcrossed into the FVB/N background using speed congenics markers from Jackson Labs. Briefly, a minimum of 8 *Tgfbr1*$^{+/-}$ animals from each generation of backcrossing were genotyped for 152 markers by the Jackson Laboratory (Bar Harbor, ME). Mice with the highest percentage of the host genome were used to backcross to the host for the next generation. Two fully congenic F6 males (99.9% FVB/N) were confirmed using a full genome wide panel of 150 SNP markers. These two males were crossed with FVB/N females to obtain pure *Tgfbr1*$^{+/-}$ mice in the FVB/N background. FVB/N mice are fairly resistant to spontaneous tumor growth however are susceptible to chemically induced carcinomas [Taketo et al., 1991;Hennings et al., 1993]. Many of the breast cancer mouse models are bred into the FVB/N strain as well, including the *MMTV-c-Neu* mouse model [Guy et al., 1992]. 100% pure FVB *Tgfbr1*$^{+/-}$ mice were crossed with FVB/N-Tg(MMTVneu)202Mul/J mice, and female virgin progeny were kept for studies.

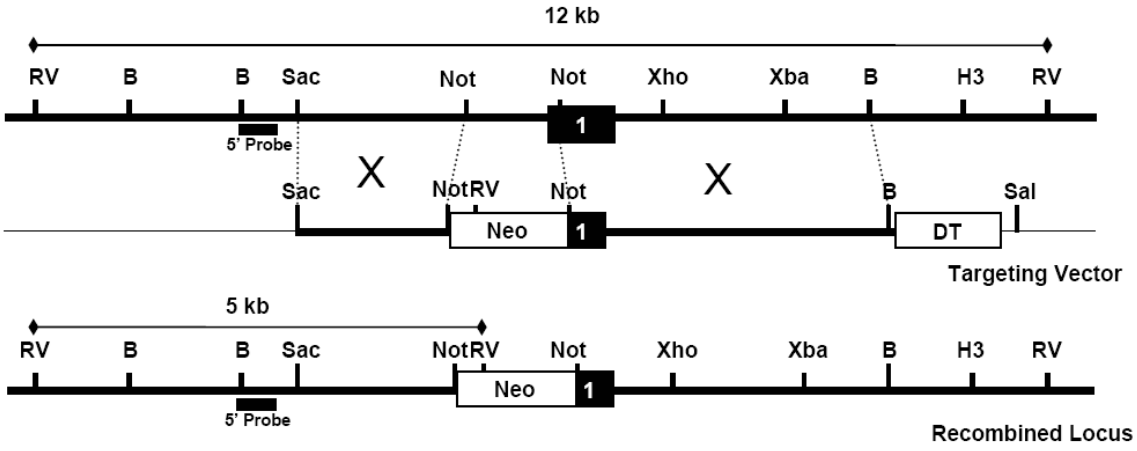


**Figure 3.1 Generation of a novel *Tgfbr1* exon knockout mouse model**

A classical knockout vector has been designed to insert a Neomycin resistance cassette (Neo) into a Not I site located immediately after the start codon, thus interrupting the *Tgfbr1* open reading frame after 6 amino acids and removing 1.1kb of mouse genomic sequence immediately upstream of this Not I site. Following transfection and selection of 129SvIm embryonic stem (ES) cells, we have successfully obtained KO clones that have been karyotyped and injected into C57BL/6 blastocysts. Germline transmission from the resulting chimeras has been obtained and a colony established. F3 *Tgfbr1*$^{+/-}$ mice were backcrossed into the FVB/N background and a fully 100% backcrossed FVB *Tgfbr1*$^{+/-}$ colony was established.

### *Neu;Tgfbr1*$^{+/-}$ mammary glands have more ductal branching than *Neu* mice

TGF-β is an important mediator of mammary gland development. Mammary glands were collected from 10, 12, and 40 week virgin female *Neu* and *Neu;Tgfbr1*$^{+/-}$ mice. *Neu;Tgfbr1*$^{+/-}$ mice had more ductal branching than the *Neu* mice at every age (Figure 3.2). Ductal growth was similar in the *Neu* and *Neu;Tgfbr1*$^{+/-}$ mice. We hypothesize that this increase in ductal branching may signify more proliferation in the breast tissue and lead to an increased risk for breast cancer development.

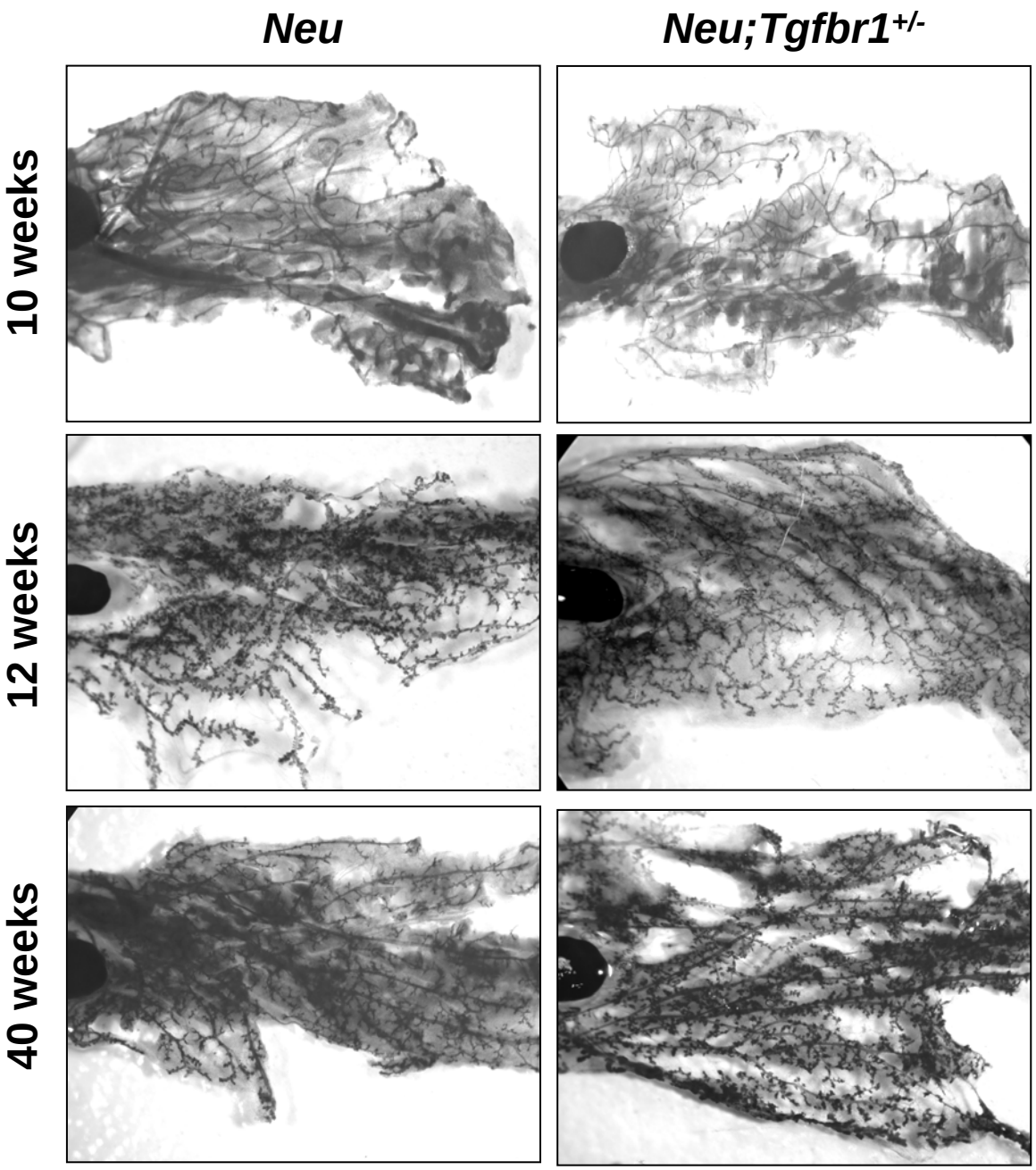


**Figure 3.2 *Neu;Tgfbr1*$^{+/-}$ mice have increased ductal branching than *Neu* mice**

Mammary gland whole mounts of the #4 mammary glands were harvested from mice at 10,12, and 40 weeks of age. *Neu;Tgfbr1*$^{+/-}$ mice have more ductal branching at each of the time points compared to *Neu* mice. Epithelial ductal growth does not appear to be affected by *Tgfbr1*$^{+/-}$.

**$Tgfbr1^{+/-}$ decreases the latency of Neu induced tumors**

Transgenic mice carrying the *MMTV-c-Neu* proto-oncogene develop focal mammary tumors with latancy period of about 205 days [Guy et al., 1992]. To determine the effect of haploinsufficiency of *Tgfbr1* on Neu induced tumor development, mice heterozygous for *Tgfbr1* were crossed with *MMTV-Neu* mice. *Neu* mice had a tumor latency of 220 days, however the *Neu;*$Tgfbr1^{+/-}$ mice developed tumors with a shorter latency of 171 days (P=0.004) (Figure 3.3.A). The hazard ratio for *Neu;*$Tgfbr1^{+/-}$ is 0.39 (95% CI= 0.1631-0.5975).

Mice were sacrificed 80 days after the initial tumor palpation or at the earliest sign of morbidity in compliance with the protocol. The overall tumor burden was not affected by *Tgfbr1* haploinsufficiency in the *Neu* mouse model. *Neu* mice developed on average 3.7 ± 0.53 tumors per mouse with a range of between 1 and 11 tumors per mouse. *Neu;*$Tgfbr1^{+/-}$ mice developed 3.5 ± 0.38 tumors per mouse, with a range of 1 to 8 tumors per mouse (p=0.78) (Figure 3.3.B). Tumor burden was also evaluated by assessing total tumor volume per mouse. Volume for each individual tumor was calculated by the formula [$V=(\text{length}/2) \times (\text{width})^2$] [Muraoka et al., 2003] and the volume of each tumor on a mouse was added to achieve the total tumor burden. There was no difference in tumor volume between *Neu* and *Neu;*$Tgfbr1^{+/-}$ mice: the average volume was 3563 ± 498 $mm^3$ and 4093 ± 450 $mm^3$ for *Neu* and *Neu;*$Tgfbr1^{+/-}$ mice, respectively (p=0.44) (Figure 3.3.C).

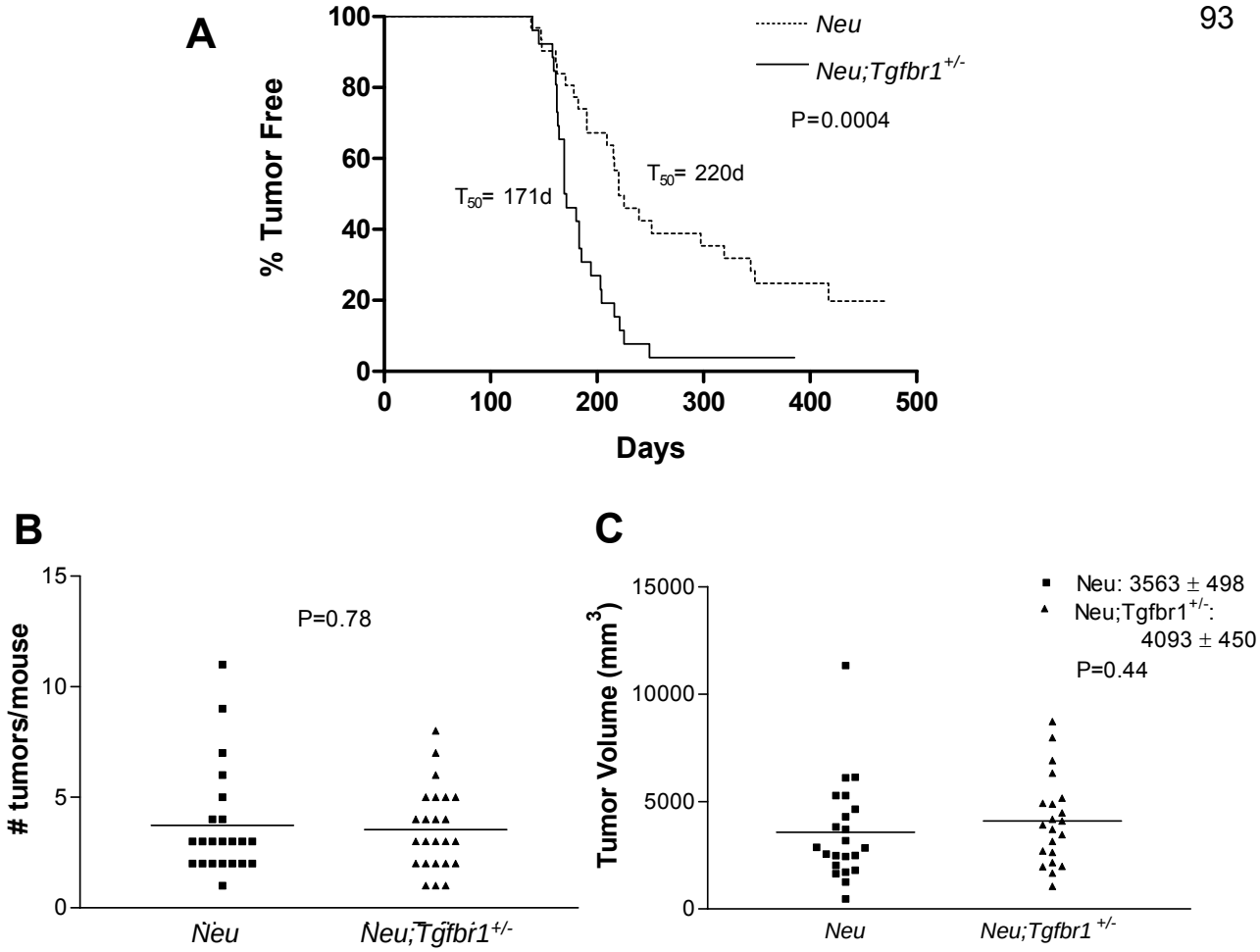


**Figure 3.3 *Tgfbr1*$^{+/-}$ reduces tumor latency in *Neu* mice but does not affect tumor burden**

*A.* *Neu* and *Neu;Tgfbr1*$^{+/-}$ virgin females were monitored twice weekly by palpation for tumor development. The average latency for *Neu* mice was 220 days but for *Neu;Tgfbr1*$^{+/-}$ mice the latency decreased to 171days (P=0.0004). n=31 for *Neu* and n=26 for *Neu;Tgfbr1*$^{+/-}$.

(B & C) Tumor burden was assessed after 80 days following initial tumor formation by assessing the total number of tumors per mouse (B) and total tumor volumes per mouse (C). Volume was calculated by the formula [(1/2)*length*width*height] for each tumor and the total volume of all the tumors were added together for each mouse to obtain total tumor volume. There was no difference between the two groups on tumor burden. n=22 for *Neu* and n=21 for *Neu;Tgfbr1*$^{+/-}$.

Tumor cells are frequently resistant to the effects of TGF-β mediated growth inhibition [Hanahan and Weinberg, 2000]. To test the effects of TGF-β mediated growth inhibition on these mammary tumors, primary tumor cell cultures were established. *Neu* tumor cells were 32.3 ± 3.53% growth inhibited, and *Neu;Tgfbr1*$^{+/-}$ tumor cells were 21.9 ± 1.36% growth inhibited after TGF-β treatment (Figure 3.4). The TGF-β-mediated growth inhibition of *Neu;Tgfbr1*$^{+/-}$ tumor cells was 32.2% lower than that of *Neu* tumor cells (p=0.007).

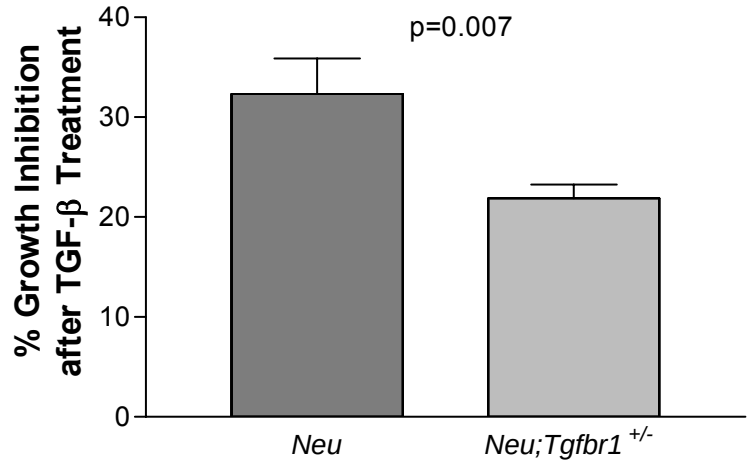


**Figure 3.4 Cells derived from *Neu;Tgfbr1*$^{+/-}$ tumors have a decreased growth inhibitory response after TGF-β treatment compared to cells derived from *Neu* tumors**

Primary cell cultures were made from tumors and TGF-β-mediated growth inhibition was assessed by $^{3}$H-thymidine incorporation. *Neu* n=6, and *Neu;Tgfbr1*$^{+/-}$ n=9. P=0.007, student t-test.

**$Tgfbr1^{+/-}$ may enhance metastasis**

TGF-β enhances the ability for tumor cells to metastasize [Jakowlew, 2006]. To determine if $Tgfbr1^{+/-}$ altered the ability for metastasis to occur in the *Neu* mouse model, visible lung surface metastases were quantitated based on inspection of the lungs under a microscope. Seventy percent of *Neu;*$Tgfbr1^{+/-}$ mice (14 out of 20) developed surface lung metastases and 36.4% of *Neu* mice (8 out of 22) developed visible surface lung metastases. This difference was almost significant (P=0.061) (Figure 3.5.A). Although *Neu;*$Tgfbr1^{+/-}$ mice had a higher incidence of visible metastases, there was no difference in the number of visible lesions per mouse between the two groups. The average number of lesions per mouse for *Neu* is 7.6 ± 2.49, and for *Neu;*$Tgfbr1^{+/-}$ is 3.8 ± 0.95; P=0.102 (Figure 3.5.B).

A critical step before cell seeding in the lungs is the ability for tumor cells to escape from the tumor and enter the blood stream [Wyckoff et al., 2000]. The presence of circulating tumor cells was assessed by collecting the blood by heart puncture, plating the serum and buffy coat laysers in tissue culture plates and assessing colony formation after 7 days in culture. Four out of 15 *Neu* blood samples (26.7%) formed colonies in plates, while 10 out of 18 *Neu;*$Tgfbr1^{+/-}$ blood samples (55.6%) formed colonies. This difference was also non-significant (P=0.187), possibly due to an inadequate number of mice (Figure 3.5.C).

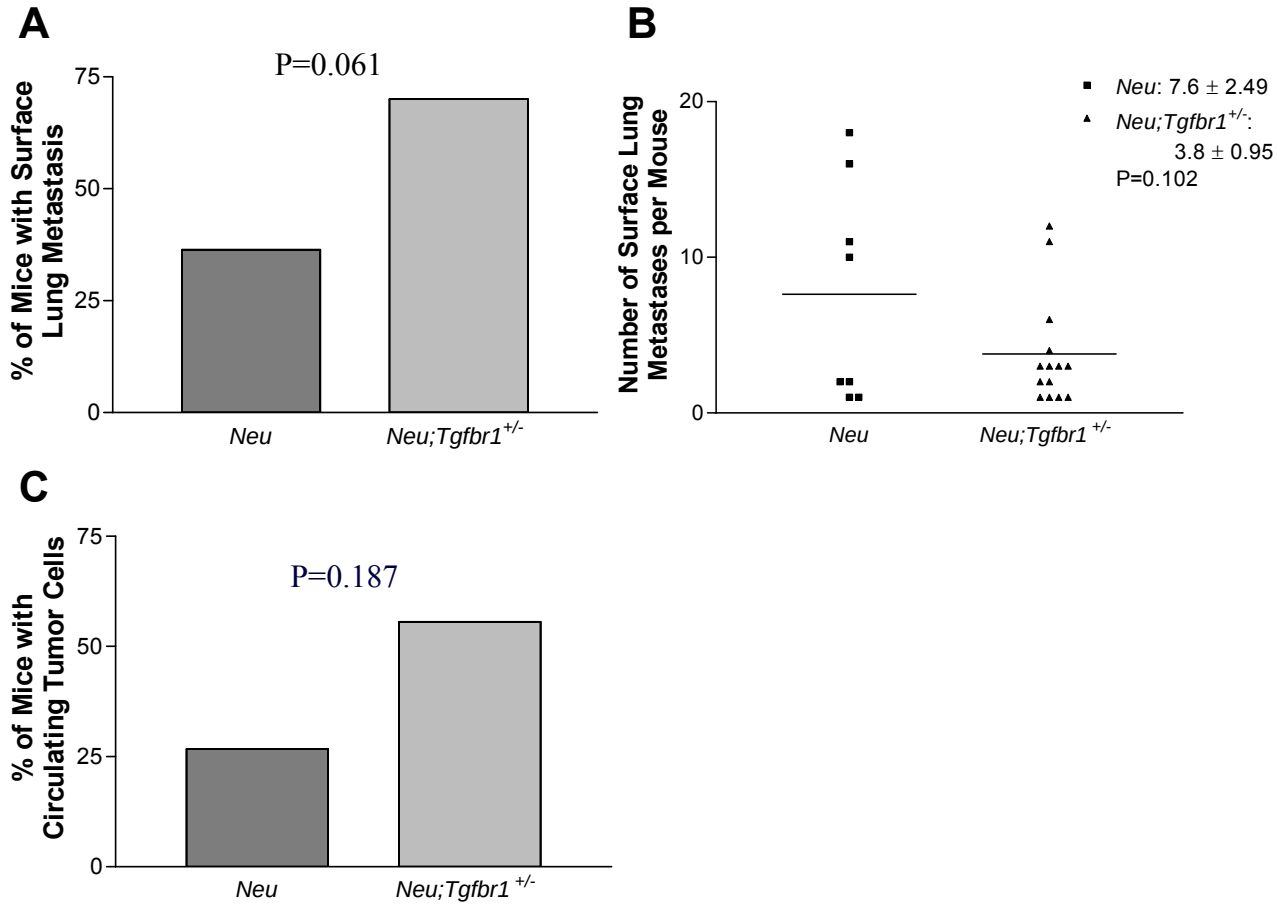


**Figure 3.5 *Neu;Tgfbr1*$^{+/-}$ may enhance lung metastasis in *Neu* mice**

A. 70% of *Neu;Tgfbr1*$^{+/-}$ mice (14/20) develop surface lung metastases. 36.4% of *Neu* mice develop surface lung metastases (8/22). P=0.061

B. The differences between number of surface lung metastases per mouse does not significantly change between *Neu* and *Neu;Tgfbr1*$^{+/-}$ mice (P=0.102).

C. Blood was collected from mice and the serum and buffy coat layers were plated in medium. After 7 days, the plates were stained with H&E and colonies counted to determine the number of circulating tumor cells. 26.7% of *Neu* mice have circulating tumor cells (4/15) and 55.6% of *Neu;Tgfbr1*$^{+/-}$ mice (10/18) have circulating tumor cells. P=0.187.

**TGF-β signaling is decreased in *Neu;Tgfbr1*$^{+/-}$ mice**

i next looked at the effect of *Tgfbr1*$^{+/-}$ on TGF-β signaling. Decreased expression of *Tgfbr1* in tumors was confirmed by western blotting (Figure 3.6.A). To determine the effect of *Tgfbr1* haploinsufficiency on TGF-β signaling in tumors, pSmad2 and pSmad3 levels were assessed. Because the growth inhibitory effects of TGF-β signaling pathway are predominantly mediated by the Smads, pSmad2 and pSmad3 can be used as surrogate markers for TGF-β signaling. Western blotting showed that phosphorylated Smad2 decreased in *Neu;Tgfbr1*$^{+/-}$ tumors compared to the *Neu*$^{+/-}$ tumors, however the levels of normal Smad2 were also decreased, indicating that lack of pSmad2 could be due to a decrease in the amount of available Smad2 (Figure 3.6.B). It is possible that a decrease in the receptor activation or signaling may play a role in negative feedback of the signaling molecules such as the Smads, however this hypothesis would need to be tested further.

The level of pSmad2 and pSmad3 were then assessed by immunohistochemistry, and the results were quantitated and expressed as positive indexes. The positive index was calculated by finding the ratio of all positively stained cells in a given field compared to the total number of cells in the field for five random fields from each tumor sample. The pSmad2 positive index for *Neu* tumors was 0.3044 ± 0.026 and for *Neu;Tgfbr1*$^{+/-}$ tumors was 0.1734 ± 0.025. This corresponds to 43% decreased activation of Smad2 in *Neu;Tgfbr1*$^{+/-}$ tumors compared to *Neu* tumors ($p=0.001$) (Figure 3.7.A). The pSmad3 index was 0.2841 ± 0.042 and 0.1220 ± 0.024 for *Neu* and *Neu;Tgfbr1*$^{+/-}$ tumors, respectively, corresponding to a 57% decrease in pSmad3 ($p=0.0046$) (Figure 3.7.B). This provides strong evidence that *Tgfbr1* haploinsufficiency results in decreased TGF-β signaling in Neu-induced tumors.

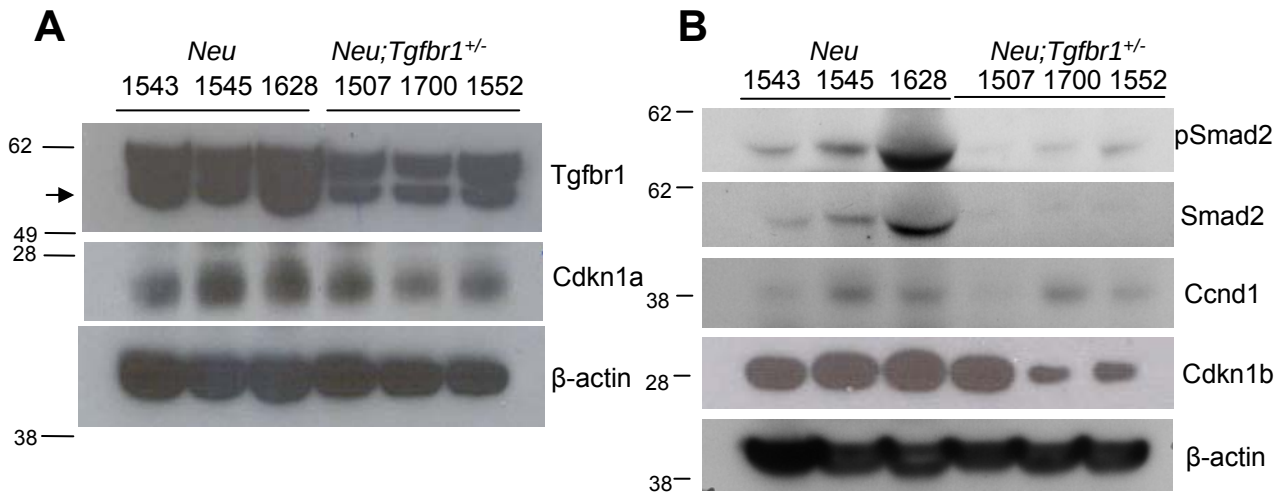


**Figure 3.6 Tumors from *Neu;Tgfbr1*$^{+/-}$ mice have lower TGF-β signaling than tumors derived from *Neu* mice**

A. Western blotting for Tgfbr1 shows decreased Tgfbr1 expression in *Neu;Tgfbr1*$^{+/-}$ mice, verifying haploinsufficiency. Cdkn1a (p27) is decreased in 2 out of the 3 *Neu;Tgfbr1*$^{+/-}$ mice. Numbers indicate mouse ID number.

B. Western blotting for TGF-β signaling components. pSmad2, Smad2, Ccnd1, and Cdkn1b are all decreased in *Neu;Tgfbr1*$^{+/-}$ mice.

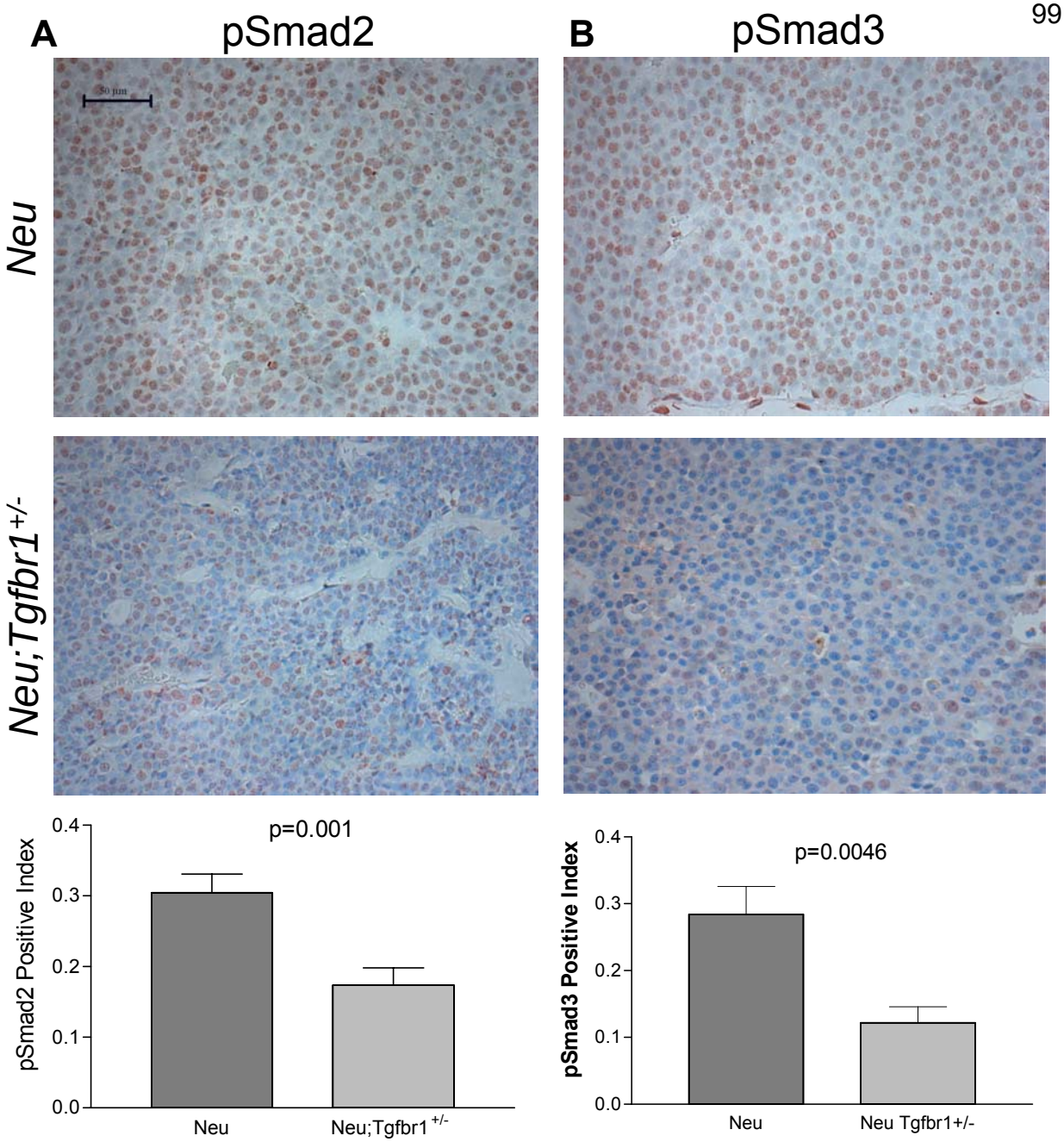


**Figure 3.7 TGF-β signaling is reduced in *Neu;Tgfbr1*$^{+/-}$ mice.**

IHC for pSmad2 (A, left panels) and pSmad3 (B, right panels). Positively stained cells were counted from five random fields from 4-5 mice. Figures are representative of all slides.

A. pSmad2 n=25 and 20 for *Neu* and *Neu;Tgfbr1*$^{+/-}$ mice, respectively. P=0.001, student t-test.

B. pSmad3, n=20 and 15 for *Neu* and *Neu;Tgfbr1*$^{+/-}$ mice, respectively. P=0.0046, student t-test.

*Neu;Tgfbr1*$^{+/-}$ tumors have lower amounts of Cdkn1b ($p27^{Kip1}$) and Cdkn1a ($p21^{Cip1}$) than the *Neu* induced tumors (Figure 3.6). Both proteins are Cdk inhibitors that are upregulated in response to TGF-β. Although these are from tumor lysates and not from cells treated with TGF-β, decreased receptor expression would lead to decreased autocrine signaling within the tumors, and lead to lower levels of TGF-β-inducible gene expression.

Western blotting also showed that Ccnd1 (Cyclin D1) expression was lower in *Neu;Tgfbr1*$^{+/-}$ tumors versus *Neu* tumors (Figure 3.6.B). These results were confirmed by IHC. Ccnd1 expression was 47.2% lower in *Neu;Tgfbr1*$^{+/-}$ tumors than *Neu* tumors (positive indexes 0.0412 ± .0006 and 0.078 ± 0.011, respectively; P=0.0099) (Figure 3.8).

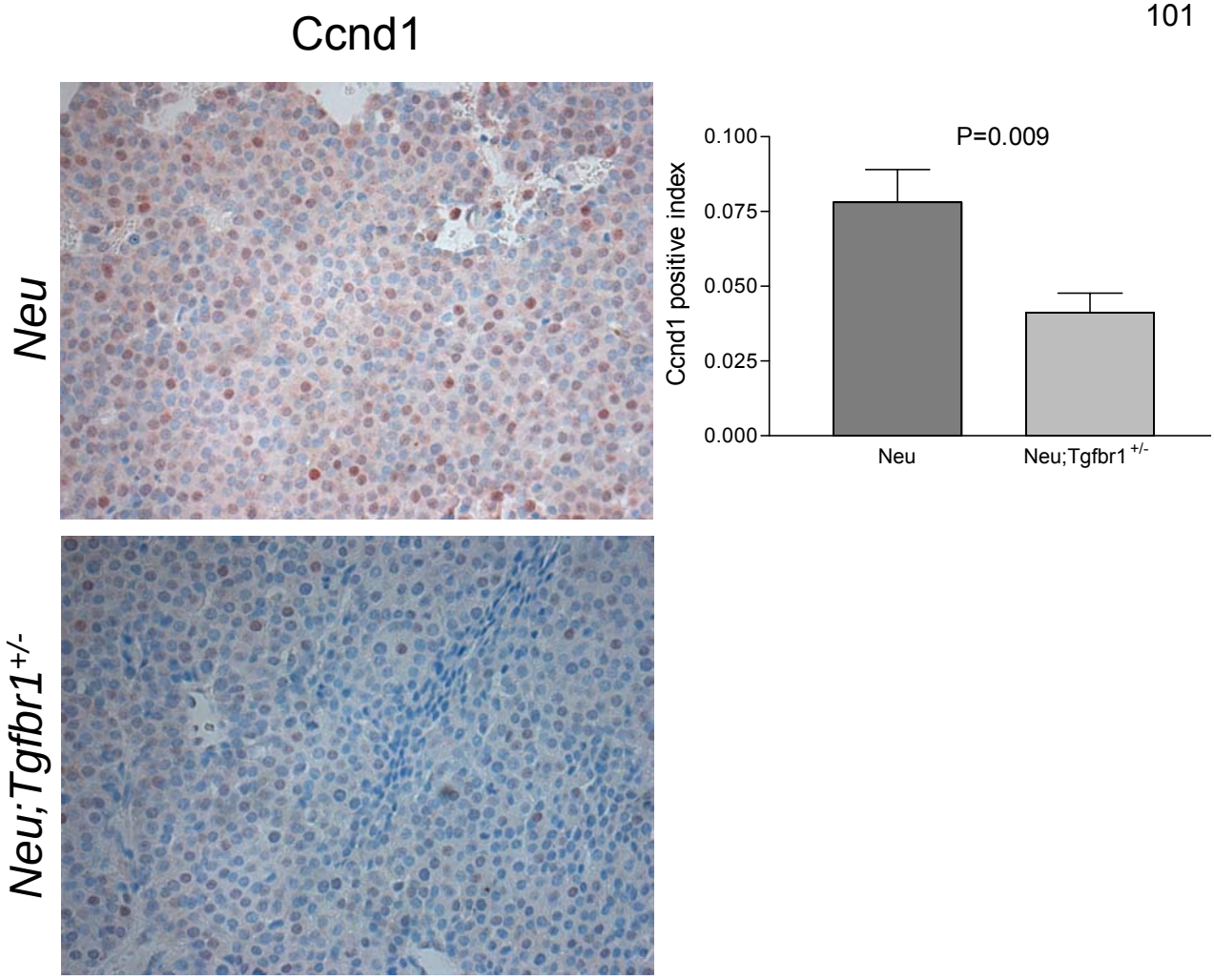


**Figure 3.8 Ccnd1 levels are decreased in *Neu;Tgfbr1*$^{+/-}$ tumors**

IHC on tumors from *Neu* and *Neu;Tgfbr1*$^{+/-}$ indicate that there is a 42.7% reduction in Ccnd1 positive cells in *Neu;Tgfbr1*$^{+/-}$ tumors verses *Neu* tumors. The positive indexes for *Neu* tumors is 0.078 ± 0.011 and for *Neu;Tgfbr1*$^{+/-}$ tumors is 0.0412 ± .0006; P=0.0099, student t-test.

**DISCUSSION**

Our lab has developed a novel mouse model of *Tgfbr1*. We then bred the *Tgfbr1*$^{+/-}$ FVB mouse to the widely used *MMTV-Neu* proto-oncogenic mouse strain to determine the effect of *Tgfbr1* haploinsufficiency on breast cancer development. Ductal elongation in the mammary gland did not appear to be affected, however there was increased ductal branching in the *Neu;Tgfbr1*$^{+/-}$ mice compared to the *Neu* mice at 10, 12, and 40 weeks of age. This is consistent with the findings that TGF-β is lowest at points of ductal branching [Nelson et al., 2006]. It is likely that this increased ductal branching is due to increased proliferation in the mammary gland, and this increase in proliferation is what stimulated a shorter latency for tumor development in the *MMTV-Neu* model. We found a significant decrease in tumor latency from 220 days to 171 days, supporting the claim that TGF-β is a tumor suppressor. Although there was a significantly shortened latency, there was no difference in tumor burden as assessed both by the number of tumors per mouse and by total volume of the tumors at 80 days after tumor initiation. This is consistent with current findings that mutations that either activate or delete the TGF-β receptors does not appear to alter tumor burden in the *c-Neu* strain [Muraoka-Cook et al., 2006;Forrester et al., 2005]. There may be differences in growth, however, in the early stages of tumor development after initiation. The data needs to be reanalyzed to assess rate of tumor growth between the two groups of mice.

There is an ongoing controversy in the field regarding the effect of TGF-β on metastases in different breast cancer mouse models. Several studies in mice show that lung metastases are increased when TGF-β is activated in the *Neu* or PyVmT model [Muraoka-Cook et al., 2004;Muraoka et al., 2003]. Additionally, when Tgfbr1 is constitutively activated in mammary glands, lung metastasis is enhanced [Muraoka-Cook et al., 2006;Siegel et al., 2003]. The model

whereby *Tgfbr2* is conditionally deleted in the mammary epithelial cells of PyVmT mice shows high similarities to our findings, that tumor latency is decreased but without significant changes in tumor burden [Forrester et al., 2005]. Forrester et al. also demonstrated that pulmonary metastasis is increased in *Tgfbr2*$^{-/-}$ mice, creating a conundrum about whether TGF-β signaling is required for lung metastasis to occur, and by what mechanism [Forrester et al., 2005]. Our data supports Forrester's findings that decreased receptor activation increases metastasis. Therefore it seems plausible that the ligand and receptor may have different effects on tumor cells' ability to metastasize.

Yang and colleagues report that Gr-1+CD11b+ myeloid cells are recruited to tumors where *Tgfbr2* is deleted. Infiltrating Gr-1+CD11b+ cells increased TGF-β production in the *Tgfbr2*$^{-/-}$ tumors which may have an effect on how the tumor cells evade detection from the host immune system. Gr-1+CD11b+ cells also promote tumor invasion and metastasis by increasing MMP production [Yang et al., 2008]. Additionally, 3 pro-inflammatory cytokines, Cxcl1, Cxcl5, and Ptgs2 (formally known as Cox2), which are downregulated in response to TGF-β in tumors from PyVmT control mice, are significantly upregulated in tumors from *PyVmT;Tgfbr2*$^{-/-}$ mice [Bierie et al., 2008]. These cytokines are involved in recruiting F4/80$^{+}$ bone marrow-derived inflammatory cells to the invading tumor front as well, indicating that deletion of *Tgfbr2* causes a pro-inflammatory response that recruits cells that promote tumor metastasis [Condeelis and Pollard, 2006]. It is not known whether deletion of *Tgfbr1* would have the same effect of recruiting myeloid cells to the tumor microenvironment.

*Neu;Tgfbr1*$^{+/-}$ tumors have decreased expression of Cdkn1b, a cyclin dependent kinase inhibitor, which is consistent with both Neu signaling and decreased TGF-β signaling, each of which leads to increased cell growth. Ccnd1 expression is also lower in *Neu;Tgfbr1*$^{+/-}$ tumors,

consistent with a previous *PyVmT;Tgfbr2*$^{-/-}$ breast cancer mouse model [Bierie et al., 2008]. Ccnd1 is often amplified or overexpressed in mammary tumors [Buckley et al., 1993;Gillett et al., 1994]. Neu expression also leads to upregulation of Ccnd1 through ERK and PI3K/AKT signaling [Lenferink et al., 2001]. CCND1 plays an important role in breast cancer development and the implication for the decreased expression of Ccnd1 that we see in our *Neu;Tgfbr1*$^{+/-}$ is uncertain at this time. Further investigation into the expression of Cdk4, Cdk6, and downstream targets of Ccnd1, such as pRB and E2F, may shed more light on the mechanism underlying Ccnd1 downregulation observed in *Neu;Tgfbr1*$^{+/-}$ mice.

We recently discovered that *TGFBR1**6A is present in 50% of colorectal cancer patients who have ASE of TGFBR1, and although *TGFBR1**6A is not a cause of ASE, *6A and ASE are in linkage disequilibrium [Valle et al., 2008]. We speculate that if this phenomenon also occurs in breast cancer patients, then the data presented in this chapter provide a possible rationale for why *TGFBR1**6A carriers have an increased risk for breast cancer when compared to non-carriers. If *6A carriers are more likely to have decreased TGF-β receptor signaling (from TGFBR1 ASE), then the epithelial cells will be primed to not only have less TGF-β mediated growth inhibitory responses from lack of receptor signaling, but also to have TGF-β mediated growth stimulatory properties of *6A (Figure 3.9). The combination of decreased TGF-β signaling resulting from the deletion of one TGF-β receptor and expression of the *Neu* proto-oncogene may be the additional "hit" that the mammary epithelial cells needs to initiate tumor formation at a faster pace than it normally would. Additionally tumors that harbor both ASE of TGFBR1 and *6A may be more aggressive due to the ability for ASE to stimulate more lung metastasis (as seen in the *Neu;Tgfbr1*$^{+/-}$ mouse model) and the ability for *6A to confer higher migratory and invasive properties to the tumor cells (Figure 3.10).

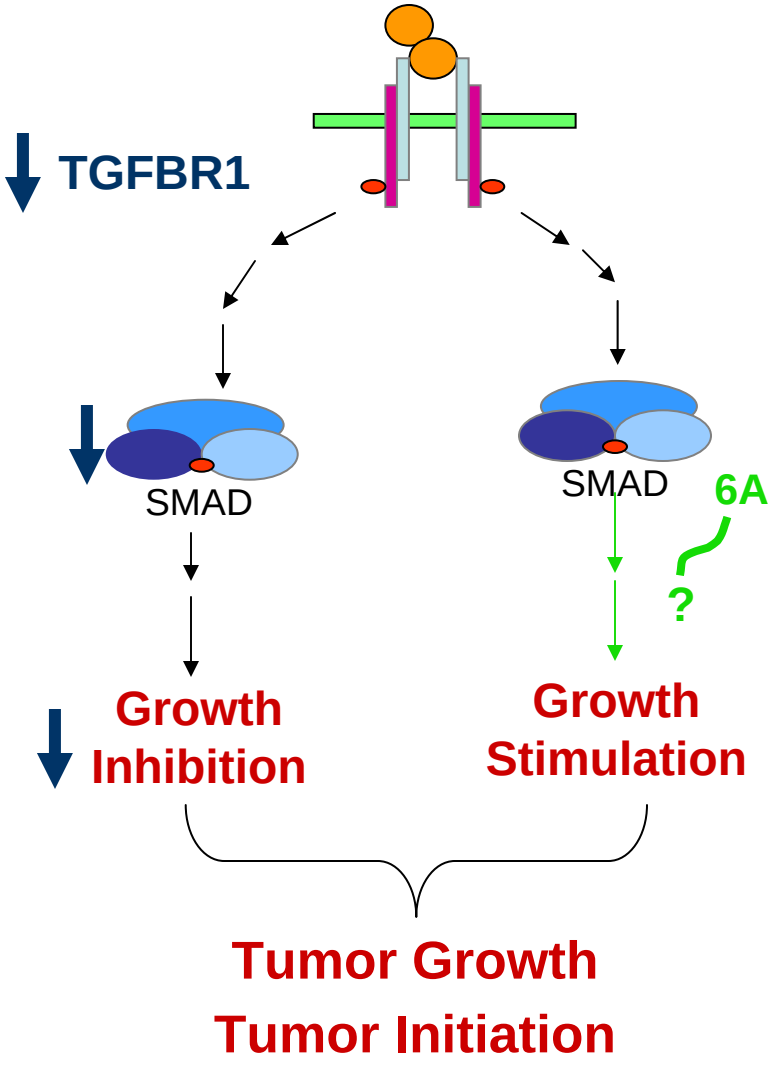


**Figure 3.9 Decreased TGFBR1 may cooperate with *TGFBR1**6A to enhance tumor formation**

Decreased TGFBR1 protein levels leads to decreased SMAD signaling and decreased TGF-β-mediated growth inhibition. Additionally, I have shown that TGFBR1*6A expression results in TGF-β-mediated growth stimulation of breast cancer cells. If patients harbor both receptor variants, then cells will have two mechanisms for providing a growth advantage to tumors.

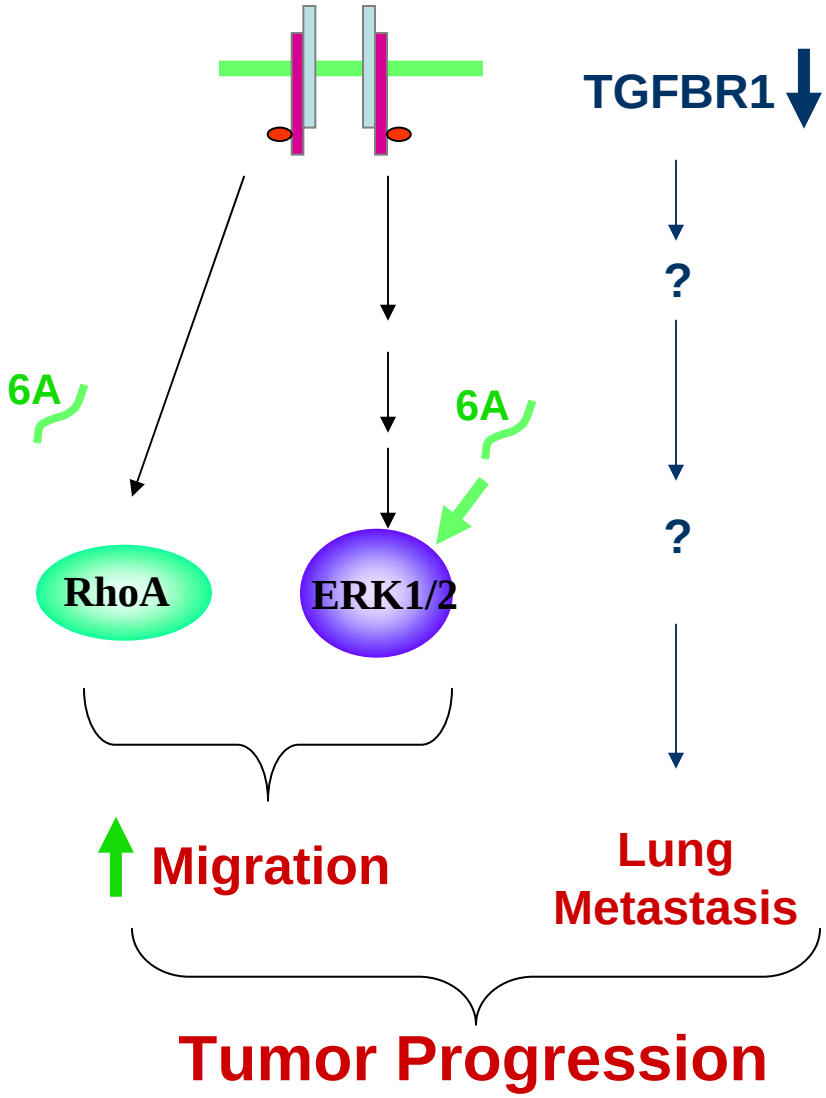


**Figure 3.10 Decreased TGFBR1 may cooperate with *TGFBR1**6A to enhance tumor progression**

TGFBR1*6A expression enhances migration and invasion of breast cancer cells. Additionally, the decreased Tgfbr1 levels in the *MMTV-Neu;Tgfbr1*$^{+/-}$ mouse model enhances lung metastasis, through an unknown mechanism. If this model replicates ASE of TGFBR1 in humans, then combining the effects of decreased TGFBR1 signaling with *6A expression, would result in rapid or aggressive tumor progression.

# CHAPTER 4

# DISCUSSION

TGF-β is a cytokine with diverse functions and complex effects. The main function of TGF-β is to induce growth inhibition in normal epithelial cells [Roberts et al., 1985]. In normal epithelial cells, TGF-β also functions to maintain the tissue architecture, induce apoptosis, guard the immune system, and protect the genomic stability [Massague, 1998]. Often, cancer cells gain resistance to TGF-β signaling, and acquire gain-of-function effects in the tumor cell. Such roles include inducing EMT, angiogenesis, increasing migratory properties of cells, escaping immunosurveillance system, and inducing metastases in late stage cancer development [Derynck et al., 2001]. Tumors often secrete excess TGF-β into the microenvironment spurring its own positive feedback loop on tumor growth [Derynck et al., 1987].

Mutations in *TGFBR2* and SMADs are rare in breast cancer, although evidence of decreased expression or mutations in breast tumors have been found [Xie et al., 2002;Lucke et al., 2001]. Two polymorphisms in *TGFBR1* have been identified that are associated with increased risk of breast cancer: *Int7G24A* and *TGFBR1**6A. The *Int7G24A* polymorphism may increase the risk for invasive breast cancer (OR 2.61, 95% CI 1.65-4.11) [Chen et al., 2006]. However, a study in Swedish familial and sporadic breast cancer did not reveal an association between *Int7G24A* and breast cancer risk or stage of tumor [Song et al., 2007]. Additional studies need to be done on this polymorphism to determine the involvement in breast cancer risk and development. Conversely, 14 studies have been completed that investigate the risk of breast cancer and *TGFBR1**6A. Completed analyses of these 14 cases reveal that *TGFBR1**6A increases the risk of breast cancer 15% in carriers compared to women who have 2 normal

alleles. Homozygosity increases the risk of breast cancer 40% (see Table 1.1.A and 1.1.B in Intro).

I have demonstrated that *6A acts in breast cancer cells to stimulate growth in the presence of TGF-β and also to enhance migration and invasion, independently of TGF-β. These effects of *6A are hypothesized to be mediated through a secondary effect from the signal sequence, and not through alteration in receptor signaling, as the mature receptors are identical [Pasche et al., 2005]. Kinase-deficient *6A cells also displayed growth stimulation after TGF-β and decreased FN1 and ARHGAP5 gene expression when compared to the *9A expressing cells, supporting the notion that *6A effects are receptor-independent. Further support for non-receptor mediated effects comes from identical canonical SMAD signaling in *6A and *9A MCF-7 cells after TGF-β treatment.

The fact that serum could stimulate more migration and invasion than TGF-β alone indicates that other serum components may synergize with cellular changes due to *6A and are responsible for the increase in migration. Hepatocyte growth factor (HGF), CXCL12, and EGF also induce MCF-7 cell migration [Elliott et al., 2002;Mosadegh et al., 2008;Meng et al., 2005]. EGF induction of cell motility is through the RAS/RAF/MEK/ERK signaling pathway [Garcia et al., 2006]. HGF, on the other hand, induces migration in MDA-MB-231 breast cancer cells and in human melanoma cell lines through PI3K/AKT signaling [Lee et al., 2008;Ye et al., 2008]. *6A is able to induce ERK activation independently of TGF-β treatment, which leads to the notion that EGF may be one of the possible serum components that can enhance migration in *6A cells. Treating the MCF-7*6A cells with an EGFR inhibitor (for example: PD168393, AG1478, Gefitinib, or Erlotinib) in the transwell chamber may indicate whether there is a link between *6A, EGFR signaling, and cell migration.

Our lab, in collaboration with Albert de la Chapelle, have shown that allele-specific expression (ASE) of *TGFBR1* occurs in 10-20% of sporadic colorectal cancer (CRC) patients and in 1-2% of the general population [Valle et al., 2008]. ASE in *TGFBR1* confers an increased risk for CRC (OR 8.7; 95% CI 2.6-29.1). ASE is dominantly inherited and segregates in families, and *TGFBR1* ASE results in reduced expression of TGFBR1 and SMAD signaling. Additionally, about 50% of patients with *TGFBR1* ASE harbor the *6A mutation. Although *6A is not a cause for *TGFBR1* ASE, *6A is likely in linkage disequilibrium with one of the putative mutations that cause ASE. Our lab is currently investigating whether *TGFBR1* ASE is also found in breast cancer.

We have developed a *Tgfbr1* haploinsufficient model that could be used to determine the impact of decreased TGF-β signaling at the receptor level on tumor development. The knockout construct was generated by inserting a Neomycin cassette into the signal sequence domain on exon 1, thus inactivating TGFBR1 at the place where the *6A mutation is located. I bred these FVB *Tgfbr1*$^{+/-}$ mice to the FVB *MMTV-c-Neu* proto-oncogene strain to determine how decreased TGF-β signaling would affect breast cancer development.

These *Neu;Tgfbr1*$^{+/-}$ mice developed multiple tumors with a shorter latency then the *Neu* induced tumors, supporting the role for *Tgfbr1* being a tumor suppresser. Surprisingly, our results also suggest that *Tgfbr1* haploinsufficiency may increase lung metastasis in the *Neu* model, which is contrary to the current dogma that increased TGF-β activity accelerates metastasis.

TGF-β signaling is implicated in breast cancer metastasis. It has been shown that in MCF-10A normal breast epithelial cells, expression of ERBB2 cooperates with TGF-β signaling to induce motility through RAC even though MCF-10A cells retain their TGF-β mediated

growth inhibitory properties. TGF-β is unable to stimulate migration in MCF-10A cells without ERBB2 expression [Ueda et al., 2004;Seton-Rogers et al., 2004].

As discussed previously in the intro and in Chapter 3, several mouse models exist to support the claim that TGF-β induces metastasis. In the MMTV-*c-Neu* (proto-oncogene) mouse model, constitutive activity of either Tgfb1 or Tgfbr1 in the mammary gland increased lung metastasis [Muraoka et al., 2003;Muraoka-Cook et al., 2006]. Tgfb1 activation was also shown to enhance lung metastases over 10 fold in *MMTV-PyVmT* induced tumors [Muraoka-Cook et al., 2004]. In the activated *Neu* model, active Tgfbr1 increases extravascular lung metastasis when allowed to signal via the Shc adaptor protein on Neu [Siegel et al., 2003]. Likewise, decreased TGF-β signaling from deleting *Tgfbr2* in the activated *Neu* mice reduced the number of extravascular lung metastasis by half [Siegel et al., 2003]. Each of these mouse models supports the conclusions that TGF-β signaling enhances lung metastasis.

However, Moses and colleagues developed a PyVmT mouse model that had a conditional null *Tgfbr2* produced by mating to MMTV-*Cre* mice. These mice also had an increase in lung metastasis, which is counter-intuitive to the above mentioned mouse models [Forrester et al., 2005;Bierie et al., 2008]. These mice matched the phenotype that we saw in our *Neu;Tgfbr1*$^{+/-}$ mice.

One of TGF-β's major tasks is to maintain the immune system homeostasis. Malignant tumor cells secrete TGF-β that acts on non-malignant cells in the tumor as well as distal cells in the host to suppress the tumor immune response and allow the tumor to continue to grow, invade, and metastasize (reviewed in [Teicher, 2007;Derynck et al., 2001]). It is believed that abrogating *Tgfbr2* in mammary carcinomas promote metastasis in the PyVmT breast cancer mouse model by increasing Cxcl5 production, which then recruits Gr-1+CD11b+ myeloid cells to the tumor

microenvironment by binding to Cxcr2. The Gr-1+CD11b+ cells also express Pf4 (previously named Cxcl4) which interacts with Cxcl12 (previously known as Sdf-1) in the tumor microenvironment [Yang et al., 2008]. Gr-1+CD11b+ cells infiltrate tumors and produce MMP9 thereby promoting angiogenesis [Yang et al., 2004]. Gr-1+CD11b+ cells produce high levels of TGF-β. Previous reports demonstrate that *Tgfbr2* deletion results in increased TGF-β present in the stroma [Lu et al., 2006], and Yang identified the Gr-1+CD11b+ cells that were recruited to the stroma as the source of increased TGF-β production [Yang et al., 2008]. Additionally tumors from *PyVmT;Tgfbr2*$^{-/-}$ mice had increased expression of Cxcl1 and Ptgs2, which recruit F4/80$^{+}$ bone marrow-derived cells to infiltrate the tumor, thus promoting metastasis [Bierie et al., 2008]. Cxcl5 production was also stimulated by *Tgfbr2* loss in *MMTV-c-neu* tumor cells as well, indicating that this increase in metastasis by *Tgfbr2* deletion may not be specific to tumors induced by PyVmT [Yang et al., 2008].

It has been proposed that tumors are heterogeneous in nature, meaning that different cell populations have different fates. Some cells are destined to remain in the primary tumor to proliferate and continue growing, while others are meant to metastasize and go to specific distal sites [Shipitsin et al., 2007]. The ability for the tumor cell to escape from the tumor and enter the blood stream is not enough for metastasis to occur. The metastasizing tumor cell must be able to select and adhere to the new site and grow in a new microenvironment [Fidler and Kripke, 1977]. Breast cancer preferentially metastasizes to the lungs and bone, but it also metastasizes to the liver and brain [Minn et al., 2005b]. Specific gene signatures were identified that can predict where a tumor cell will metastasize to. Genes that regulate breast cancer metastasis to the bone include *IL11* (Interleukin 11), *PF4*, *CTGF*, and *MMP1* [Kang et al., 2003;Minn et al., 2005b].

Moreover, SMAD4 contributes to osteolytic bone metastasis and is essential for IL11 induction [Kang et al., 2005].

There is a separate gene signature that has been identified for tumor cells metastasizing to the lungs. Genes that are upregulated include: *EREG* (Epiregulin, a broad-specificity ligand for ERBB family of receptors), *CXCL1*, *MMP1*, *ID1* (transcriptional inhibitor of cell differentiation and senescence), and *PTGS2* [Minn et al., 2005a]. An elegant study by Padua and colleagues recently showed that TGF-β activity, as measured by a TGF-β response signature (TBRS), in primary breast tumors is associated with an increased occurrence of lung, but not bone, metastasis in ER- tumors. When this TBRS in ER- tumors was compared to the lung metastasis signature (LMS) described in Minn et al. [Minn et al., 2005a], tumors that were positive for both TBRS and LMS had a high risk of pulmonary relapse. Two genes were present in both the TBRS and LMS: *ANGPTL4* (Angiopoietin like 4), and *NEDD9*, which encodes an adaptor protein implicated in focal contact formation and cell motility [Oike et al., 2004;Kim et al., 2006]. However, only *ANGPTL4* was upregulated consistently in a panel of tumors that were TBRS+. Although *ANGPTL4* was upregulated in all 13 malignant pleural cell samples tested regardless of ER, PR, or ERBB2 status, upregulation was much higher in ER- cells than ER+ cells. ANGPTL4 primes TGF-β treated cells for seeding in the lung, and knocking down *ANGPTL4* by shRNA reduces the amount of lung metastasis after injecting TGF-β-pretreated metastatic cells into mice [Padua et al., 2008].

MMTV-*Neu* mice have been shown to be ER+ [Sacco et al., 1998]. Although TGF-β induced increase in lung metastasis requires ANGPTL4 for cell seeding, upregulation of ANGPTL4 was much higher in ER- cells [Padua et al., 2008]. Thus, difference in ER status of the tumors may provide another possible mechanism for an increase in lung metastasis that we

saw that is not dependant on TGF-β activity. We can't rule out the idea that TGF-β production is not upregulated in these *Neu;Tgfbr1*$^{+/-}$ mice. Loss of *Tgfbr2* in intestinal epithelial cells in an *Apc* background enhanced intestinal tumor formation and results in increased TGF-β1 secretion in the tumor, and increased TGF-β1 secretion from the tumor-derived cell lines [Munoz et al., 2006]. Furthermore, when MCF-7 and MDA-MB-468 (which are SMAD4 deficient) cells were treated with the TGFBR1 inhibitor, SB431542, TGF-β was still able to induce migration, showing that TGF-β mediated migration is not through the SMAD4 pathway, and can occur without TGFBR1 signaling [Imamichi et al., 2005]. Additionally, TGF-β induced migration was dependent on ERK 1/2, JNK, and RHOA/ROCK signaling, and Imamichi et al. also showed that TGF-β can stimulate the ERK signaling cascade independently of TGFBR1 [Imamichi et al., 2005]. Thus, if TGF-β secretion is activated in the *Neu;Tgfbr1*$^{+/-}$ mice, TGF-β signaling could still occur by circumventing the canonical receptor activation-signaling pathway and activating the ERK/MAPK pathways to enhance tumor cell motility. Increased TGF-β could also stimulate an invasive front on tumors through signaling to the immune system to attract myeloid or bone marrow-derived immune cells.

## Future Directions

The results we obtained from the *Neu;Tgfbr1*$^{+/-}$ mouse model provide a strong rationale for the notion that constitutively decreased TGF-β signaling is associated with increased susceptibility to breast cancer, as we recently demonstrated with *TGFBR1* ASE in CRC risk. The link between *6A and *TGFBR1* ASE needs to be further investigated to see if there is a link between *6A and decreased TGFBR1 expression. I think that the first logical next step would be to assess TGF-β activation and secretion in both the animal model and in a cohort of cells

expressing TGFBR1*6A. TGF-β secretion could be enhanced due to a positive feedback loop initiated from either *6A expression or *Tgfbr1*$^{+/-}$, and excess TGF-β production would correlate with the increase in metastasis in the mouse model. As discussed above, blocking EGFR activation from available EGF in the serum may also prove insightful in the mechanism of how *6A enhances MCF-7 cell migration independently of TGF-β.

In the mouse model a more in-depth look at the mammary gland development needs to be done to see if there is a link between the increased ductal branching that we observe in *Neu;Tgfbr1*$^{+/-}$ mammary glands and proliferation and/or apoptosis. Mammary gland development also needs to be extended to include analysis of pregnancy, lactation, and post-lactation gland morphology. Additionally, the lungs from *Neu* and *Neu;Tgfbr1*$^{+/-}$ mice need to be fully sectioned and stained so that micro-metastasis can be evaluated and the full extent of *Tgfbr1* haploinsufficiency on metastasis can be assessed. The lung metastasis can be further stained with antibodies for Neu, Pcna, and TGF-β signaling markers to determine how the metastatic tumors correlate with the primary tumor. The primary mammary tumors should be further evaluated for growth (Pcna or Ki67) and apoptosis as well as for the presence of infiltrating inflammatory cells. Lysates from tumors can be further used to look at MAPK signaling factors such as ERK, p38, and JNK.

It is important to note that in cells transfected with TGFBR1*6A, the phenotypic observations are due to the presence of the signal sequence, as the mature receptor is identical to the wildtype receptor. The question as to how the *6A signal peptide exerts its functions is still unanswered. Does the signal peptide bind to another molecule in the cell? Is it capable of having signaling abilities of its own? We have discussed the use of a yeast two-hybrid screen to determine if the *6A signal peptide can bind to other proteins in the cell. If *6A can interact

with proteins that are different from those with which *9A interacts, then we could have a better understanding of how functional interactions can alter cell phenotypes. If the peptide binds to another protein, the interaction could cause structural alterations in the proteins that may induce active or inhibitory conformational states. Developing an antibody against *6A and *9A has also been discussed, however because only 3 alanines separate the two forms, it would be difficult to develop a good antibody that could distinguish between the two forms. It is also not possible to tag the signal sequence with a marker (such as myc, HA, Flag, or GST) because the tag would alter the signal sequence cleavage recognition site. Our lab has recently generated TAT-HA constructs containing either the *9A and *6A signal sequence. This TAT-derived system allows for direct transfection of cells with either the *6A or the *9A signal sequence peptides to determine the direct effect of expressing the signal peptide in the cell.

In conclusion, my thesis work has been in two areas that are instrumental to the field of breast cancer. 1) I have provided functional roles for how TGFBR1*6A can influence breast cancer. Previous to my joining the lab all we knew was that epidemiologically, TGFBR1*6A was potentially associated with breast cancer risk. (Back in 2004, the risk factor for breast cancer was increased 34% for *6A carriers and 169% for homozygotes, compared to *9A/*9A individuals [Zhang et al., 2005]). As of 2008, the correlation for *6A and breast cancer risk has changed to 15% increased risk for breast cancer in *6A carriers and 40% increased risk for homozygotes but *6A remains strongly associated with breast cancer risk. Here I show evidence that TGFBR1*6A can alter the TGF-β-mediated growth properties of breast cancer cells as well as enhance the ability for the cells to migrate and invade independently of TGF-β. A plausible mechanism for the ability for *6A to enhance migration was also demonstrated. Two crucial

mediators of migration and invasion, *ARHGAP5* and *FN1*, were downregulated in *6A cells compared to the *9A cells. ERK activation was also enhanced in *6A cells, which could lead to enhanced migratory signaling [Rosman et al., 2008]. 2) I have shown that *Tgfbr1* haploinsufficiency significantly enhanced tumor formation in the *MMTV-Neu* mouse model. Evidence also indicates that metastasis may be enhanced as well in *Neu;Tgfbr1*$^{+/-}$ mice compared to *Neu* mice. Allele-specific expression of *TGFBR1* has been shown to increase the risk of colorectal cancer [Valle et al., 2008], and our lab is currently in the process of determining if *TGFBR1* ASE is found in breast cancer patients. This *Neu;Tgfbr1*$^{+/-}$ mouse model is therefore a novel mouse model to study *TGFBR1* ASE in breast cancer development.

# CHAPTER 5

# METHODS

**Cell lines and conditions**

MCF-7 cells were cultured in RPMI 1640 (Invitrogen Corp., Grand Island, NY) supplemented with 10% heat inactivated FBS (Hyclone, Logan, Utah), 2 mM L-glutamine (Invitrogen Corp., Grand Island, NY), non-essential amino acids, 1,000 units/ml penicillin, 10,000 µg/ml streptomycin, 0.006 mg/ml human recombinant insulin (Sigma, St. Louis, MO), and 0.5 mcg/ml amphotericin B (Biologos Inc., Montgomery, AL).

Starvation media was identical except 0.5 ug/ml BSA was substituted for 10% FBS. MCF-7 cells were stably transfected with pIRES-TGFBR1*6A-HA/FLAG, pIRES-TGFBR1*9A-HA/FLAG, or vector alone and selected for with 0.5 mg/ml G418 [Pasche et al., 2005].

NIH-3T3 cells were cultured in DMEM (Invitrogen Corp., Grand Island, NY) supplemented with 10% heat inactivated FBS, 1,000 units penicillin/streptomycin, amphotericin B, 2 mM L-Glutamine, and 1 mg/ml G418 for pIRES selection.

Phoenix cells (ATCC, Mannassas, VA) were cultured according to ATCC recommendations: DMEM (Invitrogen Corp., Grand Island, NY) supplemented with 10% heat inactivated FBS, 1,000 units penicillin/streptomycin, amphotericin B, 2 mM L-Glutamine.

MCF-10A cells were grown in: DMEM/F12 (Invitrogen, Grand Island, NY), 5% heat inactivated Horse serum (Hyclone, Logan, Utah), penicillin/streptomycin, 20 ng/ml EGF (Invitrogen, Grand Island, NY), 100 ng/ml Cholera toxin (Sigma, St. Louis, MO), 10 µg/ml insulin (Sigma, St. Louis, MO), 0.5 µg/ml hydrocortisone (Sigma, St. Louis, MO), and 0.5 mcg/ml amphotericin B (Biologos Inc., Montgomery, AL).

**Plasmid constructs**

TGFBR1-HA-Flag (TGFBR1*9A-HA-Flag) and TGFBR1*6A-HA-Flag were constructed in the pIRES vector (BD Biosciences, Clontech) as described previously [Pasche et al., 2005]. TGFBR1-HA-Flag and TGFBR1*6A-HA-Flag were excised from pCMV5-TGFBR1-HA-Flag or pCMV5- TGFBR1*6A-HA-Flag, respectively, using EcoRV and BamHI restriction enzymes and inserted into the pBABE vector, linearized with EcoRI and blunt ended with Klenow. pBABE, pBABE-RasV12, were gifts from Dr. Vince Cryns, Northwestern University, Chicago, IL. The pSBE4-lux vector was a gift from Dr. Bert Vogelstein, Johns Hopkins, Baltimore, MD. The 3TP-Lux vector was a gift from Dr. Joan Massagué, Sloan-Kettering, NY.

**Transfections**

pIRES-TGFBR1-HA-Flag, pIRES-TGFBR1*6A-HA-Flag or pIRES-*6AK-HA-Flag were stably transfected into MCF-7 cells as previously described. Stable clones from each *6A and *9A lines were chosen based on similar levels of TGFBR1 and HA expression. We refer to *9A-5, *9A-9, *6A-5, *6A-1 and *6AK15 as *9A-low, *9A-high, *6A-low, *6A-intermediate (*6A-int), and *6AK-intermediate (*6AK-int) [Pasche et al., 2005].

pBABE, pBABE-TGFBR1-HA-Flag, or pBABE-TGFBR1*6A-HA-Flag was transfected into MCF-7 cells using retroviral transfection. The pBABE vectors were transfected into Phoenix cells using FuGENE6 (Roche Applied Science; Indianapolis, IN). Viral-containing media was collected 48 hrs after transfection, filter sterilized, and treated with 4 ng/ml polybrene (Sigma, St Louis, MO). The clones were selected for in 1 μg/ml puromycin. We selected two low expressing and high expressing clones for each experiment. We refer to these cells in the text as pBABE, pBABE*9A-low, pBABE-*9A-high, pBABE*6A-low, and pBABE*6A-high.

NIH-3T3 cells were stably transfected with pIRES-TGFBR1-HA-Flag or pIRES- TGFBR1*6A-HA-Flag using FuGENE6 (Roche Applied Science; Indianapolis, IN) and selected for with 1 mg/ml G418.

pBABE, or pBABE-HRasV$^{12}$ was transiently transfected into the NIH-3T3*9A or *6A stably transfected cells by viral transfection. The pBABE vector was mixed with 125 mM $CaCl_2$ and HBS and transfected into phoenix cells. Two days after transfection, the viral supernatant was collected, filter sterilized, and treated with 4 ng/ml polybrene (Sigma, St Louis, MO). NIH-3T3 cells were incubated with the viral supernatant for 24 hrs then refed with complete media.

MCF-10A cells were transfected with pBABE, pBABE-TGFBR1*9A-Flag/HA, or pBABE-TGFBR1*6A-Flag/HA using retroviral transfection. Briefly, 2x10$^6$ Phoenix cells were plated in flasks, and the following day transfected with 3 μg of the appropriate vector DNA using FuGene6 according to the manufacturer's protocol (Roche). Twenty-four hr after transfection, the media was replaced with fresh media and viral supernatant was collected 48 hr after

transfection. The viral supernatant was spun down for 5 min at 1000 rpm to remove cell debris, diluted 1:8 in MCF-10A growth media, and polybrene (Sigma, St. Louis, MO) was added at a final concentration of 8 μg/ml. MCF-10A cells plated at a density of $5x10^5$ cells were fed with the viral cocktail. Twenty-four hr after viral transfection, media was replaced, and selection media (1 μg/ml puromycin) was added 48 hr after transfection.

**Luciferase Assays**

MCF-7 cells stably transfected with pIRES, pIRES-TGFBR1-HA-Flag, and pIRES-TGFBR1*6A-HA-Flag were plated in triplicate at a density of $1.5x10^5$ cells per well in a 6-well plate (BD Falcon; Bedford, MA) a day before being transfected with either the pSBE4-lux vector [Zawel et al., 1998] or 3TP-lux vector [Carcamo et al., 1995] using FuGENE6 (Roche Applied Science; Indianapolis, IN) according to the manufacturer's recommendations. Twenty-four hr after transfection, cells were treated with 100 pM TGF-β1 (R&D Systems, Minneapolis, Minn) for 18 hr, then harvested for the luciferase assay using the protocol from the Luciferase Assay Systems kit (Promega; Madison, WI) using the Flash 'n Glow system (Berthold Technologies, Bad Wildbad, Germany).

**Migration assay**

MCF-7 cells were grown to 80% confluence then serum starved overnight prior to setting up the experiment. Cells were washed twice in Dulbecco's PBS, and harvested from the plate using 0.5M EDTA, pH 6.8. The cells were collected and resuspended in starvation media. We used twenty-four well transwell chambers (BD BioCoat Control Inserts from BD Biosciences, Bedford, MA) with 8.0 μm pore size polycarbonate membrane for this experiment. The cells

were plated at a density of $5x10^4$ cells/well in 0.5 ml in the upper well, which was placed into a lower well containing one of the following conditions: complete media + 5ng/ml TGF-β, or complete growth media (10% FBS). After 24 hr at 37°C, 5% $CO_2$ incubator for 24 hr, the experiment was stopped by wiping the cells from the well with a cotton swab and fixed and stained using the Diff-Quik kit (Dade-Behring, Newark, DE). Migration was quantitated by counting 12 fields at a magnification of 400x. Each experiment was repeated in triplicate and the results averaged. Statistical analysis was performed using the student t-test.

**Invasion Assay**

The invasion assay was identical to the above migration assay except that inserts were coated with 100 µl Matrigel (BD Bioscience, Bedford, MA), diluted to 1mg/ml in DMEM. The experiment was stopped after 72 hr using the same method as above.

**Scratch Wound**

MCF-7 cells were plated in 12 well plates. At 100% confluence, cells were scratched with a 10 µl pipet tip in the shape of a cross. The cells were fed with complete media (10% FBS) or 1 µg/ml anti-TGF-β (clone: 1D11, cat #MAB1835, R&D Systems, Minneapolis, MN) in complete media. Pictures were taken with a Nikon camera fitted to a microscope eyepiece either above the intersection or to the left of the intersection. The gaps were measured and calculated into percent wound closure. Results shown are representative from 4 experiments.

MCF-10A cells, MCF-10A pBABE vector only, pBABE-TGFBR1*9A or pBABE-TGFBR1*6A were plated in 12 well plates. The cells were grown to 100% confluence, scratched using a 10 µl

pipet tip in the shape of a cross, and fed with the following: normal growth media (5% serum), growth media + 2.5 ng/ml TGFB1 (R&D Systems), or 1 μg/ml anti-TGFB (R&D Systems). Pictures were taken with a Nikon camera fitted to a microscope eyepiece either above the intersection. The gaps were measured and calculated into percent wound closure. Results are the average of 2 experiments.

**Growth Inhibition Assay**

NIH 3T3 or MCF-7 cells were plated at a density of $1x10^5$ cells in a 6 well plate, and allowed to adhere overnight. Cells were treated with 100 pM TGF-β1 for 18 hr, before addition of $^3$H-thymidine (Amersham, Piscataway, NJ) for an additional 4 hrs. After the 4 hr incubation, cells were washed with ice-cold PBS, fixed for 1 hr with 95% methanol, re-washed in PBS, and lysed with 0.2 N NaOH. $^3$H-thymidine incorporation was measured using the Beckman Coulter scintillation counter (Fullerton, CA).

**Oncogenic Assessment**

To test foci formation, NIH-3T3 cells and stable clones were plated in 60 mm plates at a density of $1x10^5$ cells/plate. The media was replaced every 2-3 days and grown for 21 days. The cells were fixed with methanol and stained with methylene blue. To determine whether *6A enhances H-Ras$V^{12}$ transformation of NIH-3T3 cells, a transient transfection using pBABE-H-Ras$V^{12}$ was performed 24 hr after plating the NIH-3T3 clones.

To assay colony formation, NIH-3T3 cells and stable clones were plated at a density of $1x10^5$ cells in 0.3% agar, and laid overtop 0.6% agar. The plates were incubated at 37ºC in 5% $CO_2$, and monitored every 2-3 days for colony formation up to 28 days.

**Gene Array**

MCF-7-TGFBR1-HA-Flag and MCF-7-TGFBR1*6A-HA-Flag cells expressing similar amounts of transgene were grown in complete media. RNA was collected from each cell line in triplicate using the RNeasy protect Mini kit (Qiagen, Valencia, CA). RNA quality was confirmed using the 2100 Bioanalyzer from Agilent (Santa Clara, CA). The microarray was performed using the Affymetrix GeneChip® Human Genome U133 Plus 2.0 Array and carried out according to the protocols by Affymetrix, Inc (Affymetrix, Santa Clara, CA). The array was read on the Affymetrix GeneChip Scanner 3000. The microarray data consist of 54,675 probe sets and were normalized using RMA algorithm [Irizarry et al., 2003]. Once normalized, genes that were both up- or down-regulated 1.5 fold in *6A cells over *9A cells, and had a p-value of <0.01 using t-test, were uploaded into the Ingenuity Pathway Analysis software (Ingenuity Systems, Redwood City, CA). The Ingenuity Pathway Analysis software sorts the genes into their appropriate signaling pathways, from which we were able to search through genes specifically involved in migration and invasion pathways. This search led to the identification of two genes that were downregulated in *6A cells compared to *9A cells that would directly lead to an increase in migration. Results of the gene array were then confirmed using real-time PCR.

**Real-time PCR**

RNA was collected from cells using the RNeasy protect Mini kit (Qiagen, Valencia, CA). Reverse transcription was carried out using 2 µg RNA in a 100 µl reaction volume using the TaqMan Reverse Transcription Kit (Applied Biosystems, Branchburg, NJ). 2 µl of cDNA was combined with 10 µM of each forward and reverse primer, 50 µM of the TaqMan probe, and TaqMan Fast Universal PCR Master Mix (2x) (Applied Biosystems, Foster City, CA). We used the following primers and TaqMan probes for ARHGAP5, fibronectin 1 (FN1), and glyceraldehyde -3-phopshate dehydrogenase (GAPDH):

ARHGAP5 sense primer (5'- AGCCCAATTCCTGCCAATAAG-3'),

antisense primer (5'-AGGAAGGGTGAAAGAATAAGATCCA-3'),

TaqMan probe (5'-FAM TGACTTGAGAATTCTCATGTGCGCCAT QSY7-3'),

FN1 sense primer (5'-TCGCCATCAGTAGAAGGTAGCA-3'),

antisense primer (5'-TACTTTCTTGATTTTCTTCCACAGCATA-3'),

TaqMan probe (5'-FAM TCAACCTTCCTGAAACTGCAAACTCCGTC QSY7-3'),

GAPDH sense primer (5'-GAAGGTGAAGGTCGGAGTC -3'),

antisense primer (5'-GAAGATGGTGATGGGATTC-3'),

TaqMan probe (5'- FAM CAAGCTTCCCGTTCTCAGCC QSY7-3'). Polymerase chain reaction amplification and detection was performed on the Applied Biosystems 7500/7500 Fast PCR system (Applied Biosystems, Foster City, CA). The *ARHGAP5* and *FN1* transcripts were quantitated relative to *GAPDH* by Comparative $C_T$ method following the Applied Biosystems protocol.

**Western Blots**

Cells were grown to 80% confluence, serum starved overnight, treated with 100 pM TGF-β1 for 18 hr, and lysates collected. Cells were lysed, boiled, and separated by SDS-PAGE. Proteins were transferred to a nitrocellulose membrane, blocked in 5% milk in TBST, and immunoblotted with the appropriate antibodies. Fibronectin (EP5): sc-8422, RhoA (26C4): sc-418, JNK1 (C-17): sc-474, p38 (N-20): sc-728 were obtained from Santa Cruz Biotechnology, Santa Cruz, CA; phospho-Smad2 (Ser465/467) (Cell Signaling Technology, Danvers, MA); Smad4/DPC4, Akt/PKB; phopsho-Akt1/PKBα (Ser463), phospho-SAPK/JNK (Thr183/Tyr185), phospho-p38 MAP Kinase (Thr180/Tyr182), phospho-p44/42 MAP Kinase (Thr202/Tyr204), and p44/42 MAP Kinase were obtained from Upstate Cell Signaling Solutions, Lake Placid, NY.; anti-p190-B RhoGAP [EP489Y] (Novus Biologicals, Littleton, CO); and α-tubulin (Sigma-Aldrich, Inc; St. Louis, MO). Secondary antibodies were peroxidase-conjugated affinity purified anti rabbit or mouse IgG (Rockland, Gilbertsville, PA), and detection was performed with ECL Plus Western Blotting Detection System (Amersham Applied Biosystems, England). Densitometry analysis was done using UN-SCAN-IT software (Silk Scientific Inc., Orem, Utah).

**RhoA-GTPase Activity Assays**

Pull down assays to detect GTP-bound RhoA were done as described previously [Ren et al., 1999;Ren and Schwartz, 2000]. A fusion protein containing the Rho binding domain (RBD) for rhotekin [Reid et al., 1996] and a glutathione S-transferase (GST) was used (a kind gift from Dr. Martin A. Schwartz, Scripps Institute, La Jolla, CA). Cells were serum starved overnight before being fed with complete media (10% FBS). After 10 min, cells were washed twice in ice cold Tris-buffered saline (TBS), and lysed in lysis buffer (50 mM Tris, pH 7.2, 1% Triton X-100,

0.5% sodium deoxycholate, 0.1% SDS, 500 mM NaCl, 10 mM $MgCl_2$, 1 mM DTT, 10 µg/ml each of leupeptin, aprotinin, and 1 mM PMSF). Cell lysates were incubated at 4ºC for 60 min with the RBD-GST pre-coupled to agarose-glutathione beads (Sigma, St. Louis, MO) to precipitate the GST-bound RhoA. The product was separated on a 13% SDS-PAGE gel, transferred to nitrocellulose, and immunoblotted with a RhoA antibody (Santa Cruz Biotechnology, Santa Cruz, CA). A second gel ran with lysates prior to immunoprecipitated was probed with RhoA and α-tubulin (Sigma-Aldrich, St. Louis, MO) as a loading control.

The amount of GTP-bound RhoA was also assessed using the G-LISA™ RhoA Activation Assay Biochem Kit™ (Absorbance Based) (Cytoskeleton, Inc, Denver, CO) according to the kit's protocol, which came with all the reagents used in the assay. Briefly, 2 days after plating $1x10^6$ cells in a 100 mm dish, the cells were serum starved overnight, and refed the following day with fresh complete media (10% FBS) for various early time points. Cells were washed once with ice cold PBS and lysed in the lysis buffer supplied in the kit. Protein concentration was evaluated using the Precision Red Advanced Protein Assay Reagent, and 1.75 µg of protein was used for the assay. The absorbance was measured at 490 nm using a microplate spectrophotometer.

**Generation of *Tgfbr1*$^{+/-}$ Mice**

A classical knockout vector was generated by inserting a Neomycin resistance cassette (Neo) into a Not I site located immediately after the start codon in exon 1 and removing 1.1kb of mouse genomic sequence immediately upstream of this Not I site. Following transfection and selection of 129SvIm embryonic stem (ES) cells, KO clones were karyotyped and injected into

C57BL/6 blastocysts. Germline transmission from the resulting chimeras was obtained and a colony established. F3 *Tgfbr1*$^{+/-}$ mice were backcrossed into the FVB/N background using speed congenics markers. Briefly, a minimum of 8 *Tgfbr1*$^{+/-}$ animals from each generation of backcrossing were genotyped for 152 markers by the Jackson Laboratory (Bar Harbor, ME). Mice with the highest percentage of the host genome were used to backcross to the host for the next generation. Two fully congenic F6 males (99.9% FVB/N) were confirmed using a full genome wide panel of 150 SNP markers (Jackson laboratory, Bar Harbor, ME). These two males were crossed with FVB/N females to obtain pure *Tgfbr1*$^{+/-}$ mice in the FVB/N background.

*Tgfbr1*$^{+/-}$ genotyping was confirmed by PCR analysis using the following set of 3 primers: 5'-AGACCCCAGCTCTTAGCCCCCA -3', 5'-GAGACGCTCCACCCACCTTCCC-3', and 5'-GAAGCTGACTCTAGAGGATCCC-3'. PCR amplification results in 2 bands in *Tgfbr1*$^{+/-}$ mice (240 bp and 314 bp, corresponding to the knocked-out and WT *Tgfbr1* allele, respectively).

100% pure FVB *Tgfbr1*$^{+/-}$ mice were crossed with FVB/N-Tg(MMTVneu)202Mul/J mice (Jackson Laboratory). MMTVneu genotyping was confirmed according to Jackson lab protocols using the following primers: 5'-TTTCCTGCAGCAGCCTACGC-3' and 5'-CGGAACCCACATCAGGCC-3'. The *Tgfbr1*$^{+/-}$ genotype was confirmed as described above. Virgin female mice were saved and used for experiments. Mice were monitored twice weekly by palpation for tumor formation. Tumors were measured once weekly. Volumes were calculated using the formula [$V=(\text{length}/2) \times (\text{width})^2$] [Muraoka et al., 2003]. Mice were sacrificed according to protocol at 80 d post tumor formation or at the earliest signs of morbidity.

**Isolation and Culture of Primary Tumor Cells**

Primary tumors were digested for 1 hr at 37ºC using 2 mg/ml Collagenase B (Roche Applied Science; Indianapolis, IN) in DMEM/F12 media (50:50; Gibco, Invitrogen, Grand Island, NY). Dissociated cells were spun down, washed 3 times in PBS, and plated in DMEM/F12 media supplemented with 10% heat inactivated FBS (Hyclone, Logan, Utah), 2 mm L-glutamine (Invitrogen Corp., Grand Island, NY), 1,000 units/ml penicillin, 10,000 μg/ml streptomycin (Invitrogen Corp., Grand Island, NY), and 50 ng/ml insulin (Sigma, St. Louis, MO). Cells were maintained in 37ºC at 5% $CO_2$.

**Assessing Metastasis**

To assess lung metastasis, lungs were dissected from the mouse and observed under a microscope for visible signs of lung metastasis. Lungs were then fixed in formalin to be further sectioned to count micro-metastases.

**Assessing Circulating Tumor Cells**

Tumor cell entry into the blood stream is an important step in the metastatic process. It has been determined that the tumor cell density found in the blood correlates with the number of single tumor cells in the lungs or metastases [Wyckoff et al., 2000] To assess the intravasation of tumor cells into the blood stream, 1 ml of blood was collected by heart puncture with a needle coated with heparin, to prevent clotting upon collection. The blood was centrifuged at 5000 rpm for 5 min, and the serum and buffy coat layers were plated in DMEM supplemented with 10% heat inactivated FBS (Hyclone, Grand Rapid, Utah). 24 hr after plating, the media was washed and

changed. 7 days after plating, the plates were fixed with 95% methanol and stained with methylene blue. The colonies from each plate were counted [Wyckoff et al., 2000].

**Histological analysis**

Right no. 4 mammary glands, tumors, and lungs were dissected and fixed in 10% formalin. Paraffin-embedded tissues were sectioned at 5μm, deparaffinized, rehydrated, and used for H&E or IHC studies. H&E staining was performed on an automated processor using hematoxylin and eosin. For immunohistochemistry, antigen retrieval was performed in boiling water bath in antigen retrieval buffer (1:10 dilution, S1699 Dako EnVision System, Carpinteria, CA) for 30 min, and the protocol was done using the EnVision+System-HRP kit (using DAB or AEC as the substrate chromagen) (Dako, Carpinteria, CA). After primary antibody incubation, the sections were then incubated with HRP labeled polymer (cat # K4008, Dako EnVision System, Carpinteria, CA) for 30 min and the immunocomplexes were detected with AEC+chromogen (cat # K4008, Dako EnVision System, Carpinteria, CA) or DAB+ (cat # 3468, Dako EnVision System, Carpinteria, CA) and counterstained with hematoxylin (cat # MHS1-100ML, Sigma, St Louis, MO) Antibodies used are as follows: CyclinD1 (sc-8396, Santa Cruz Biotechnology, Santa Cruz, CA), pSmad2 (#3101, Cell Signaling Technologies, Danvers, MA). Anti-pSmad3 was a gift from Dr. Koichi Matsuzaki, Kanzai Medical University, Osaka, Japan [Yamagata et al., 2005;Sekimoto et al., 2007]. The positive stained indexes were calculated by counting the number of positive stained cells in five random fields from 4 mice and dividing by the total number of cells in the field.

**Mammary Gland Whole Mount:**

The #4 mammary gland was harvested from virgin female mice at 10, 12, and 40 weeks of age, and fixed in formalin for 2 d. The glands were washed with water, then 3 successive acetone washes, the last one overnight. The next day, the glands were washed with PBS, and digested with 0.1 mg/ml Collagenase (Sigma C6885, St. Louis, MO) for 5 hr at 37ºC. They were washed again in PBS and stained overnight in Carmine Aluminum (0.5% w/v Carmine (Fisher Acros), and 0.5% w/v aluminum potassium sulfate (Acros)). The outer membrane was removed from the mammary gland. The glands were then spread on glass slides, mounted in Entellan mounting medium, and photographed.

**Western Blotting**

Tumors and mammary glands were collected and immediately snapped-frozen on dry-ice/ethanol. Tissues were taken, ground up in dry ice using a mortar and pestel, and then resuspended in lysis buffer: TNT buffer (10 mM Tris pH 8.0, 1% Triton X-100, 1 mM EDTA, 150 mM NaCl), supplemented with Phosphatase Inhibitor Cocktails 1 and 2, and Protease Inhibitor Cocktail (Sigma, St. Louis, MO). Lysates were centrifuged at 14000xg for 15 min. Total protein (10 μg) was separated by SDS-PAGE, and transferred onto nitrocellulose, and immunoblotted with the following antibodies: TGFBR1 (sc-398), Neu (sc-284), CyclinD1 (sc-8396) (Santa Cruz Biotechnology, Santa Cruz, CA); pSmad2 (#3101), Smad2 (#3122) (Cell Signaling Technologies, Danvers, MA); β-Actin (Sigma, St. Louis, MO). Secondary antibodies were from Rockland (Gilbertsville, PA).

# Reference List

(2000) The exon 13 duplication in the BRCA1 gene is a founder mutation present in geographically diverse populations. The BRCA1 Exon 13 Duplication Screening Group. *Am J Hum Genet* **67**: 207-212

NCCN Clinical Practice Guidelines in Oncology. http://www.nccn.org/professionals/physician_gls/PDF/genetics_screening.pdf . 2006. NCCN. Ref Type: Electronic Citation

Andrieu N, Goldgar DE, Easton DF, Rookus M, Brohet R, Antoniou AC, Peock S, Evans G, Eccles D, Douglas F, Nogues C, Gauthier-Villars M, Chompret A, Van Leeuwen FE, Kluijt I, Benitez J, Arver B, Olah E, Chang-Claude J (2006) Pregnancies, breast-feeding, and breast cancer risk in the International BRCA1/2 Carrier Cohort Study (IBCCS). *J Natl Cancer Inst* **98**: 535-544

Antoniou A, Pharoah PD, Narod S, Risch HA, Eyfjord JE, Hopper JL, Loman N, Olsson H, Johannsson O, Borg A, Pasini B, Radice P, Manoukian S, Eccles DM, Tang N, Olah E, Anton-Culver H, Warner E, Lubinski J, Gronwald J, Gorski B, Tulinius H, Thorlacius S, Eerola H, Nevanlinna H, Syrjakoski K, Kallioniemi OP, Thompson D, Evans C, Peto J, Lalloo F, Evans DG, Easton DF (2003) Average Risks of Breast and Ovarian Cancer Associated with BRCA1 or BRCA2 Mutations Detected in Case Series Unselected for Family History: A Combined Analysis of 22 Studies. *Am J Hum Genet* **72**:

Ao M, Williams K, Bhowmick NA, Hayward SW (2006) Transforming Growth Factor-{beta} Promotes Invasion in Tumorigenic but not in Nontumorigenic Human Prostatic Epithelial Cells. *Cancer Res* **66**: 8007-8016

Arnold SF, Tims E, Bluman EM, McGrath BE (1999) Regulation of transforming growth factor beta1 by radiation in cells of two human breast cancer cell lines. *Radiat Res* **152**: 487-492

Bakin AV, Tomlinson AK, Bhowmick NA, Moses HL, Arteaga CL (2000) Phosphatidylinositol 3-kinase function is required for transforming growth factor beta-mediated epithelial to mesenchymal transition and cell migration. *J Biol Chem* **275**: 36803-36810

Baldus SE, Schwarz E, Lohrey C, Zapatka M, Landsberg S, Hahn SA, Schmidt D, Dienes HP, Schmiegel WH, Schwarte-Waldhoff I (2005) Smad4 deficiency in cervical carcinoma cells. *Oncog* **24**: 810-819

Baxter SW, Choong DY, Eccles DM, Campbell IG (2002) Transforming Growth Factor beta Receptor 1 Polyalanine Polymorphism and Exon 5 Mutation Analysis in Breast and Ovarian Cancer. *Cancer Epidemiol Biomarkers Prev* **11**: 211-214

Bhowmick NA, Ghiassi M, Bakin A, Aakre M, Lundquist CA, Engel ME, Arteaga CL, Moses HL (2001) Transforming growth factor-beta1 mediates epithelial to mesenchymal transdifferentiation through a RhoA-dependent mechanism. *Mol Biol Cell* **12**: 27-36

Bian Y, Caldes T, Wijnen J, Franken P, Vasen H, Kaklamani V, Nafa K, Peterlongo P, Ellis N, Baron JA, Burn J, Moeslein G, Morrison PJ, Chen Y, Ahsan H, Watson P, Lynch HT, de la CA, Fodde R, Pasche B (2005) TGFBR1*6A may contribute to hereditary colorectal cancer. *J Clin Oncol* **23**: 3074-3078

Bian Y, Kaklamani V, Reich J, Pasche B (2003) TGF-beta signaling alterations in cancer. *Cancer Treat Res* **115**: 73-94

Bierie B, Stover DG, Abel TW, Chytil A, Gorska AE, Aakre M, Forrester E, Yang L, Wagner KU, Moses HL (2008) Transforming growth factor-beta regulates mammary carcinoma cell survival and interaction with the adjacent microenvironment. *Cancer Res* **68**: 1809-1819

Birch JM, Hartley AL, Tricker KJ, Prosser J, Condie A, Kelsey AM, Harris M, Jones PH, Binchy A, Crowther D, . (1994) Prevalence and diversity of constitutional mutations in the p53 gene among 21 Li-Fraumeni families. *Cancer Res* **54**: 1298-1304

Bottinger EP, Factor VM, Tsang ML, Weatherbee JA, Kopp JB, Qian SW, Wakefield LM, Roberts AB, Thorgeirsson SS, Sporn MB (1996) The recombinant proregion of transforming growth factor beta1 (latency-associated peptide) inhibits active transforming growth factor beta1 in transgenic mice. *Proc Natl Acad Sci U S A* **93**: 5877-5882

Boulton SJ (2006) Cellular functions of the BRCA tumour-suppressor proteins. *Biochem Soc Trans* **34**: 633-645

Breast Cancer Association Consortium (2006) Commonly studied single-nucleotide polymorphisms and breast cancer: results from the Breast Cancer Association Consortium. *J Natl Cancer Inst* **98**: 1382-1396

Brown PD, Wakefield LM, Levinson AD, Sporn MB (1990) Physicochemical activation of recombinant latent transforming growth factor-beta's 1, 2, and 3. *Growth Factors* **3**: 35-43

Buck MB, Pfizenmaier K, Knabbe C (2004) Antiestrogens induce growth inhibition by sequential activation of p38 mitogen-activated protein kinase and transforming growth factor-beta pathways in human breast cancer cells. *Mol Endocrinol* **18**: 1643-1657

Buckley MF, Sweeney KJ, Hamilton JA, Sini RL, Manning DL, Nicholson RI, deFazio A, Watts CK, Musgrove EA, Sutherland RL (1993) Expression and amplification of cyclin genes in human breast cancer. *Oncog* **8**: 2127-2133

Caceres M, Guerrero J, Martinez J (2005) Overexpression of RhoA-GTP induces activation of the Epidermal Growth Factor Receptor, dephosphorylation of focal adhesion kinase and increased motility in breast cancer cells. *Exp Cell Res* **309**: 229-238

Cantor SB, Bell DW, Ganesan S, Kass EM, Drapkin R, Grossman S, Wahrer DC, Sgroi DC, Lane WS, Haber DA, Livingston DM (2001) BACH1, a novel helicase-like protein, interacts directly with BRCA1 and contributes to its DNA repair function. *Cell* **105**: 149-160

Caraci F, Gili E, Calafiore M, Failla M, La RC, Crimi N, Sortino MA, Nicoletti F, Copani A, Vancheri C (2008) TGF-beta1 targets the GSK-3beta/beta-catenin pathway via ERK activation in the transition of human lung fibroblasts into myofibroblasts. *Pharmacol Res* **57**: 274-282

Carcamo J, Zentella A, Massague J (1995) Disruption of transforming growth factor beta signaling by a mutation that prevents transphosphorylation within the receptor complex. *Mol Cell Biol* **15**: 1573-1581

Chen CR, Kang Y, Massague J (2001) Defective repression of c-myc in breast cancer cells: A loss at the core of the transforming growth factor beta growth arrest program. *Proc Natl Acad Sci U S A* **98**: 992-999

Chen T, Carter D, Garrigue-Antar L, Reiss M (1998) Transforming growth factor beta type I receptor kinase mutant associated with metastatic breast cancer. *Cancer Res* **58**: 4805-4810

Chen T, de Vries EG, Hollema H, Yegen HA, Vellucci VF, Strickler HD, Hildesheim A, Reiss M (1999) Structural alterations of transforming growth factor-beta receptor genes in human cervical carcinoma. *Int J Cancer* **82**: 43-51

Chen T, Jackson C, Costello B, Singer N, Colligan B, Douglass L, Pemberton J, Deddens J, Graff JR, Carter JH (2004) An intronic variant of the TGFBR1 gene is associated with carcinomas of the kidney and bladder. *Int J Cancer* **112**: 420-425

Chen T, Jackson CR, Link A, Markey MP, Colligan BM, Douglass LE, Pemberton JO, Deddens JA, Graff JR, Carter JH (2006) Int7G24A variant of transforming growth factor-beta receptor type I is associated with invasive breast cancer. *Clin Cancer Res* **12**: 392-397

Chen YG, Liu F, Massague J (1997) Mechanism of TGFbeta receptor inhibition by FKBP12. *EMBO J* **16**: 3866-3876

Christensen L, Nielsen M, Andersen J, Clemmensen I (1988) Stromal fibronectin staining pattern and metastasizing ability of human breast carcinoma. *Cancer Res* **48**: 6227-6233

Condeelis J, Pollard JW (2006) Macrophages: obligate partners for tumor cell migration, invasion, and metastasis. *Cell* **124**: 263-266

Cox A, Dunning AM, Garcia-Closas M, Balasubramanian S, Reed MW, Pooley KA, Scollen S, Baynes C, Ponder BA, Chanock S, Lissowska J, Brinton L, Peplonska B, Southey MC, Hopper JL, McCredie MR, Giles GG, Fletcher O, Johnson N, dos SS, I, Gibson L, Bojesen SE, Nordestgaard BG, Axelsson CK, Torres D, Hamann U, Justenhoven C, Brauch H, Chang-Claude J, Kropp S, Risch A, Wang-Gohrke S, Schurmann P, Bogdanova N, Dork T, Fagerholm R, Aaltonen K, Blomqvist C, Nevanlinna H, Seal S, Renwick A, Stratton MR, Rahman N, Sangrajrang S, Hughes D, Odefrey F, Brennan P, Spurdle AB, Chenevix-Trench G, Beesley J,

Mannermaa A, Hartikainen J, Kataja V, Kosma VM, Couch FJ, Olson JE, Goode EL, Broeks A, Schmidt MK, Hogervorst FB, Van't Veer LJ, Kang D, Yoo KY, Noh DY, Ahn SH, Wedren S, Hall P, Low YL, Liu J, Milne RL, Ribas G, Gonzalez-Neira A, Benitez J, Sigurdson AJ, Stredrick DL, Alexander BH, Struewing JP, Pharoah PD, Easton DF (2007) A common coding variant in CASP8 is associated with breast cancer risk. *Nat Genet* **39**: 352-358

Crawford SE, Stellmach V, Murphyullrich JE, Ribeiro SF, Lawler J, Hynes RO, Boivin GP, Bouck N (1998) Thrombospondin-1 is a major activator of tgf-beta-1 in vivo. *Cell* **93**: 1159-1170

Datto MB, Li Y, Panus JF, Howe DJ, Xiong Y, Wang XF (1995) Transforming Growth-Factor-Beta Induces the Cyclin-Dependent Kinase Inhibitor P21 Through A P53-Independent Mechanism. *Proceedings of the National Academy of Sciences of the United States of America* **92**: 5545-5549

de Jong JS, van Diest PJ, van d, V, Baak JP (1998) Expression of growth factors, growth-inhibiting factors, and their receptors in invasive breast cancer. II: Correlations with proliferation and angiogenesis. *J Pathol* **184**: 53-57

De Larco JE, Todaro GJ (1978) Growth factors from murine sarcoma virus-transformed cells. *Proc Natl Acad Sci U S A* **75**: 4001-4005

Deng CX (2006) BRCA1: cell cycle checkpoint, genetic instability, DNA damage response and cancer evolution. *Nucleic Acids Res* **34**: 1416-1426

Derynck R, Akhurst RJ, Balmain A (2001) TGF-beta signaling in tumor suppression and cancer progression. *Nat Genet* **29**: 117-129

Derynck R, Gelbart WM, Harland RM, Heldin CH, Kern SE, Massague J, Melton DA, Mlodzik M, Padgett RW, Roberts AB, Smith J, Thomsen GH, Vogelstein B, Wang XF (1996) Nomenclature: vertebrate mediators of TGFbeta family signals. *Cell* **87**: 173

Derynck R, Goeddel DV, Ullrich A, Gutterman JU, Williams RD, Bringman TS, Berger WH (1987) Synthesis of messenger RNAs for transforming growth factors alpha and beta and the epidermal growth factor receptor by human tumors. *Cancer Res* **47**: 707-712

Di Guglielmo GM, Le RC, Goodfellow AF, Wrana JL (2003) Distinct endocytic pathways regulate TGF-beta receptor signalling and turnover. *Nat Cell Biol* **5**: 410-421

Dubois CM, Blanchette F, Laprise MH, Leduc R, Grondin F, Seidah NG (2001) Evidence that furin is an authentic transforming growth factor-beta1-converting enzyme. *Am J Pathol* **158**: 305-316

Dubois CM, Laprise MH, Blanchette F, Gentry LE, Leduc R (1995) Processing of transforming growth factor beta 1 precursor by human furin convertase. *J Biol Chem* **270**: 10618-10624

Dumont N, Bakin AV, Arteaga CL (2003) Autocrine transforming growth factor-beta signaling mediates Smad-independent motility in human cancer cells. *J Biol Chem* **278**: 3275-3285

Dunning AM, Ellis PD, McBride S, Kirschenlohr HL, Healey CS, Kemp PR, Luben RN, Chang-Claude J, Mannermaa A, Kataja V, Pharoah PD, Easton DF, Ponder BA, Metcalfe JC (2003) A transforming growth factorbeta1 signal peptide variant increases secretion in vitro and is associated with increased incidence of invasive breast cancer. *Cancer Res* **63**: 2610-2615

Elliott BE, Hung WL, Boag AH, Tuck AB (2002) The role of hepatocyte growth factor (scatter factor) in epithelial-mesenchymal transition and breast cancer. *Can J Physiol Pharmacol* **80**: 91-102

Elliott RL, Blobe GC (2005) Role of transforming growth factor Beta in human cancer. *J Clin Oncol* **23**: 2078-2093

Enns C (2001) Overview of protein trafficking in the secretory and endocytic pathways. *Curr Protoc Cell Biol* **Chapter 15**: Unit

Evers B, Jonkers J (2006) Mouse models of BRCA1 and BRCA2 deficiency: past lessons, current understanding and future prospects. *Oncog* **25**: 5885-5897

Fanayan S, Firth SM, Baxter RC (2002) Signaling through the Smad pathway by insulin-like growth factor-binding protein-3 in breast cancer cells. Relationship to transforming growth factor-beta 1 signaling. *J Biol Chem* **277**: 7255-7261

Feigelson HS, Patel AV, Diver WR, Stevens VL, Thun MJ, Calle EE (2006) Transforming growth factor beta receptor type I and transforming growth factor beta1 polymorphisms are not associated with postmenopausal breast cancer. *Cancer Epidemiol Biomarkers Prev* **15**: 1236-1237

Feng XH, Lin X, Derynck R (2000) Smad2, Smad3 and Smad4 cooperate with Sp1 to induce p15(Ink4B) transcription in response to TGF-beta. *EMBO J* **19**: 5178-5193

Fenig E, Kanfi Y, Wang Q, Beery E, Livnat T, Wasserman L, Lilling G, Yahalom J, Wieder R, Nordenberg J (2001) Role of transforming growth factor beta in the growth inhibition of human breast cancer cells by basic fibroblast growth factor. *Breast Cancer Res Treat* **70**: 27-37

Fidler IJ, Kripke ML (1977) Metastasis results from preexisting variant cells within a malignant tumor. *Science* **197**: 893-895

Fontana L, Chen Y, Prijatelj P, Sakai T, Fassler R, Sakai LY, Rifkin DB (2005) Fibronectin is required for integrin alphavbeta6-mediated activation of latent TGF-beta complexes containing LTBP-1. *FASEB J* **19**: 1798-1808

Forrester E, Chytil A, Bierie B, Aakre M, Gorska AE, Sharif-Afshar AR, Muller WJ, Moses HL (2005) Effect of conditional knockout of the type II TGF-beta receptor gene in mammary

epithelia on mammary gland development and polyomavirus middle T antigen induced tumor formation and metastasis. *Cancer Res* **65**: 2296-2302

Fritz G, Brachetti C, Bahlmann F, Schmidt M, Kaina B (2002) Rho GTPases in human breast tumours: expression and mutation analyses and correlation with clinical parameters. *Br J Cancer* **87**: 635-644

Fritz G, Just I, Kaina B (1999) Rho GTPases are over-expressed in human tumors. *Int J Cancer* **81**: 682-687

Fujii D, Brissenden JE, Derynck R, Francke U (1986) Transforming growth factor beta gene maps to human chromosome 19 long arm and to mouse chromosome 7. *Somat Cell Mol Genet* **12**: 281-288

Gad S, Caux-Moncoutier V, Pages-Berhouet S, Gauthier-Villars M, Coupier I, Pujol P, Frenay M, Gilbert B, Maugard C, Bignon YJ, Chevrier A, Rossi A, Fricker JP, Nguyen TD, Demange L, Aurias A, Bensimon A, Stoppa-Lyonnet D (2002) Significant contribution of large BRCA1 gene rearrangements in 120 French breast and ovarian cancer families. *Oncog* **21**: 6841-6847

Gajdusek CM, Luo Z, Mayberg MR (1993) Basic fibroblast growth factor and transforming growth factor beta-1: synergistic mediators of angiogenesis in vitro. *J Cell Physiol* **157**: 133-144

Garcia R, Franklin RA, McCubrey JA (2006) EGF induces cell motility and multi-drug resistance gene expression in breast cancer cells. *Cell Cycle* **5**: 2820-2826

Garrigue-Antar L, Munoz-Antonia T, Antonia SJ, Gesmonde J, Vellucci VF, Reiss M (1995) Missense mutations of the transforming growth factor beta type II receptor in human head and neck squamous carcinoma cells. *Cancer Res* **55**: 3982-3987

Gentry LE, Lioubin MN, Purchio AF, Marquardt H (1988) Molecular events in the processing of recombinant type 1 pre-pro-transforming growth factor beta to the mature polypeptide. *Mol Cell Biol* **8**: 4162-4168

Gentry LE, Nash BW (1990) The pro domain of pre-pro-transforming growth factor beta 1 when independently expressed is a functional binding protein for the mature growth factor. *Biochemistry* **29**: 6851-6857

Gillett C, Fantl V, Smith R, Fisher C, Bartek J, Dickson C, Barnes D, Peters G (1994) Amplification and overexpression of cyclin D1 in breast cancer detected by immunohistochemical staining. *Cancer Res* **54**: 1812-1817

Gobbi H, Arteaga CL, Jensen RA, Simpson JF, Dupont WD, Olson SJ, Schuyler PA, Plummer WD, Jr., Page DL (2000) Loss of expression of transforming growth factor beta type II receptor correlates with high tumour grade in human breast in-situ and invasive carcinomas. *Histopathology* **36**: 168-177

Gobbi H, Dupont WD, Simpson JF, Plummer WD, Jr., Schuyler PA, Olson SJ, Arteaga CL, Page DL (1999) Transforming growth factor-beta and breast cancer risk in women with mammary epithelial hyperplasia. *J Natl Cancer Inst* **91**: 2096-2101

Gotzmann J, Mikula M, Eger A, Schulte-Hermann R, Foisner R, Beug H, Mikulits W (2004) Molecular aspects of epithelial cell plasticity: implications for local tumor invasion and metastasis. *Mutat Res* **566**: 9-20

Grau AM, Wen W, Ramroopsingh DS, Gao YT, Zi J, Cai Q, Shu XO, Zheng W (2007) Circulating transforming growth factor-beta-1 and breast cancer prognosis: results from the Shanghai Breast Cancer Study. *Breast Cancer Res Treat*

Graus-Porta D, Beerli RR, Daly JM, Hynes NE (1997) ErbB-2, the preferred heterodimerization partner of all ErbB receptors, is a mediator of lateral signaling. *EMBO J* **16**: 1647-1655

Greenberg RA (2008) Recognition of DNA double strand breaks by the BRCA1 tumor suppressor network. *Chromosoma* **117**: 305-317

Guy CT, Webster MA, Schaller M, Parsons TJ, Cardiff RD, Muller WJ (1992) Expression of the neu protooncogene in the mammary epithelium of transgenic mice induces metastatic disease. *Proc Natl Acad Sci U S A* **89**: 10578-10582

Hanahan D, Weinberg RA (2000) The hallmarks of cancer. *Cell* **100**: 57-70

Hennings H, Glick AB, Lowry DT, Krsmanovic LS, Sly LM, Yuspa SH (1993) FVB/N mice: an inbred strain sensitive to the chemical induction of squamous cell carcinomas in the skin. *Carcinogenesis* **14**: 2353-2358

Hishida A, Iwata H, Hamajima N, Matsuo K, Mizutani M, Iwase T, Miura S, Emi N, Hirose K, Tajima K (2003) Transforming growth factor B1 T29C polymorphism and breast cancer risk in Japanese women. *Breast Cancer* **10**: 63-69

Horii Y, Takei H, Koibuchi Y, Horiguchi J, Maemura M, Iino Y, Morishita Y (2006) The regulatory effect of tamoxifen on fibronectin expression in estrogen-dependent MCF-7 breast carcinoma cells. *Oncol Rep* **15**: 1191-1195

Huang C, Jacobson K, Schaller MD (2004) MAP kinases and cell migration. *J Cell Sci* **117**: 4619-4628

Huse M, Muir TW, Xu L, Chen YG, Kuriyan J, Massague J (2001) The TGF beta receptor activation process: an inhibitor- to substrate-binding switch. *Mol Cell* **8**: 671-682

Iavarone A, Massague J (1997) Repression of the CDK activator Cdc25A and cell-cycle arrest by cytokine TGF-beta in cells lacking the CDK inhibitor p15. *Nature* **387**: 417-422

Iglesias M, Frontelo P, Gamallo C, Quintanilla M (2000) Blockade of Smad4 in transformed keratinocytes containing a Ras oncogene leads to hyperactivation of the Ras-dependent Erk

signalling pathway associated with progression to undifferentiated carcinomas. *Oncog* **19**: 4134-4145

Imamichi Y, Waidmann O, Hein R, Eleftheriou P, Giehl K, Menke A (2005) TGF beta-induced focal complex formation in epithelial cells is mediated by activated ERK and JNK MAP kinases and is independent of Smad4. *Biol Chem* **386**: 225-236

Irizarry RA, Bolstad BM, Collin F, Cope LM, Hobbs B, Speed TP (2003) Summaries of Affymetrix GeneChip probe level data. *Nucleic Acids Res* **31**: e15

Jakowlew SB (2006) Transforming growth factor-beta in cancer and metastasis. *Cancer Metastasis Rev* **25**: 435-457

Jemal A, Siegel R, Ward E, Murray T, Xu J, Smigal C, Thun MJ (2006) Cancer Statistics, 2006. *Ca: a Cancer Journal for Clinicians* **56**: 106-130

Jin Q, Hemminki K, Grzybowska E, Klaes R, Soderberg M, Zientek H, Rogozinska-Szczepka J, Utracka-Hutka B, Pamula J, Pekala W, Forsti A (2004) Polymorphisms and haplotype structures in genes for transforming growth factor beta1 and its receptors in familial and unselected breast cancers. *Int J Cancer* **%20;112**: 94-99

Jullien P, Berg TM, Lawrence DA (1989) Acidic cellular environments: activation of latent TGF-beta and sensitization of cellular responses to TGF-beta and EGF. *Int J Cancer* **43**: 886-891

Kadouri L, Hubert A, Rotenberg Y, Hamburger T, Sagi M, Nechushtan C, Abeliovich D, Peretz T (2007) Cancer risks in carriers of the BRCA1/2 Ashkenazi founder mutations. *J Med Genet* **44**: 467-471

Kaklamani V, Pasche B (2005) Transforming Growth Factor Beta and breast cancer. *Cancer Treat Res* **126**: 129-156

Kaklamani VG, Hou N, Bian Y, Reich J, Offit K, Michel LS, Rubinstein WS, Rademaker A, Pasche B (2003) TGFBR1*6A and cancer risk: a meta-analysis of seven case-control studies. *J Clin Oncol* **21**: 3236-3243

Kaklamani VG, Baddi L, Liu J, Rosman D, Phukan S, Bradley C, Hegarty C, McDaniel B, Rademaker A, Oddoux C, Ostrer H, Michel LS, Huang H, Chen Y, Ahsan H, Offit K, Pasche B (2005) Combined Genetic Assessment of Transforming Growth Factor-{beta} Signaling Pathway Variants May Predict Breast Cancer Risk. *Cancer Res* **65**: 3454-3461

Kang Y, He W, Tulley S, Gupta GP, Serganova I, Chen CR, Manova-Todorova K, Blasberg R, Gerald WL, Massague J (2005) Breast cancer bone metastasis mediated by the Smad tumor suppressor pathway. *Proc Natl Acad Sci U S A* **102**: 13909-13914

Kang Y, Siegel PM, Shu W, Drobnjak M, Kakonen SM, Cordon-Cardo C, Guise TA, Massague J (2003) A multigenic program mediating breast cancer metastasis to bone. *Cancer Cell* **3**: 537-549

Kaspar M, Zardi L, Neri D (2006) Fibronectin as target for tumor therapy. *Int J Cancer* **118**: 1331-1339

Kelsey JL, Gammon MD, John EM (1993) Reproductive factors and breast cancer. *Epidemiol Rev* **15**: 36-47

Kim M, Gans JD, Nogueira C, Wang A, Paik JH, Feng B, Brennan C, Hahn WC, Cordon-Cardo C, Wagner SN, Flotte TJ, Duncan LM, Granter SR, Chin L (2006) Comparative oncogenomics identifies NEDD9 as a melanoma metastasis gene. *Cell* **125**: 1269-1281

King MC, Marks JH, Mandell JB (2003) Breast and Ovarian Cancer Risks Due to Inherited Mutations in BRCA1 and BRCA2. *Science* **302**: 643-646

Krippl P, Langsenlehner U, Renner W, Yazdani-Biuki B, Wolf G, Wascher TC, Paulweber B, Bahadori B, Samonigg H (2003) The L10P polymorphism of the transforming growth factor-beta 1 gene is not associated with breast cancer risk. *Cancer Lett* **201**: 181-184

Lalloo F, Varley J, Ellis D, Moran A, O'dair L, Pharoah P, Evans DG (2003) Prediction of pathogenic mutations in patients with early-onset breast cancer by family history. *Lancet* **361**: 1101-1102

Lalloo F, Varley J, Moran A, Ellis D, O'dair L, Pharoah P, Antoniou A, Hartley R, Shenton A, Seal S, Bulman B, Howell A, Evans DG (2006) BRCA1, BRCA2 and TP53 mutations in very early-onset breast cancer with associated risks to relatives. *Eur J Cancer* **42**: 1143-1150

Lanigan F, O'Connor D, Martin F, Gallagher WM (2007) Molecular links between mammary gland development and breast cancer. *Cell Mol Life Sci* **64**: 3159-3184

Lawrence DA, Pircher R, Jullien P (1985) Conversion of a high molecular weight latent beta-TGF from chicken embryo fibroblasts into a low molecular weight active beta-TGF under acidic conditions. *Biochem Biophys Res Commun* **133**: 1026-1034

Lee WJ, Chen WK, Wang CJ, Lin WL, Tseng TH (2008) Apigenin inhibits HGF-promoted invasive growth and metastasis involving blocking PI3K/Akt pathway and beta 4 integrin function in MDA-MB-231 breast cancer cells. *Toxicol Appl Pharmacol* **226**: 178-191

Lehmann K, Janda E, Pierreux CE, Rytomaa M, Schulze A, McMahon M, Hill CS, Beug H, Downward J (2000) Raf induces TGFbeta production while blocking its apoptotic but not invasive responses: a mechanism leading to increased malignancy in epithelial cells. *Genes Dev* **14**: 2610-2622

Lenferink AE, Busse D, Flanagan WM, Yakes FM, Arteaga CL (2001) ErbB2/neu kinase modulates cellular p27(Kip1) and cyclin D1 through multiple signaling pathways. *Cancer Res* **61**: 6583-6591

Leu JI, Crissey MA, Taub R (2003) Massive hepatic apoptosis associated with TGF-beta1 activation after Fas ligand treatment of IGF binding protein-1-deficient mice. *J Clin Invest* **111**: 129-139

Levy L, Hill CS (2005) Smad4 dependency defines two classes of transforming growth factor {beta} (TGF-{beta}) target genes and distinguishes TGF-{beta}-induced epithelial-mesenchymal transition from its antiproliferative and migratory responses. *Mol Cell Biol* **25**: 8108-8125

Li DM, Sun H (1998) PTEN/MMAC1/TEP1 suppresses the tumorigenicity and induces G1 cell cycle arrest in human glioblastoma cells. *Proc Natl Acad Sci U S A* **95**: 15406-15411

Li FP, Fraumeni JF, Jr., Mulvihill JJ, Blattner WA, Dreyfus MG, Tucker MA, Miller RW (1988) A cancer family syndrome in twenty-four kindreds. *Cancer Res* **48**: 5358-5362

Li W, Zhu T, Guan KL (2004) Transformation potential of Ras isoforms correlates with activation of phosphatidylinositol 3-kinase but not ERK. *J Biol Chem* **279**: 37398-37406

Lichtenstein P, Holm NV, Verkasalo PK, Iliadou A, Kaprio J, Koskenvuo M, Pukkala E, Skytthe A, Hemminki K (2000) Environmental and heritable factors in the causation of cancer - Analyses of cohorts of twins from Sweden, Denmark, and Finland. *nejm* **343**: 78-85

Liu F, Pouponnot C, Massague J (1997) Dual role of the smad4/dpc4 tumor suppressor in tgf-beta-inducible transcriptional complexes. *Genes Dev* **11**: 3157-3167

Lo RS, Wotton D, Massague J (2001) Epidermal growth factor signaling via Ras controls the Smad transcriptional co-repressor TGIF. *EMBO J* **20**: 128-136

Lu SL, Herrington H, Reh D, Weber S, Bornstein S, Wang D, Li AG, Tang CF, Siddiqui Y, Nord J, Andersen P, Corless CL, Wang XJ (2006) Loss of transforming growth factor-beta type II receptor promotes metastatic head-and-neck squamous cell carcinoma. *Genes Dev* **20**: 1331-1342

Lucke CD, Philpott A, Metcalfe JC, Thompson AM, Hughes-Davies L, Kemp PR, Hesketh R (2001) Inhibiting mutations in the transforming growth factor beta type 2 receptor in recurrent human breast cancer. *Cancer Res* **61**: 482-485

Luo K (2004) Ski and SnoN: negative regulators of TGF-beta signaling. *Curr Opin Genet Dev* **14**: 65-70

Marchand LL, Haiman CA, van den Berg D, Wilkens LR, Kolonel LN, Henderson BE (2004) T29C Polymorphism in the Transforming Growth Factor {beta}1 Gene and Postmenopausal Breast Cancer Risk: The Multiethnic Cohort Study. *Cancer Epidemiol Biomarkers Prev* **13**: 412-415

Markowitz S, Wang J, Myeroff L, Parsons R, Sun L, Lutterbaugh J, Fan RS, Zborowska E, Kinzler KW, Vogelstein B, . (1995) Inactivation of the type II TGF-beta receptor in colon cancer cells with microsatellite instability. *Science* **268**: 1336-1338

Massague J (1998) TGF-beta signal transduction. *Annu Rev Biochem* **67**: 753-791

Massague J (2008) TGFbeta in Cancer. *Cell* **134**: 215-230

Mazoyer S (2005) Genomic rearrangements in the BRCA1 and BRCA2 genes. *Hum Mutat* **25**: 415-422

McMahon GA, Dignam JD, Gentry LE (1996) Structural characterization of the latent complex between transforming growth factor beta 1 and beta 1-latency-associated peptide. *Biochem J* **313 ( Pt 1)**: 343-351

Meijers-Heijboer H, van den OA, Klijn J, Wasielewski M, de SA, Oldenburg R, Hollestelle A, Houben M, Crepin E, van Veghel-Plandsoen M, Elstrodt F, van DC, Bartels C, Meijers C, Schutte M, McGuffog L, Thompson D, Easton D, Sodha N, Seal S, Barfoot R, Mangion J, Chang-Claude J, Eccles D, Eeles R, Evans DG, Houlston R, Murday V, Narod S, Peretz T, Peto J, Phelan C, Zhang HX, Szabo C, Devilee P, Goldgar D, Futreal PA, Nathanson KL, Weber B, Rahman N, Stratton MR (2002) Low-penetrance susceptibility to breast cancer due to CHEK2(*)1100delC in noncarriers of BRCA1 or BRCA2 mutations. *Nat Genet* **31**: 55-59

Meng S, Chen Z, Munoz-Antonia T, Wu J (2005) Participation of both Gab1 and Gab2 in the activation of the ERK/MAPK pathway by epidermal growth factor. *Biochem J* **391**: 143-151

Minn AJ, Gupta GP, Siegel PM, Bos PD, Shu W, Giri DD, Viale A, Olshen AB, Gerald WL, Massague J (2005a) Genes that mediate breast cancer metastasis to lung. *Nature* **436**: 518-524

Minn AJ, Kang Y, Serganova I, Gupta GP, Giri DD, Doubrovin M, Ponomarev V, Gerald WL, Blasberg R, Massague J (2005b) Distinct organ-specific metastatic potential of individual breast cancer cells and primary tumors. *J Clin Invest* **115**: 44-55

Miyazono K, Hellman U, Wernstedt C, Heldin CH (1988) Latent high molecular weight complex of transforming growth factor beta 1. Purification from human platelets and structural characterization. *J Biol Chem* **263**: 6407-6415

Mosadegh B, Saadi W, Wang SJ, Jeon NL (2008) Epidermal growth factor promotes breast cancer cell chemotaxis in CXCL12 gradients. *Biotechnol Bioeng* **100**: 1205-1213

Moser AR, Pitot HC, Dove WF (1990) A dominant mutation that predisposes to multiple intestinal neoplasia in the mouse. *Science* **247**: 322-324

Muller WJ, Sinn E, Pattengale PK, Wallace R, Leder P (1988) Single-step induction of mammary adenocarcinoma in transgenic mice bearing the activated c-neu oncogene. *Cell* **54**: 105-115

Munger JS, Huang XZ, Kawakatsu H, Griffiths MD, Dalton SL, Wu JF, Pittet JF, Kaminski N, Garat C, Matthay MA, Rifkin DB, Sheppard D (1999) The integrin alpha v beta 6 binds and activates latent TGF beta 1: A mechanism for regulating pulmonary inflammation and fibrosis. *Cell* **96**: 319-328

Munoz NM, Upton M, Rojas A, Washington MK, Lin L, Chytil A, Sozmen EG, Madison BB, Pozzi A, Moon RT, Moses HL, Grady WM (2006) Transforming growth factor beta receptor type II inactivation induces the malignant transformation of intestinal neoplasms initiated by Apc mutation. *Cancer Res* **66**: 9837-9844

Muraoka RS, Koh Y, Roebuck LR, Sanders ME, Brantley-Sieders D, Gorska AE, Moses HL, Arteaga CL (2003) Increased malignancy of Neu-induced mammary tumors overexpressing active transforming growth factor beta1. *Mol Cell Biol* **23**: 8691-8703

Muraoka-Cook RS, Kurokawa H, Koh Y, Forbes JT, Roebuck LR, Barcellos-Hoff MH, Moody SE, Chodosh LA, Arteaga CL (2004) Conditional overexpression of active transforming growth factor beta1 in vivo accelerates metastases of transgenic mammary tumors. *Cancer Res* **64**: 9002-9011

Muraoka-Cook RS, Shin I, Yi JY, Easterly E, Barcellos-Hoff MH, Yingling JM, Zent R, Arteaga CL (2006) Activated type I TGFbeta receptor kinase enhances the survival of mammary epithelial cells and accelerates tumor progression. *Oncog* **25**: 3408-3423

Nagy P, Vereb G, Sebestyen Z, Horvath G, Lockett SJ, Damjanovich S, Park JW, Jovin TM, Szollosi J (2002) Lipid rafts and the local density of ErbB proteins influence the biological role of homo- and heteroassociations of ErbB2. *J Cell Sci* **115**: 4251-4262

Nelson CM, Vanduijn MM, Inman JL, Fletcher DA, Bissell MJ (2006) Tissue geometry determines sites of mammary branching morphogenesis in organotypic cultures. *Science* **314**: 298-300

Nevanlinna H, Bartek J (2006) The CHEK2 gene and inherited breast cancer susceptibility. *Oncog* **25**: 5912-5919

ntonelli-Orlidge A, Saunders KB, Smith SR, D'Amore PA (1989) An activated form of transforming growth factor beta is produced by cocultures of endothelial cells and pericytes. *Proc Natl Acad Sci U S A* **86**: 4544-4548

O'Connell P, Albertsen H, Matsunami N, Taylor T, Hundley JE, Johnson-Pais TL, Reus B, Lawrence E, Ballard L, White R, . (1994) A radiation hybrid map of the BRCA1 region. *Am J Hum Genet* **54**: 526-534

Oft M, Heider KH, Beug H (1998) TGFbeta signaling is necessary for carcinoma cell invasiveness and metastasis. *Curr Biol* **8**: 1243-1252

Oft M, Peli J, Rudaz C, Schwarz H, Beug H, Reichmann E (1996) TGF-beta1 and Ha-Ras collaborate in modulating the phenotypic plasticity and invasiveness of epithelial tumor cells. *Genes Dev* **10**: 2462-2477

Oike Y, Yasunaga K, Suda T (2004) Angiopoietin-related/angiopoietin-like proteins regulate angiogenesis. *Int J Hematol* **80**: 21-28

Okadome T, Oeda E, Saitoh M, Ichijo H, Moses HL, Miyazono K, Kawabata M (1996) Characterization of the interaction of FKBP12 with the transforming growth factor-beta type I receptor in vivo. *J Biol Chem* **271**: 21687-21690

Olayioye MA, Neve RM, Lane HA, Hynes NE (2000) The ErbB signaling network: receptor heterodimerization in development and cancer. *EMBO J* **19**: 3159-3167

Padua D, Zhang XH, Wang Q, Nadal C, Gerald WL, Gomis RR, Massague J (2008) TGFbeta primes breast tumors for lung metastasis seeding through angiopoietin-like 4. *Cell* **133**: 66-77

Park SH (2005) Fine tuning and cross-talking of TGF-beta signal by inhibitory Smads. *J Biochem Mol Biol* **38**: 9-16

Pasche B, Kolachana P, Nafa K, Satagopan J, Chen YG, Lo RS, Brener D, Yang D, Kirstein L, Oddoux C, Ostrer H, Vineis P, Varesco L, Jhanwar S, Luzzatto L, Massague J, Offit K (1999) TbetaR-I(6A) is a candidate tumor susceptibility allele. *Cancer Res* **59**: 5678-5682

Pasche B, Luo Y, Rao PH, Nimer SD, Dmitrovsky E, Caron P, Luzzatto L, Offit K, Cordon-Cardo C, Renault B, Satagopan JM, Murty VV, Massague J (1998) Type I transforming growth factor beta receptor maps to 9q22 and exhibits a polymorphism and a rare variant within a polyalanine tract. *Cancer Res* **58**: 2727-2732

Pasche B, Kaklamani VG, Hou N, Young T, Rademaker A, Peterlongo P, Ellis N, Offit K, Caldes T, Reiss M, Zheng T (2004) TGFBR1*6A and Cancer: A Meta-Analysis of 12 Case-Control Studies. *J Clin Oncol* **22**: 756-758

Pasche B, Knobloch TJ, Bian Y, Liu J, Phukan S, Rosman D, Kaklamani V, Baddi L, Siddiqui FS, Frankel W, Prior TW, Schuller DE, Agrawal A, Lang J, Dolan ME, Vokes EE, Lane WS, Huang CC, Caldes T, Di Cristofano A, Hampel H, Nilsson I, von Heijne G, Fodde R, Murty VVVS, de la Chapelle A, Weghorst CM (2005) Somatic Acquisition and Signaling of TGFBR1*6A in Cancer. *JAMA: The Journal of the American Medical Association* **294**: 1634-1646

Pechkovsky DV, Scaffidi AK, Hackett TL, Ballard J, Shaheen F, Thompson PJ, Thannickal VJ, Knight DA (2008) Transforming growth factor beta1 induces alphavbeta3 integrin expression in human lung fibroblasts via a beta3 integrin-, c-Src-, and p38 MAPK-dependent pathway. *J Biol Chem* **283**: 12898-12908

Petrij-Bosch A, Peelen T, van VM, van ER, Olmer R, Drusedau M, Hogervorst FB, Hageman S, Arts PJ, Ligtenberg MJ, Meijers-Heijboer H, Klijn JG, Vasen HF, Cornelisse CJ, van ', V,

Bakker E, van Ommen GJ, Devilee P (1997) BRCA1 genomic deletions are major founder mutations in Dutch breast cancer patients. *Nat Genet* **17**: 341-345

Pierce DF, Jr., Gorska AE, Chytil A, Meise KS, Page DL, Coffey RJ, Jr., Moses HL (1995) Mammary tumor suppression by transforming growth factor beta 1 transgene expression. *Proc Natl Acad Sci U S A* **92**: 4254-4258

Pierce DF, Jr., Johnson MD, Matsui Y, Robinson SD, Gold LI, Purchio AF, Daniel CW, Hogan BL, Moses HL (1993) Inhibition of mammary duct development but not alveolar outgrowth during pregnancy in transgenic mice expressing active TGF-beta 1. *Genes Dev* **7**: 2308-2317

Pierreux CE, Nicolas FJ, Hill CS (2000) Transforming growth factor beta-independent shuttling of Smad4 between the cytoplasm and nucleus. *Mol Cell Biol* **20**: 9041-9054

Pietenpol JA, Holt JT, Stein RW, Moses HL (1990) Transforming growth factor beta 1 suppression of c-myc gene transcription: role in inhibition of keratinocyte proliferation. *Proc Natl Acad Sci U S A* **87**: 3758-3762

Pille JY, Denoyelle C, Varet J, Bertrand JR, Soria J, Opolon P, Lu H, Pritchard LL, Vannier JP, Malvy C, Soria C, Li H (2005) Anti-RhoA and anti-RhoC siRNAs inhibit the proliferation and invasiveness of MDA-MB-231 breast cancer cells in vitro and in vivo. *Mol Ther* **11**: 267-274

Pinto M, Oliveira C, Cirnes L, Carlos MJ, Ramires M, Nogueira A, Carneiro F, Seruca R (2003) Promoter methylation of TGFbeta receptor I and mutation of TGFbeta receptor II are frequent events in MSI sporadic gastric carcinomas. *J Pathol* **200**: 32-38

Rahimi RA, Leof EB (2007) TGF-beta signaling: a tale of two responses. *J Cell Biochem* **102**: 593-608

Rapoport TA, Jungnickel B, Kutay U (1996) Protein transport across the eukaryotic endoplasmic reticulum and bacterial inner membranes. *Annu Rev Biochem* **65**: 271-303

Reid T, Furuyashiki T, Ishizaki T, Watanabe G, Watanabe N, Fujisawa K, Morii N, Madaule P, Narumiya S (1996) Rhotekin, a new putative target for Rho bearing homology to a serine/threonine kinase, PKN, and rhophilin in the rho-binding domain. *J Biol Chem* **271**: 13556-13560

Ren XD, Kiosses WB, Schwartz MA (1999) Regulation of the small GTP-binding protein Rho by cell adhesion and the cytoskeleton. *EMBO J* **18**: 578-585

Ren XD, Schwartz MA (2000) Determination of GTP loading on Rho. *Methods Enzymol* **325**: 264-272

Ridley AJ (2001) Rho GTPases and cell migration. *J Cell Sci* **114**: 2713-2722

Roberts AB, Anzano MA, Wakefield LM, Roche NS, Stern DF, Sporn MB (1985) Type beta transforming growth factor: a bifunctional regulator of cellular growth. *Proc Natl Acad Sci U S A* **82**: 119-123

Roberts AB, Sporn MB, Assoian RK, Smith JM, Roche NS, Wakefield LM, Heine UI, Liotta LA, Falanga V, Kehrl JH, . (1986) Transforming growth factor type beta: rapid induction of fibrosis and angiogenesis in vivo and stimulation of collagen formation in vitro. *Proc Natl Acad Sci U S A* **83**: 4167-4171

Rohlfs EM, Puget N, Graham ML, Weber BL, Garber JE, Skrzynia C, Halperin JL, Lenoir GM, Silverman LM, Mazoyer S (2000) An Alu-mediated 7.1 kb deletion of BRCA1 exons 8 and 9 in breast and ovarian cancer families that results in alternative splicing of exon 10. *Genes Chromosomes Cancer* **28**: 300-307

Rosman DS, Phukan S, Huang CC, Pasche B (2008) TGFBR1*6A enhances the migration and invasion of MCF-7 breast cancer cells through RhoA activation. *Cancer Res* **68**: 1319-1328

Sacco MG, Gribaldo L, Barbieri O, Turchi G, Zucchi I, Collotta A, Bagnasco L, Barone D, Montagna C, Villa A, Marafante E, Vezzoni P (1998) Establishment and characterization of a new mammary adenocarcinoma cell line derived from MMTV neu transgenic mice. *Breast Cancer Res Treat* **47**: 171-180

Safina A, Vandette E, Bakin AV (2006) ALK5 promotes tumor angiogenesis by upregulating matrix metalloproteinase-9 in tumor cells. *Oncog*

Saha D, Datta PK, Beauchamp RD (2001) Oncogenic ras represses transforming growth factor-beta /Smad signaling by degrading tumor suppressor Smad4. *J Biol Chem* **276**: 29531-29537

Saharinen J, Hyytiainen M, Taipale J, Keski-Oja J (1999) Latent transforming growth factor-beta binding proteins (LTBPs)--structural extracellular matrix proteins for targeting TGF-beta action. *Cytokine Growth Factor Rev* **10**: 99-117

Sato Y, Rifkin DB (1989) Inhibition of endothelial cell movement by pericytes and smooth muscle cells: activation of a latent transforming growth factor-beta 1-like molecule by plasmin during co-culture. *J Cell Biol* **109**: 309-315

Sato Y, Tsuboi R, Lyons R, Moses H, Rifkin DB (1990) Characterization of the activation of latent TGF-beta by co-cultures of endothelial cells and pericytes or smooth muscle cells: a self-regulating system. *J Cell Biol* **111**: 757-763

Schmierer B, Hill CS (2007) TGFbeta-SMAD signal transduction: molecular specificity and functional flexibility. *Nat Rev Mol Cell Biol* **8**: 970-982

Schultz-Cherry S, Murphy-Ullrich JE (1993) Thrombospondin causes activation of latent transforming growth factor-beta secreted by endothelial cells by a novel mechanism. *J Cell Biol* **122**: 923-932

Seal S, Thompson D, Renwick A, Elliott A, Kelly P, Barfoot R, Chagtai T, Jayatilake H, Ahmed M, Spanova K, North B, McGuffog L, Evans DG, Eccles D, Easton DF, Stratton MR, Rahman N (2006) Truncating mutations in the Fanconi anemia J gene BRIP1 are low-penetrance breast cancer susceptibility alleles. *Nat Genet*

Secker GA, Shortt AJ, Sampson E, Schwarz QP, Schultz GS, Daniels JT (2008) TGFbeta stimulated re-epithelialisation is regulated by CTGF and Ras/MEK/ERK signalling. *Exp Cell Res* **314**: 131-142

Sekelsky JJ, Newfeld SJ, Raftery LA, Chartoff EH, Gelbart WM (1995) Genetic characterization and cloning of mothers against dpp, a gene required for decapentaplegic function in Drosophila melanogaster. *Genetics* **139**: 1347-1358

Sekimoto G, Matsuzaki K, Yoshida K, Mori S, Murata M, Seki T, Matsui H, Fujisawa J, Okazaki K (2007) Reversible Smad-dependent signaling between tumor suppression and oncogenesis. *Cancer Res* **67**: 5090-5096

Seton-Rogers SE, Lu Y, Hines LM, Koundinya M, LaBaer J, Muthuswamy SK, Brugge JS (2004) Cooperation of the ErbB2 receptor and transforming growth factor beta in induction of migration and invasion in mammary epithelial cells. *Proc Natl Acad Sci U S A* **101**: 1257-1262

Shi Y, Massague J (2003) Mechanisms of TGF-beta signaling from cell membrane to the nucleus. *Cell* **113**: 685-700

Shipitsin M, Campbell LL, Argani P, Weremowicz S, Bloushtain-Qimron N, Yao J, Nikolskaya T, Serebryiskaya T, Beroukhim R, Hu M, Halushka MK, Sukumar S, Parker LM, Anderson KS, Harris LN, Garber JE, Richardson AL, Schnitt SJ, Nikolsky Y, Gelman RS, Polyak K (2007) Molecular definition of breast tumor heterogeneity. *Cancer Cell* **11**: 259-273

Siegel PM, Massague J (2003) Cytostatic and apoptotic actions of TGF-beta in homeostasis and cancer. *Nat Rev Cancer* **3**: 807-821

Siegel PM, Shu W, Cardiff RD, Muller WJ, Massague J (2003) Transforming growth factor {beta} signaling impairs Neu-induced mammary tumorigenesis while promoting pulmonary metastasis. *PNAS* **100**: 8430

Silberstein GB, Flanders KC, Roberts AB, Daniel CW (1992) Regulation of mammary morphogenesis: evidence for extracellular matrix-mediated inhibition of ductal budding by transforming growth factor-beta 1. *Dev Biol* **152**: 354-362

Slamon DJ, Clark GM, Wong SG, Levin WJ, Ullrich A, McGuire WL (1987) Human breast cancer: correlation of relapse and survival with amplification of the HER-2/neu oncogene. *Science* **235**: 177-182

Slamon DJ, Godolphin W, Jones LA, Holt JA, Wong SG, Keith DE, Levin WJ, Stuart SG, Udove J, Ullrich A, . (1989) Studies of the HER-2/neu proto-oncogene in human breast and ovarian cancer. *Science* **244**: 707-712

Smith HS, Riggs JL, Mosesson MW (1979) Production of fibronectin by human epithelial cells in culture. *Cancer Res* **39**: 4138-4144

Soltoff SP, Cantley LC (1996) p120cbl is a cytosolic adapter protein that associates with phosphoinositide 3-kinase in response to epidermal growth factor in PC12 and other cells. *J Biol Chem* **271**: 563-567

Song B, Margolin S, Skoglund J, Zhou X, Rantala J, Picelli S, Werelius B, Lindblom A (2007) TGFBR1(*)6A and Int7G24A variants of transforming growth factor-beta receptor 1 in Swedish familial and sporadic breast cancer. *Br J Cancer* **97**: 1175-1179

Stratton MR, Rahman N (2008) The emerging landscape of breast cancer susceptibility. *Nat Genet* **40**: 17-22

Su LK, Kinzler KW, Vogelstein B, Preisinger AC, Moser AR, Luongo C, Gould KA, Dove WF (1992) Multiple intestinal neoplasia caused by a mutation in the murine homolog of the APC gene. *Science* **256**: 668-670

Sun PD, Davies DR (1995) The cystine-knot growth-factor superfamily. *Annu Rev Biophys Biomol Struct* **24**: 269-291

Syrjakoski K, Kuukasjarvi T, Auvinen A, Kallioniemi OP (2004) CHEK2 1100delC is not a risk factor for male breast cancer population. *Int J Cancer* **108**: 475-476

Takei H, Iino Y, Horiguchi J, Maemura M, Koibuchi Y, Nagaoka H, Yokoe T, Oyama T, Morishita Y (1998) Angiogenesis and stromal fibronectin expression in invasive breast carcinoma. *Int J Oncol* **12**: 517-523

Taketo M, Schroeder AC, Mobraaten LE, Gunning KB, Hanten G, Fox RR, Roderick TH, Stewart CL, Lilly F, Hansen CT, . (1991) FVB/N: an inbred mouse strain preferable for transgenic analyses. *Proc Natl Acad Sci U S A* **88**: 2065-2069

Teicher BA (2007) Transforming growth factor-beta and the immune response to malignant disease. *Clin Cancer Res* **13**: 6247-6251

Ten DP, Geurts van Kessel AH, Foulkes JG, Le Beau MM (1988) Transforming growth factor type beta 3 maps to human chromosome 14, region q23-q24. *Oncog* **3**: 721-724

Thiery JP (2003) Epithelial-mesenchymal transitions in development and pathologies. *Curr Opin Cell Biol* **15**: 740-746

Tian F, Byfield SD, Parks WT, Stuelten CH, Nemani D, Zhang YE, Roberts AB (2004) Smad-binding defective mutant of transforming growth factor beta type I receptor enhances tumorigenesis but suppresses metastasis of breast cancer cell lines. *Cancer Res* **64**: 4523-4530

Tsou HC, Teng DH, Ping XL, Brancolini V, Davis T, Hu R, Xie XX, Gruener AC, Schrager CA, Christiano AM, Eng C, Steck P, Ott J, Tavtigian SV, Peacocke M (1997) The role of MMAC1

mutations in early-onset breast cancer: causative in association with Cowden syndrome and excluded in BRCA1-negative cases. *Am J Hum Genet* **61**: 1036-1043

Tsukazaki T, Chiang TA, Davison AF, Attisano L, Wrana JL (1998) SARA, a FYVE domain protein that recruits Smad2 to the TGFbeta receptor. *Cell* **95**: 779-791

Tzahar E, Waterman H, Chen X, Levkowitz G, Karunagaran D, Lavi S, Ratzkin BJ, Yarden Y (1996) A hierarchical network of interreceptor interactions determines signal transduction by Neu differentiation factor/neuregulin and epidermal growth factor. *Mol Cell Biol* **16**: 5276-5287

Ueda Y, Wang S, Dumont N, Yi JY, Koh Y, Arteaga CL (2004) Overexpression of HER2 (erbB2) in human breast epithelial cells unmasks transforming growth factor beta-induced cell motility. *J Biol Chem* **279**: 24505-24513

Ueki N, Nakazato M, Ohkawa T, Ikeda T, Amuro Y, Hada T, Higashino K (1992) Excessive production of transforming growth-factor beta 1 can play an important role in the development of tumorigenesis by its action for angiogenesis: validity of neutralizing antibodies to block tumor growth. *Biochim Biophys Acta* **1137**: 189-196

Urtreger A, Porro F, Puricelli L, Werbajh S, Baralle FE, Bal de Kier JE, Kornblihtt AR, Muro AF (1998) Expression of RGD minus fibronectin that does not form extracellular matrix fibrils is sufficient to decrease tumor metastasis. *Int J Cancer* **78**: 233-241

Urtreger AJ, Werbajh SE, Verrecchia F, Mauviel A, Puricelli LI, Kornblihtt AR, Bal de Kier Joffe ED (2006) Fibronectin is distinctly downregulated in murine mammary adenocarcinoma cells with high metastatic potential. *Oncol Rep* **16**: 1403-1410

Valle L, Serena-Acedo T, Liyanarachchi S, Hampel H, Comeras I, Li Z, Zeng Q, Zhang HT, Pennison MJ, Sadim M, Pasche B, Tanner SM, de la CA (2008) Germline Allele-specific Expression of TGFBR1 Confers an Increased Risk of Colorectal Cancer. *Science*

Wain HM, Bruford EA, Lovering RC, Lush MJ, Wright MW, Povey S (2002) Guidelines for human gene nomenclature. *Genomics* **79**: 464-470

Wakefield LM, Smith DM, Flanders KC, Sporn MB (1988) Latent transforming growth factor-beta from human platelets. A high molecular weight complex containing precursor sequences. *J Biol Chem* **263**: 7646-7654

Walsh T, Casadei S, Coats KH, Swisher E, Stray SM, Higgins J, Roach KC, Mandell J, Lee MK, Ciernikova S, Foretova L, Soucek P, King MC (2006) Spectrum of mutations in BRCA1, BRCA2, CHEK2, and TP53 in families at high risk of breast cancer. *JAMA* **295**: 1379-1388

Wang D, Kanuma T, Mizunuma H, Takama F, Ibuki Y, Wake N, Mogi A, Shitara Y, Takenoshita S (2000) Analysis of specific gene mutations in the transforming growth factor-beta signal transduction pathway in human ovarian cancer. *Cancer Res* **60**: 4507-4512

Watanabe M, Masuyama N, Fukuda M, Nishida E (2000) Regulation of intracellular dynamics of Smad4 by its leucine-rich nuclear export signal. *EMBO Rep* **1**: 176-182

Wei YY, Chen YJ, Hsiao YC, Huang YC, Lai TH, Tang CH (2008) Osteoblasts-derived TGF-beta1 enhance motility and integrin upregulation through Akt, ERK, and NF-kappaB-dependent pathway in human breast cancer cells. *Mol Carcinog* **47**: 526-537

Wilkins JA, Sansom OJ (2008) C-Myc is a critical mediator of the phenotypes of Apc loss in the intestine. *Cancer Res* **68**: 4963-4966

Wooster R, Bignell G, Lancaster J, Swift S, Seal S, Mangion J, Collins N, Gregory S, Gumbs C, Micklem G (1995) Identification of the breast cancer susceptibility gene BRCA2. *Nature* **378**: 789-792

Wotton D, Lo RS, Lee S, Massague J (1999) A Smad transcriptional corepressor. *Cell* **97**: 29-39

Wrana JL, Attisano L, Carcamo J, Zentella A, Doody J, Laiho M, Wang XF, Massague J (1992) TGF beta signals through a heteromeric protein kinase receptor complex. *Cell* **71**: 1003-1014

Wu MH, Chou YC, Yu JC, Yu CP, Wu CC, Chu CM, Yang T, Lai CH, Hsieh CY, You SL, Chen CJ, Sun CA (2006) Hormonal and body-size factors in relation to breast cancer risk: a prospective study of 11,889 women in a low-incidence area. *Ann Epidemiol* **16**: 223-229

Wyckoff JB, Jones JG, Condeelis JS, Segall JE (2000) A critical step in metastasis: in vivo analysis of intravasation at the primary tumor. *Cancer Res* **60**: 2504-2511

Xie W, Mertens JC, Reiss DJ, Rimm DL, Camp RL, Haffty BG, Reiss M (2002) Alterations of Smad signaling in human breast carcinoma are associated with poor outcome: a tissue microarray study. *Cancer Res* **62**: 497-505

Yamagata H, Matsuzaki K, Mori S, Yoshida K, Tahashi Y, Furukawa F, Sekimoto G, Watanabe T, Uemura Y, Sakaida N, Yoshioka K, Kamiyama Y, Seki T, Okazaki K (2005) Acceleration of Smad2 and Smad3 phosphorylation via c-Jun NH(2)-terminal kinase during human colorectal carcinogenesis. *Cancer Res* **65**: 157-165

Yang EY, Moses HL (1990) Transforming growth factor beta 1-induced changes in cell migration, proliferation, and angiogenesis in the chicken chorioallantoic membrane. *J Cell Biol* **111**: 731-741

Yang L, DeBusk LM, Fukuda K, Fingleton B, Green-Jarvis B, Shyr Y, Matrisian LM, Carbone DP, Lin PC (2004) Expansion of myeloid immune suppressor Gr+CD11b+ cells in tumor-bearing host directly promotes tumor angiogenesis. *Cancer Cell* **6**: 409-421

Yang L, Huang J, Ren X, Gorska AE, Chytil A, Aakre M, Carbone DP, Matrisian LM, Richmond A, Lin PC, Moses HL (2008) Abrogation of TGF beta signaling in mammary carcinomas recruits Gr-1+CD11b+ myeloid cells that promote metastasis. *Cancer Cell* **13**: 23-35

Yarden Y, Sliwkowski MX (2001) Untangling the ErbB signalling network. *Nat Rev Mol Cell Biol* **2**: 127-137

Ye M, Hu D, Tu L, Zhou X, Lu F, Wen B, Wu W, Lin Y, Zhou Z, Qu J (2008) Involvement of PI3K/Akt signaling pathway in hepatocyte growth factor-induced migration of uveal melanoma cells. *Invest Ophthalmol Vis Sci* **49**: 497-504

Yokota M, Ichihara S, Lin TL, Nakashima N, Yamada Y (2000) Association of a t29 -> C polymorphism of the transforming growth factor-beta 1 gene with genetic susceptibility to myocardial infarction in Japanese. *Circulation* **101**: 2783-2787

Yoshida K, Miki Y (2004) Role of BRCA1 and BRCA2 as regulators of DNA repair, transcription, and cell cycle in response to DNA damage. *Cancer Sci* **95**: 866-871

Zavadil J, Bitzer M, Liang D, Yang YC, Massimi A, Kneitz S, Piek E, Bottinger EP (2001) Genetic programs of epithelial cell plasticity directed by transforming growth factor-beta. *Proc Natl Acad Sci U S A* **98**: 6686-6691

Zavadil J, Bottinger EP (2005) TGF-beta and epithelial-to-mesenchymal transitions. *Oncog* **24**: 5764-5774

Zawel L, Dai JL, Buckhaults P, Zhou S, Kinzler KW, Vogelstein B, Kern SE (1998) Human Smad3 and Smad4 are sequence-specific transcription activators. *Mol Cell* **1**: 611-617

Zhang HT, Fei QY, Chen F, Qi QY, Zou W, Wang JC, Zhang RM, Tao SH, Chen XF, Luo ZW (2003) Mutational analysis of the transforming growth factor beta receptor type I gene in primary non-small cell lung cancer. *Lung Cancer* **40**: 281-287

Zhang HT, Zhao J, Zheng SY, Chen XF (2005) Is TGFBR1*6A Really Associated With Increased Risk of Cancer? *J Clin Oncol* **23**: 7743-7744

Ziv E, Cauley J, Morin PA, Saiz R, Browner WS (2001) Association between the T29-->C polymorphism in the transforming growth factor beta1 gene and breast cancer among elderly white women: The Study of Osteoporotic Fractures. *JAMA* **285**: 2859-2863

Zuo Y, Shields SK, Chakraborty C (2006) Enhanced intrinsic migration of aggressive breast cancer cells by inhibition of Rac1 GTPase. *Biochem Biophys Res Commun* **351**: 361-367

# VITA

**Name:** Diana Susan Rosman

**Born**: Morristown, New Jersey, USA

**Education:**

**Northwestern University**, Chicago, IL
Integrated Graduate Program in Life Sciences,
PhD, Cell and Molecular Biology; Concentration in Cancer Biology, September 2003-present.
GPA 3.3/4.0 Graduation, September, 2008

**Boston University**, Boston, MA
B.A. Biochemistry and Molecular Biology, College of Arts and Science, May 2003.
GPA 3.39/4.0 Cum Laude

**Grants and Awards**

**Northwestern University**

- Katten Muchin Rosenman Travel Scholarship Program, The Robert H. Lurie Comprehensive Cancer Center of Northwestern University, 2007
- Chicago Baseball Cancer Charities Fellowship, Northwestern University, 2005-2006.
- Malkin Scholar, Northwestern University, 2005

**Boston University**

- Funded Research Opportunity Grant, Boston University Undergraduate Research Opportunities Program, 2003

**Research Experience**

**Northwestern University**, Chicago, IL September 2003-present
Graduate Student Department of Medicine, Division of Hematology/ Oncology, Advisor: Boris Pasche, MD, PhD

- Identified, designed, and developed a personalized thesis project titled: The Role of *TGFBR1* Variants in Breast Cancer
- Techniques included: cell culture ▪ mouse work (breeding, measuring tumors, dissecting) ▪ migration and invasion transwell assays ▪ western blotting ▪ growth inhibition assays ▪ immunofluorescence ▪ immunohistochemistry ▪ isolation of cells from mouse embryos and tissues ▪ PCR ▪ gene array
- Designed and presented two posters for two different conferences
- Presented a lecture for the departmental Tumor Cell Biology weekly seminar series

**Boston University**, Boston, MA September 2002- May 2003
Undergraduate Research Student (Biology Department, Advisor: Thomas D. Gilmore, PhD)

- Investigating a project entitled "The Characterization of a Synthetic Derivative of a Fungal Compound That Has Increased Bioactivity"
- Techniques included: gel electrophoresis ▪ Western blotting ▪ cell culturing

**Pfizer Global Research and Development**, Groton, CT May 2002 – August 2002

Summer Associate (Cancer Department)

- Designed and presented a poster examining the effects of a small molecule anti-angiogenesis inhibitor on endothelial/tumor cell apoptosis
- Techniques included: immunohistochemistry ▪ immunoprecipitation ▪ western blotting ▪ fluorescence activated cell sorting (FACS) analysis ▪ cell culturing ▪ vascular permeability assays
- Observed i*n vivo* techniques: tumor cell injections (xenographs) ▪ dosing ▪ collection of organs and tumors in nude mice

**Boston University**, Boston, MA January 2002 – May 2002

Undergraduate Research Student (Center for Advanced Biotechnology**,** Advisor: Cassandra Smith, PhD**)**

- Investigated a project titled "Assessment of Tissue-Specific Changes in *Rattus norvegicus*"
- Techniques included: isolation, amplification, fingerprinting, and analysis of Rat DNA

**University of Florida**, Gainesville, FL June 2001 – August 2001

Undergraduate Research Student (Department of Zoology, Advisor: Peter Piermarini, PhD)

- Investigated a project to determine how the change in water salinity affects Na,K- ATPase expression in the gills of sting rays
- Techniques included: Western blotting ▪ immunohistochemistry

## Publications

Zeng, Q., Phukan, S., Xu, Y., Sadim, M., **Rosman, D.S**., Liao, J., Zhang, M., Yang, G-Y., Huang, C-C., Valle, L., Di Cristofano, A., de la Chapelle, A., Pasche, B. *Tgfbr1* haploinsufficiency is a potent modifier of colon cancer development. Manuscript in preparation.

**Rosman, D.S.,** Phukan, S., Huang, C., Pasche, B. (2008) *TGFBR1**6A enhances the migration and invasion of MCF-7 breast cancer cells through RhoA activation. *Cancer Research*, 68(5): 1319-1328.

**Rosman, D.S.,** Kaklamani, V., Pasche, B. (2007) New insights into Breast Cancer genetics and impact on patient management. *Current Treatment Options in Oncology*, 8:61-73.

Liang, M.C., Bardhan, S., Pace, E.A., **Rosman, D**., Beutler, J.A, Porco, J.A., Gilmore, T.D. (2006) Inhibition of transcription factor NF-kappaB signaling proteins IKKbeta and p65 through specific cystein residues by epoxyquinone A monomer: a correlation with its anti-cancer cell growth activity. *Biochemical Pharmacology,* 71(5):634-45.

Pasche, B., Knobloch, T.J., Bian, Y, Liu, J., Phukan, S., **Rosman, D**.*, Kaklamani, V., Baddi, L., Siddiqui, F.S., Frankel, W.L., Prior, T.W., Schuller, D.E., Agrawal, A., Lang, J., Dolan, E., Vokes, E.E., Lane, W.S., Huang, C.-C., Caldes, T., Di Cristofano, A., Hampel, H., Nilsson, I., von Heijne, G., Fodde, R., Murty, V.V.V.S., de la Chapelle, A., Weghorst, C.W. (2005) Somatic acquisition and signaling of *TGFBR1**6A in cancer. *Journal of the American Medical Association,* 294:1634-1646. (* shared second authorship)

Kaklamani, V., Baddi, L., Liu, J., **Rosman, D**., Bradley, C., Hegarty, C., McDaniel, B., Rademaker, A., Oddoux, C., Ostrer, H., Michel, L., Chen, Y., Ahsan, H., Offit, K., Pasche, B. (2005) Combined genetic assessment of common TGF-β signaling pathway variants may predict breast cancer risk. *Cancer Research,* 65:3454-3461.

Kaklamani, V., Baddi, L., **Rosman, D.**, Liu, J., Ellis, N., Oddoux, C., Ostrer, H., Chen, Y., Ahsan, H., Offit, K**.,** Pasche, B. (2004) No major association between *TGFBR1**6A and prostate cancer. *BMC Genetics,* 5:28-41.

**Poster Presentations**

*TGFBR1**6A Enhances the migration and invasion of MCF-7 breast cancer cells through RhoA activation. **Rosman, D.S**., Huang, C.C., Pasche, B. *Keystone Symposia: TGF-ß Family in Homeostasis and Disease*. Santa Fe, NM. February 2008.

*TGFBR1**6A Enhances Migration of MCF-7 Breast Cancer Cells. **Rosman, D.S**., Pasche, B. *American Association for Cancer Research: TGF-β in Cancer and Other Diseases*. La Jolla, CA. February 2006.

**Teaching**

**Northwestern University**

Teaching assistant, Graduate Level Biochemistry, Fall 2005

- Designed and taught a class lecture
- Graded homework and exams
- Led and taught discussion and review sessions
- Individually tutored students

Lecturer, "The Role of *TGFBR1**6A in Breast Cancer" March 2007. Tumor Cell Biology Seminar Series, Hematology and Oncology departmental weekly seminar series.

Mentor, Ilya Chalik, American Cancer Society High School Intern. Summer 2005,

- Designed a project to determine the role of *TGFBR1*6A* on the mediators of TGF-β-induced growth arrest
- Trained student on proper lab techniques and how to keep an accurate lab notebook

Co-Organizer, Tumor Cell Biology Journal Club, 2005-2006

- Weekly seminar series for students in the Tumor Biology track.
- Responsibilities involved choosing monthly discussion topics and recruiting presenters