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Transforming growth factor alpha and mouse models of human breast cancer

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Transforming growth factor alpha (TGFα) is a principal molecule in the normal and neoplastic development of the mammary gland. Binding of TGFα to the epidermal growth factor receptor (EGFR), activates the EGFRs' endogenous tyrosine kinase activity and stimulates growth of the epithelium in the virgin and pregnant mouse mammary gland. TGFα expression can be detected in breast cancer cells *in vivo* and *in vitro* and overexpression can elicit partial transformation or immortalized human and rodent mammary epithelial cells. Despite evidence implicating TGFα in the development of mammary neoplasia, the actual mechanism of TGFα-induced transformation is unclear. Transgenic mouse models targeting heterologus TGFα to the mammary gland have established TGFα overexpression can induce hyperproliferation, hyperplasia and occasional carcinoma. These transgenic studies demonstrated a facilitating, proliferative role for TGFα in the development of neoplasia and implicated several oncogenes that can cooperate with TGFα to transform the mammary epithelium. From studies of EGFR signaling pathways, inhibitory and modulating agents such as anti-EGFR antibodies and specific kinases inhibitors have been used to block the action of this pathway and prevent the development of TGFα-induced neoplasia and tumor formation. Studies in Stat5a knockout mice have established that the JAK2/Stat5a pathway can facilitate the survival of the mammary epithelium and can impact the progression of TGFα-mandated mammary tumorigenesis. Together these experiments indicate that TGFα and the EGFR signaling pathway are potentially amenable to therapies for treatment of human breast disease. *Oncogene* (2000) **19,** 1085–1091.

Keywords: TGF alpha; mammary gland; transformation; cancer; mouse models; transgenic

Introduction

Development and progression of breast cancer, like many other types of human cancer, is dependent on the progressive corruption and alteration of normal signaling pathways. Signaling mechanisms that transmit growth signals are a predominant target for carcinogenic and oncogenic alterations as they often confer a growth advantage to the pre-neoplastic cell. One of the primary growth factor receptors involved in normal and neoplastic development of the mammary gland is the EGFR and its extended family of related receptors and peptide ligands (Table 1). EGFR and the ligand TGFα, have expression levels altered in the development of cancer in numerous issues. The related family of ligands that bind to these receptors; EGF, heregulin, amphiregulin, and cripto also display alterations in their expression and activity within the pre-neoplastic and transformed cell (for a review see (Dickson and Lippman, 1995; Gullick *et al*., 1999; Schroeder and Lee, 1997)). Despite the well characterized role of the EGFR and TGFα in normal mammary growth signaling, in transformation of mammary epithelial cells *in vitro* and *in vivo* and their elevated expression in some breast cancers, the specific alterations of the EGFR signaling mechanism that lead to TGFα-initiated neoplasia are poorly understood. In an attempt to elucidate these alterations, several transgenic mouse models have been generated to explore the role of TGFα and EGFR in mammary neoplasia. This review will focus on TGFα and its role in transformation of the mammary gland. In addition, it will examine those studies that have attempted to identify the mechanisms of TGFα action in the mammary gland and those molecules that are capable of cooperatively interacting with TGFα to promote tumorigenesis.

TGFα and EGFR expression coincides with normal proliferation in vivo

TGFα is structurally and functionally similar to EGF; the peptides share a 42% identity and can elicit the same biological effects in cultured mammary epithelial cells and explants (Daniel and Silberstein, 1985; Salomon *et al*., 1987; Vonderhaar, 1987). TGFα is often co-expressed with the EGFR and binding to the receptor activates the EGFRs' endogenous tyrosine kinase activity. Mammary epithelial cell lines can be stimulated to proliferate by TGFα (Smith *et al*., 1989; Zajchowski and Sager, 1991) and it can act as an autocrine growth factor in normal and immortalized human mammary epithelial cells. TGFα autocrine and proliferative activity can be blocked in these cells by an anti-EGFR antibody (Bates *et al*., 1990; Kenney *et al*., 1993).

In normal mammary gland development, TGFα and EGF transcripts can be detected in the ductal and lobuloalveolar stages. The virgin mouse mammary gland expresses TGFα in the proliferative cap cells and the stromal fibroblasts around the neck of the terminal endbud, whereas EGF expression is localized

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Table 1

Ligand	*Receptor*
EGF-Like	
EGF	EGFR
TGFα	EGFR
Amphiregulin	EGFR
Epiregulin	EGFR ErbB-4
Betacellulin	EGFR ErbB-3 ErbB-4
Heparin binding-EGF	EGFR ErbB-3 ErbB-4
Heregulin/neuregulins	
Heregulin-1 (α and β)	ErbB-3 ErbB-4
Heregulin-2 (α and β)	ErbB-3 ErbB-4
Heregulin-3	?
Heregulin-4	ErbB-4
Glial growth factor	?
Cripto	
Cripto-1	? unique receptor and erbB4
Cripto-3	?

to the luminal ductal epithelium (Snedeker *et al*., 1991). In the absence of ovarian steroids, exogenous TGFα and EGF can stimulate ductal growth of the mouse mammary epithelium suggesting that they can act independently of secondary signals (Snedeker *et al*., 1991). EGF and TGFα mRNA is present in pregnant and lactating rat and human mammary glands and increases 2–3-fold over virgin levels at pregnancy (Liscia *et al*., 1990). The EGFR is required for ductal development (Wiesen *et al*., 1999) and can be detected in both the stromal and epithelial components of the virgin mammary gland (Coleman *et al*., 1988). In the pregnant gland, a rapid increase is observed during midpregnancy precisely at the time of extensive cellular proliferation (Edery *et al*., 1985). After pregnancy, the levels of EGFR decrease significantly. The coincident expression of TGFα and its receptor with the proliferative phases of mammary epithelial growth and TGFαs' direct mitogenic effect on epithelium *in vitro* and *in vivo* confirms the functional role for this signaling dyad in the development and proliferation of the mammary epithelium.

TGFα expression is associated with human breast cancer

TGFα is expressed and mitotically active in numerous breast cancer cell lines and has been directly implicated as a modulator of transformation *in vivo* (Borellini and Oka, 1989; Daniel and Silberstein, 1985; de Jong *et al*., 1998a; Salomon *et al*., 1984; Valverius *et al*., 1989). In fact, the discovery of TGFα was based on its ability to transform retrovirally-infected cultured fibroblasts (Todaro *et al*., 1980; de Larco and Todaro, 1978). Expression of TGFα has been identified in pleural effusions from normal mammary gland (Arteaga *et al*., 1988), in invasive ductal carcinoma (Pilichowska *et al*., 1997) and correlates with increased neo-angiogensis (de Jong *et al*., 1998b) in breast tumors. Carcinomas of the breast that have higher level expression of TGFα also express high levels of EGFR, implicating a functional role for the TGFα/EGFR autocrine loop in tumors (Umekita *et al*., 1992). The correlation between levels of EGFR expression and neoplastic transformation *in vivo* is controversial (Gullick and Srinivasan, 1998). Robertson and colleagues demonstrated that 40–60% of neoplasias examined in one study displayed normal levels of EGFR expression (Robertson *et al*., 1996). Only a small proportion of breast cancers has elevated levels of EGFR (Slamon *et al*., 1987), or amplification of EGFR (Peters and Wolff, 1983). This data implies that alterations in EGFR ligands may be more influential on the activity of the EGFR than changes in the levels of receptor expression itself. Alternatively, the formation of heterodimers between EGFR and the other erbB family members may play a role in effecting differential activities in tumorigenesis of the breast.

TGFα can modulate cellular transformation in breast cancer cell lines. In the immortalized human breast cancer cell line, MCF-7, TGFα transfected cells that expressed EGFR were stimulated to grow in a colony forming assay (Ciardiello *et al*., 1990). In a parallel study with fully transformed MCF-7 cells that lacked EGFR, TGFα transfection did not effect a positive growth advantage (Clarke *et al*., 1989). Overexpression of TGFα in the immortalized mouse mammary cell line, NOG-8, generated anchorage independent growth but colonies failed to form tumors in nude mice (Shankar *et al*., 1989). Therefore, expression of TGFα is associated with the ability to stimulate growth, while overexpression is allied with partial transformation *in vitro* and correlated with appearance of carcinomas *in vivo*.

TGFα transgenic mouse models of breast cancer

Mouse models have been developed to study the role of TGFα in the transformation and development of the mammary gland. Several promoters have been utilized to target TGFα both non-specifically and specifically to the mammary gland. These mouse models have established the importance of TGFα in the early stages of neoplastic development of the mammary gland.

Initial studies demonstrating the neoplasia-promoting activity of TGFα were described in mice expressing human TGFα under the control of the zinc-inducible metallothionein (MT) promoter (Jhappan *et al*., 1990). Despite the fact that expression of the transgene was low in the mammary gland and was generally not inducible, these mice displayed increased cellular proliferation and delayed epithelial penetration of the stromal fatpad during ductal development. In a second parallel study, using the MT promoter directing the expression of rat TGFα, Sandgren and colleagues noticed that mice that had passed through multiple pregnancies developed hyperplastic nodules and dysplasia of the mammary epithelium (Sandgren *et al*., 1990). Only one mouse developed a secretory adenocarcinoma. These studies demonstrated that there was an association between the *in vivo* expression of TGFα and the development of mammary hyperplasia.

A study directing TGFα expression specifically to the mammary gland with the mouse mammary tumor virus (MMTV) promoter demonstrated unequivocally the growth and neoplasia promoting activity of TGFα (Matsui *et al*., 1990). Precocious alveolar development, hyperproliferation and hyperplasias were apparent in the mature virgin mouse. The alveolar hyperplasia was present in glands from mature virgin mice, but not in

immature virgin mice, suggesting a requirement for secondary inputs from systemic hormones for any TGFα-dependent hyperplasia to occur. Hyperplastic alveolar nodules and cysts were prominent in the multiparous animal and increased in hyperplastic and dysplastic character with the number of pregnancies. One multiparous mouse developed adenocarcinomas although none were metastatic. A second study was performed with the MMTV-TGFα mice that studied in greater detail the generation of hyperplasia and tumors (Halter *et al*., 1992). At one year, 65% of multiparous and 45% of virgin mice displayed hyperplasia. By 16 months, 40% of the multiparous and 30% of the virgin mice and mice had generated tumors. These studies demonstrated that TGFα could stimulate the proliferation of the mammary epithelium and generate proliferative late pregnancy mammary gland phenotypes in early pregnant animals. In addition, the hyperplasia, dysplasia and frank carcinoma observed with this mouse model suggested that there was a multistage nature to the progression of TGFα-initiated transformation.

A more dramatic mammary phenotype was achieved by targeting rat TGFα exclusively to the mammary gland with the WAP-TGFα transgenic mouse (Sandgren *et al*., 1995). This transgenic model achieved high level TGFα expression during pregnancy and lactation and displayed similar proliferative mammary gland phenotypes as described in the MT and MMTV transgenic models but with an increased incidence and decreased latency of tumors. In addition, the process of involution, in which the gland absorbs and reorganizes a significant portion of its epithelial structure, was delayed in these mice as it was in the original MT mice (Sandgren *et al*., 1990). Latency of tumor appearance was decreased in comparison to the MMTV-TGFα model, but still required several rounds of pregnancy for initial tumor development. A majority of the tumors were well-differentiated glandular adenomas or carcinomas (53%) and fibroadenomas (35%) and retained the glandular characteristics of late pregnant mammary epithelium. The appearance of multiple types of tumor and the retention of normal glandular morphologies suggested that these different types were stages of TGFα-dependent tumor progression. Interestingly, the levels of cyclinD1 were elevated in the WAP-TGFα transgenic glands. The targeted overexpression of this cell cycle regulatory molecule is known to effect mammary transformation (Wang *et al*., 1994) and is in a chromosomal region frequently rearranged in human breast cancer (Dickson *et al*., 1995; Gillett *et al*., 1994, 1996, 1999). This indicates that cyclinD1 may be a cooperative factor in the development of TGFα induced mammary carcinogenesis. The delay in involution was suggested as a mechanism for promoting transformation of the mammary gland as it provided an expanded population of proliferative epithelial cells that could be predisposed to transformation. This seminal paper demonstrated TGFα could transform the mammary

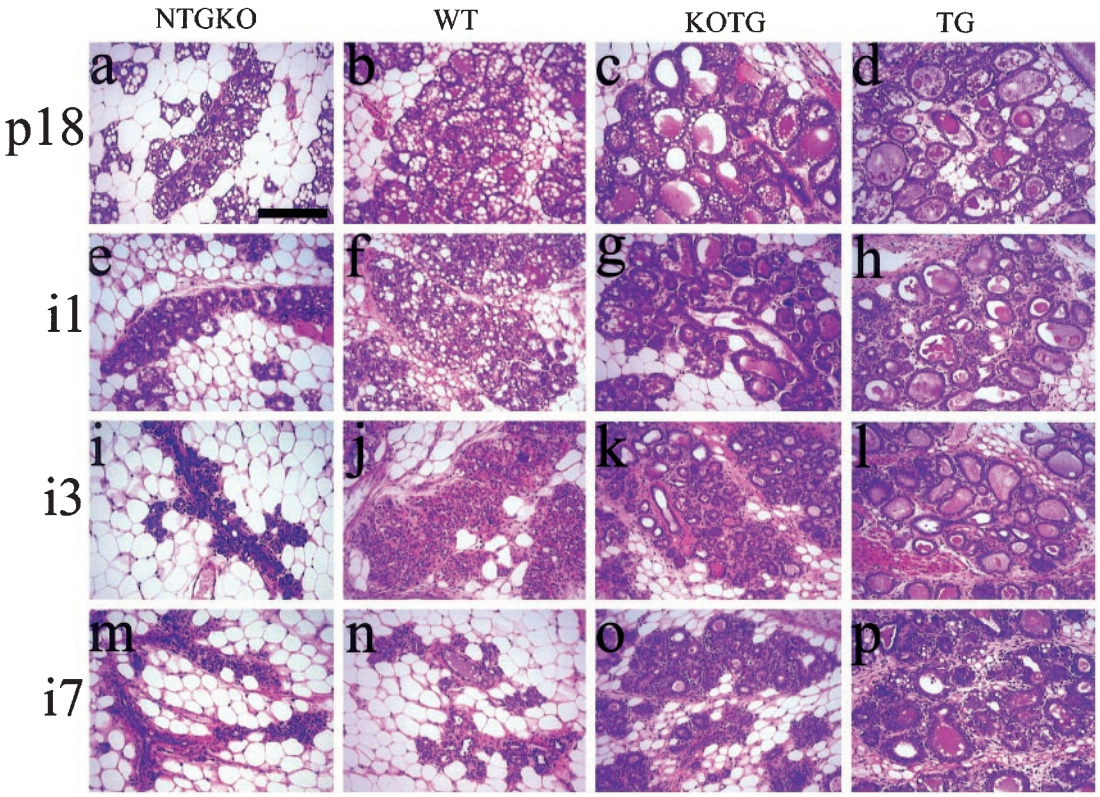

Figure 1 Involution is enhanced in the absence of Stat5a. Hemotoxylin and eosin staining of inguinal mammary glands from Stat5a null non-transgenic (NTGKO), wildtype (WT), Stat5a null TGFα transgenic (KOTG) and TGFα transgenic (TG) mice at 18 days of pregnancy (p18/a–d), days 1 (i1/e–h), 3 (i3/i–l) and 7 (i7/m–p) of involution. Samples were collected from mice in or immediately after their first pregnancy. Compare epithelial condensation at day 3 and 7 of involution in the wildtype (1-j) versus the KOTG (1-k) and TG (1-l). Magnification is defined by the bar in a $=200\ \mu$m. Reproduced with permission (Humphreys and Hennighausen, 1999)

epithelium and suggested a possible mechanism for neoplastic development. The observation that TGFα could cause a delay in involution was supported by results from a separate study with MT-TGFα mice (Smith *et al*., 1995). An increase in DNA synthesis in lactation was accompanied by a significant decrease in apoptotic cells after 2 days of involution. Involution was also affected in a mouse model utilizing WAP-TGFα and the Stat5a knockout mouse (Humphreys and Hennighausen, 1999). In the absence of Stat5a, apoptosis levels rose in the mammary gland during pregnancy and involution. This increase in apoptosis enhanced involution of the WAP-TGFα gland (Figure 1) and increased the latency of WAP-TGFα-induced tumors. This result demonstrated the WAP-TGFα-induced delay in involution and tumor formation, could be abrogated by a downstream signaling molecule that regulates cell death in the mammary epithelium.

Subsequent studies examined the role that oncogenes may play in acting cooperatively with TGFα to promote mammary transformation. The oncogene c-*myc* is overexpressed in 25–30% of breast cancer (Bonilla *et al*., 1988; Callahan and Campbell, 1989; Mariani-Costantini *et al*., 1988, 1989; Morse *et al*., 1988) and in rodent mammary epithelial cell lines transfected with myc, TGFα could cooperate to support a transformed phenotype (Telang *et al*., 1990). Mice that overexpressed both c-*myc* and TGFα in the mammary gland had an increased tumor incidence and decreased tumor latency when compared to c-*myc* transgenics (Amundadottir *et al*., 1995). A second paper utilizing the same bitransgenic mice demonstrated that the synergism between these two proteins was due to a cooperative growth stimulus and inhibition of c-*myc*-induced apoptosis by TGFα (Amundadottir *et al*., 1996). Apoptotic tumors were present exclusively in mice that expressed only c-*myc*. In cell lines derived from these tumors, exogenous TGFα could inhibit apoptosis. These data again implicated TGFα as a survival factor in the mammary epithelium. Interestingly, tumor cells from the mammary glands of these bitransgenic mice could only become apoptotic when exposed to a specific inhibitor of the EGFR kinase pathway. This result suggested that an intact TGFα/EGFR autocrine loop was required to mediate the survival effects of TGFα.

Interaction between TGFα and the proto-oncogene *neu* was examined in bitransgenic mice expressing both

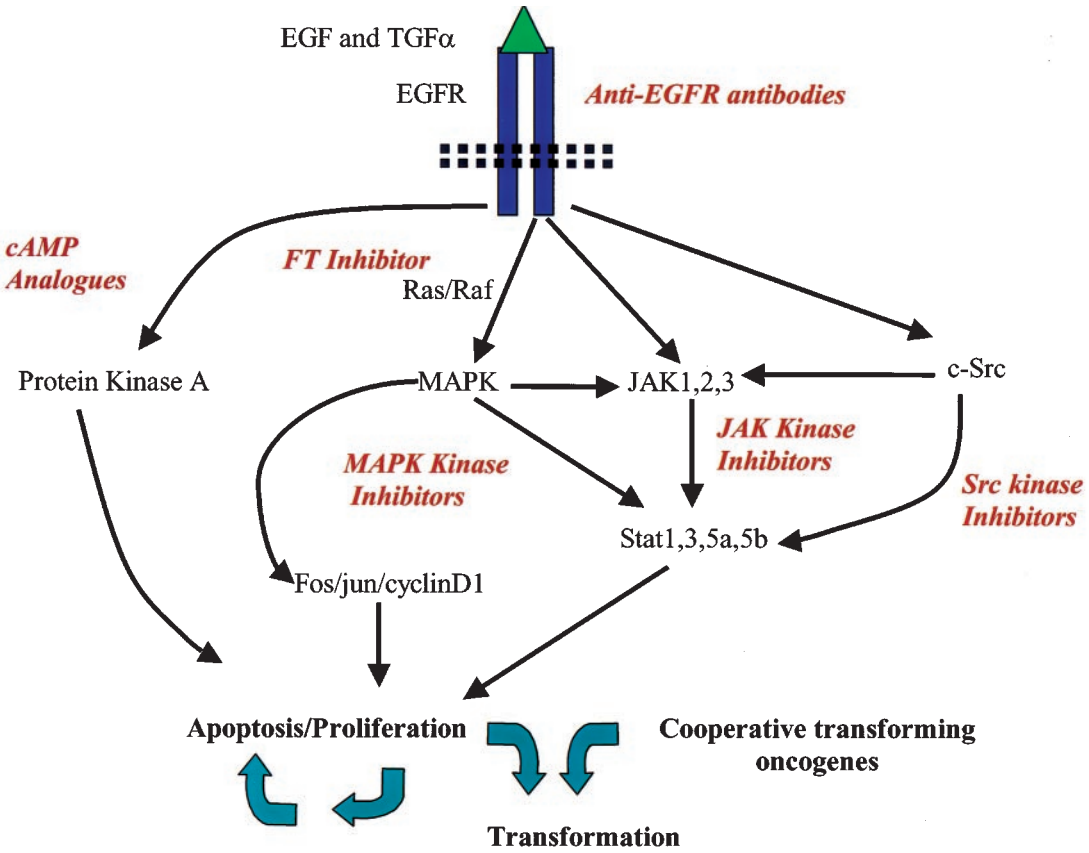

Figure 2 Model of the TGFα/EGF signal transduction pathway and EGFR signaling inhibitors that affect EGFR-mediated transformation. EGF and TGFα binding to the EGFR stimulate the activation of the endogenous receptor tyrosine kinase. The membrane-bound EGFR kinase activates one of several intracellular signal transduction pathways including the protein kinase A, Ras/Raf/MAPK, c-*src* and Jak/Stat pathways. Direct or indirect phosphorylation and activation of one or more of the Stat proteins, 1, 3, 5a, and 5b can be achieved through several of these mechanisms. The activating kinase, attributes of the targeted Stat and phosphorylated residue can elicit distinct functional consequences for the cell. Multiple intercellular pathways can lead to activation of the Stats and other nuclear factors like myc and cyclinD1. Each of these pathways can be blocked by specific inhibitors (shown in red Italics). The proliferative stimulus provided by TGFα and EGF in cooperation with transforming oncogenes can lead to cellular transformation. Consequently, a clear understanding of the mechanisms involved and the use of multifocal inhibitors of signaling intermediaries is critical for effectual inhibition of EGFR signal transduction. Some alternative and intermediary intercellular signaling molecules have been omitted for clarity

genes under the control of the MMTV promoter (Muller *et al*., 1996). The *neu* proto-oncogene is a member of the EGFR family and although it does not bind to TGFα or EGF, it can form heterodimers with the EGFR and it is often found overexpressed and amplified in human breast cancers (Slamon *et al*., 1987). Tumor latency was decreased in the bitransgenic mice, compared to the TGFα and *neu*, monotransgenic lines. At 150 days 95% of the bitransgenic mice had mammary tumors vs 6% and 35% for the TGFα and *neu* mice, respectively. Bitransgenic tumors were multifocal whereas mono-transgenic lines generated focal tumors and contained activated *neu*. This paper suggested a novel mechanism for TGFα cooperativity involving the transactivation of *neu* through the EGFR. Treatment of MMTV-TGFα mice with the tumor promoter 7, 12 dimethyl benzanthracene demonstrated that TGFα could accelerate tumor formation and the authors suggested that TGFα could act as a tumor promoter (Coffey *et al*., 1994).

These studies on the mechanism of TGFα-mediated tumorigenesis and cooperativity in the mammary gland demonstrate that the overexpression of TGFα gives the preneoplastic mammary epithelium a proliferative advantage but in and of itself is not a transforming event. Secondary events, like the activation of proto-oncogenes, can significantly increase the efficiency with which TGFα can transform the mammary epithelium. Importantly, these studies support the theory that TGFα's influence on the regulation of apoptosis in the mammary gland is a possible mechanism of promoting survival of the neoplastic cell.

Disruption of TGFα signaling and potential therapies for breast cancer

The EGFR is required for mammary gland ductal development (Wiesen *et al*., 1999; Xie *et al*., 1997) and, as described previously, is aberrantly expressed in 40% of human breast cancers and occasionally overexpressed in those tumors with poor prognosis. This receptor can interact with several distinct ligands (Table 1) and activates different intracellular and nuclear signaling pathways (Figure 2). Substrates for this type 1 receptor tyrosine kinase are numerous and demonstrate the influence of EGFR on the regulation of cellular growth. Importantly, it is clear that this receptor plays a central role in the development and progression of human breast cancer. Therefore, insight into EGFR signal transduction and the mechanism of substrate selection and activation are critical to understanding the development of breast cancer. The EGFR and its interaction with TGFα has been and continues to be a target for potential anti-cancer therapies that aim to regulate its activity and signaling mechanisms.

A recent study demonstrated that inhibition of the signal from EGFR to its intracellular signaling molecules is critical in blocking tumor progression. Treatment of MMTV-TGFα transgenic mice with a farnesyl transferase inhibitor, which blocks Ras function, demonstrated a significant regression of mammary tumors (Norgaard *et al*., 1999). This inhibitory effect was ineffectual after tumor acceleration from multipregnancy or after treatment with the carcinogen DMBA supporting the theory that that EGFR signaling is important in the initial stage of neoplastic development. The EGFR can activate the intracellular tyrosine kinases Jak1 and the transcription factors Stat1, 3 and 5b in response to growth signaling from EGF (Leonard and O'Shea, 1998). Recent data has demonstrated that growth hormone activated Ras/Raf/MAPK pathway can directly activate the normally cytokine-activated pathway of Stat5a (Pircher *et al*., 1999) and EGF can phosphorylate Stat5a through c-*src* (Olayioye *et al*., 1999). Stat5a, a prolactin-activated transcription factor, has an established role in the development and differentiation of the lactation-competent mammary gland (Liu *et al*., 1987) but is also associated with signaling in tumor cells (Hayakawa *et al*., 1998; Richer *et al*., 1998; Yu *et al*., 1997; Zhang *et al*., 1996). Stat5a-null mice interbred with WAP-TGFα transgenic mice revealed inhibition of TGFα-dependent MAPK activation and TGFα-dependent apoptosis inhibition after deletion of Stat5a. The increase in apoptosis permitted a more complete epithelial regression to occur at involution (Figure 1). A more complete regression deleted a significant number of potentially neoplastic cells and this impacted WAP-TGFα driven tumorigenesis. Stat5a null/WAP TGFα transgenic mice had an increase in tumor latency when compared to the WAP-TGFα transgenic. These mice displayed an increase in apoptosis before and during involution. These data suggested Stat5a was acting as a survival factor for the mammary epithelium by blocking the onset of apoptosis. Theoretically, the absence of Stat5a permitted apoptosis to occur and thereby diminished the pool of potential neoplastic cells that could become transformed (Humphreys and Hennighausen, 1999).

A novel mechanism of inhibition of TGFα activity was demonstrated recently in human mammary epithelial cells with an anti-metalloproteinase. Metalloproteinases are extracellular enzymes that cleave components of the extracellular matrix including the EGFR ligands; TGFα and EGF. This cleavage event releases them from the cell surface rendering the growth factor into an active form. These metalloproteinase inhibitors prevented TGFα release from the cell surface, blocked cell migration and decreased proliferation. Additionally, the metalloproteinase inhibitors reduced the growth of EGF-dependent tumor cell lines and could synergize with anti-EGFR antibodies (Dong *et al*., 1999).

Therapies for inhibiting the action of EGFR and indirectly the action of TGFα, to block tumorigenesis are being explored. Current research into the action of molecules that may have a role in regulating the TGFα signaling pathway like EGFR-specific monoclonal antibodies (Ciardiello *et al*., 1999), protein kinase A inhibitors, a combination of antibodies and chemotheraputic agents (Bianco *et al*., 1997; Ciardiello *et al*., 1996; Ciardiello and Tortora, 1998), antiestrogens like taxol, raloxifen, alone or in combination with antibodies like the humanized monoclonal antibody to *neu*; Herceptin (Brenner and Adams, 1999; Hanna *et al*., 1999; Robertson, 1998; Ross and Fletcher, 1998), provide some promising results for future treatment of breast cancer.

Conclusions

Transgenic mouse model studies have given us insight into possible mechanism of TGFα-initiated breast cancer. Overexpression of TGFα may be an early prognostic factor in the initiation of this disease and in cooperation with secondary transforming events can lead to carcinoma. Importantly, the role of TGFα in the inhibition of apoptosis during normal development could lead to a critical understanding of the role this growth factor plays in normal and neoplastic development in the mammary gland. This growth factor pathway appears to be able to promote growth in the initial phase of neoplastic transformation although it lacks the dominating transformation ability observed with some of the other EGFR-related mouse models of mammary gland disease like MMTV/erb-2 (Guy *et al*., 1992; Siegel *et al*., 1999). In combination with an understanding of TGFα signaling mechanisms, inhibitory agents, which act on the receptor-ligand interaction or on the intracellular kinases stimulated by the EGFR, could lead to practical and efficacious treatment regimens for this disease.

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