Abstract

Transforming growth factor (TGF)-βs are plausible candidate tumor suppressors in the breast. They also have oncogenic activities under certain circumstances, however. Genetically altered mouse models provide powerful tools to analyze the complexities of TGF-β action in the context of the whole animal. Overexpression of TGF-β can suppress tumorigenesis in the mammary gland, raising the possibility that use of pharmacologic agents to enhance TGF-β function locally might be an effective method for the chemoprevention of breast cancer. Conversely, loss of TGF-β response increases spontaneous and induced tumorigenesis in the mammary gland. This confirms that endogenous TGF-βs have tumor suppressor activity in the mammary gland, and suggests that the loss of TGF-β receptors seen in some human breast hyperplasias may play a causal role in tumor development.

Keywords: dominant-negative mutant receptors, mammary gland, transforming growth factor-β, transgenic mice, tumor suppressor

Introduction

The hypothesis that transforming growth factor (TGF)-βs may act as a tumor suppressors in the mammary gland has considerable intuitive appeal. TGF-βs are expressed at all stages of mammary development except lactation [1••], and they have potent growth inhibitory effects on mammary epithelial cells in vitro and in vivo [2,3]. Furthermore, many breast cancer cell lines have absent or decreased TGF-β responsiveness [4], and decreased expression of the type II TGF-β receptor in early premalignant breast lesions is correlated with increased probability of subsequent invasive disease [5••]. TGF-βs also have pro-oncogenic activities, however, and advanced human breast tumors overexpress TGF-βs [6]. This suggests that the role of TGF-βs in breast cancer is probably complex.

The potential oncogenic and tumor suppressor activities of TGF-βs are summarized in Figure 1.

We propose that TGF-βs are ‘conditional tumor suppressors’, with suppressor activity depending on a variety of factors, including the TGF-β responsiveness of the mammary epithelium, the stage in tumorigenesis, the nature of cooperating oncogenic events, and the level of expression of TGF-β ligand and receptor. It will be critical to define the precise circumstances under which TGF-βs act as tumor suppressors in the breast if we are to exploit the TGF-β system for therapy and/or prevention of breast cancer. Genetically engineered mouse models provide an invaluable experimental tool for this purpose, because TGF-β function can be manipulated in the context of the

DNR = dominant-negative mutant type II transforming growth factor-β receptor; LTR = long terminal repeat; MMTV = mouse mammary tumor virus; TGF = transforming growth factor; WAP = whey acidic protein.
Transforming growth factor (TGF-β) has tumor suppressor and oncogenic activities. Acting directly on the mammary epithelium, TGF-β could suppress tumorigenesis through a number of mechanisms, including inhibition of epithelial cell proliferation, induction of apoptosis or senescence in initiated cells, and maintenance of genomic stability. All of these activities require an intact epithelial response to TGF-β. TGF-β can also have pro-oncogenic effects. These may be either direct, or indirect via the stroma. Direct effects include the promotion of the epithelial–mesenchymal transition and invasiveness, and an increase in production of parathyroid hormone-related peptide (PTHrP). These are also dependent on an intact epithelial response directly or indirectly via the stroma. Indirect effects include the induction of angiogenesis, and suppression of the immune surveillance system. The indirect oncogenic effects are presumed to dominate when epithelial responsiveness to TGF-β is lost.

intact organism, where complex tissue interactions and regulatory cues are maintained. Also, whereas analysis of clinical samples can reveal provocative correlations between altered gene expression and tumorigenesis, animal studies allow causal mechanistic connections to be established.

Two different approaches: ‘gain-of-function’ and ‘loss-of-function’
To date, there are five mouse models that have been generated specifically to elucidate TGF-β function in the mammary gland (Table 1), and two more that give some insight into the process (TGF-β1+/− and Smad3−/− mice). Two conceptually different types of approach are used in the generation of models such as these, and we briefly consider the advantages and limitations of these before dealing with the specific results.

In the first, ‘gain-of-function’ approach, a protein is transgenically overexpressed in a target organ. This approach establishes what a protein can do in a given tissue context. It is of particular value for molecules such as TGF-β, with action that is so strongly context-dependent that the roles in vivo cannot readily be predicted from in vitro activities. The caveat, however, is that the transgenic protein is usually expressed at high levels, frequently in a cell type that might not normally express it in vivo, and it is not subject to all of the normal regulatory processes. Thus, the phenotype may not necessarily reflect the true role of the endogenous protein. In the second, ‘loss-of-function’ approach, a gene is knocked out by homologous recombination, or gene function is ablated by transgenic overexpression of antagonists or dominant-negative mutants. This approach establishes what role the endogenous protein actually does play in the tissue, with the caveat that many regulatory systems have built-in redundancy that may obscure an important role unless multiple members of a gene family are inactivated simultaneously. For example, there are three isoforms of TGF-β, all of which are expressed in the mammary gland, and it is not clear to what extent they can functionally compensate for each other.

Results from both approaches should be interpreted with care because of the possibility of confounding systemic or organismal effects. To be certain that an observed phenotype is due to a direct effect on the mammary gland, ideally all animal models should be subjected to the rigorous test of transplantation of the genetically modified mammary epithelium onto the cleared fat pad of syngeneic or immunocompromised hosts. If the original mammary phenotype is also observed in the transplant, this confirms that the genetic manipulation affected the mammary gland directly. This technique also gives useful information about stromal–epithelial interactions. Only one of the TGF-β-related models [whey acidic protein (WAP)-TGF-β1] has been subjected to this type of analysis so far, so results from the others should be considered provocative but in need of further confirmation.

Gain-of-function models
Potential for complex regulatory roles in ductal and lobular development
TGF-β1 has been targeted to the mammary gland using two different promoters, and the resulting mice showed different phenotypes [7,8], probably due to the differing temporal and spatial patterns of expression of the two promoters. The WAP promoter is very specific for the mammary epithelium, but pregnancy and lactation are required for high level expression of transgene because WAP is a milk protein. Thus, the virgin gland cannot be readily studied with this construct. In contrast, the mouse mammary tumor virus (MMTV)-long terminal repeat (LTR) promoter/enhancer element drives gene expression in the virgin as well as lactating glands. It is less specific, however, and shows expression also in other tissues such as the salivary gland, which can have potentially confounding effects.

WAP-TGF-β1 mice, in which TGF-β was targeted primarily to the secretory epithelium and its direct progenitors, showed a lactation-deficient phenotype. Ductal development of the mammary glands was not overtly impaired, but the formation of lobuloalveolar structures and the produc-
tion of milk were largely suppressed [7]. It was proposed that TGF-β had two effects: it induced apoptosis of cells in the developing alveoli; and it directly or indirectly induced accelerated senescence of the multipotential mammary stem cell compartment [9•]. In contrast, when TGF-β1 was overexpressed from the MMTV promoter/enhancer (MMTV-LTR), the mice showed impaired development of the mammary ductal epithelium, but alveolar outgrowth and lactation occurred normally from the hypoplastic ductal tree. Interestingly, the nature of the target cell may determine the mechanism of the TGF-β response. In the alveolar compartment, TGF-β induced apoptosis without effects on proliferation, whereas in the ductal compartment it inhibited proliferation.

Together the data suggest that endogenous TGF-βs could play an important role in determining the balance of cells in the ductal versus lobular progenitor compartments, and the fate of cells within those compartments. We now need to know in which compartments endogenous TGF-βs are normally expressed or activated, as this will give insight into whether they play these roles in the normal mouse. Since stem cells are the likely targets of carcinogenic events [10], it is particularly provocative that TGF-βs may be important in regulating mammary stem-cell kinetics.

For both the WAP-TGF-β1 and MMTV-TGF-β1 mice, the TGF-β1 construct that was used was a constitutively activated mutant form. It is noteworthy that no phenotype was obtained when the native latent form of TGF-β1 was over-expressed from the MMTV-LTR [8 •]. This suggests that activation rather than production of latent TGF-β is a key limiting step in controlling TGF-β activity in the normal mammary gland. Currently, the mechanism for activation of latent TGF-β in the mammary gland is unknown, and this is a very important area for further investigation.

**Protection against tumorigenesis**

What effect does overexpression of TGF-β have on tumorigenesis? To address this question, tumorigenesis was initiated in the MMTV-TGF-β1 mice either by treatment with the mammary carcinogen 7,12-dimethylbenz-[a]-anthracene or by intercrossing with mice overexpressing the oncogene TGF-α in the mammary gland [11••]. In both cases the incidence of mammary tumors was reduced in MMTV-TGF-β1 mice compared with wild-type controls. This suggests that local overexpression of TGF-β1 may suppress mammary tumorigenesis, at least in the early stages. The observations raise the exciting possibility that local enhancement of the endogenous TGF-β system, through the use of pharmacologic agents, could prevent or delay the development of breast cancer. However, enthusiasm for this approach should be tempered by recent findings that a reduction in type II TGF-β receptor levels may occur very early in the genesis of some human breast cancers [5••]. When this occurs, locally increased

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**Table 1**

Transgenic mouse models designed to address the roles of TGF-βs in the mammary gland

| Model       | Transgene     | Expression pattern | Developmental phenotype                                                                 | Tumor phenotype                                                                                     | References                          |
|-------------|---------------|--------------------|---------------------------------------------------------------------------------------|----------------------------------------------------------------------------------------------------|-------------------------------------|
| **Gain-of-function models**                                      |                |                    |                                                                                       |                                                                                                    |                                     |
| MMTV-TGF-β1<sup>5223/225</sup> | Constitutively activated form of TGF-β1 | Mammary epithelium | Decreased ductal development in young animals                                         | Inhibition of tumorigenesis induced by chemical carcinogens or oncogenes                           | [8•,11••]                          |
| WAP-TGF-β1<sup>5223/225</sup> | Constitutively activated form of TGF-β1 | Mammary epithelium, specifically in lobulo-alveolar progenitors and fate-committed daughters | Inability to lactate due to decreased maintenance of lobuloalveolar structures                   | Inhibition of tumorigenesis induced by TGF-β                                                   | [7,9•] (Smith G, unpublished data)  |
| **Loss-of-function models**                                     |                |                    |                                                                                       |                                                                                                    |                                     |
| MMTV-DNR     | Dominant-negative mutant type II TGF-β receptor | Mammary epithelium | Precocious lobuloalveolar development and production of milk proteins in virgins     | Increased spontaneous tumorigenesis in aged mice                                                   | [27•] (Moses H, unpublished data)     |
| MMTV-DNR     | Dominant-negative mutant type II TGF-β receptor | Mammary epithelium | Increased lobuloalveolar development in virgins                                       | Increased tumorigenesis in response to carcinogens                                                 | [28••]                             |
| MT-DNR       | Dominant-negative mutant type II TGF-β receptor | Mammary stroma     | Increased ductal branching                                                            | Not described                                                                                      | [29•]                              |

DNR, dominant-negative mutant type II transforming growth factor-β receptor; MMTV, mouse mammary tumor virus; WAP, whey acidic protein; MT, metallothionein promoter; TGF, transforming growth factor.
TGF-β could provide a positive selective pressure for outgrowth of initiated TGF-β-resistant clones, and might actually promote tumorigenesis rather than suppressing it [12]. This is an issue that could be addressed experimentally by crossing the MMTV-TGF-β1 mice with the MMTV-dominant-negative mutant type II transforming growth factor-β receptor (DNR) mice, which have diminished epithelial responsiveness to TGF-β (see below).

Loss-of-function models
Negative regulatory roles in normal mammary gland development
The TGF-βs signal through a pathway involving activation of serine–threonine kinase receptors and downstream signal transduction molecules termed ‘Smads’ [13]. Germline knockouts for TGF-β2 and TGF-β3, the type II TGF-β receptor, and the signal transduction components Smad2, and Smad4 are all embryonic or perinatally lethal, which is consistent with critical roles for TGF-β family members during development [14–17,18*]. Of the knockouts on this pathway that have been generated to date, the only ones that survive birth are the TGF-β1-null and the Smad3-null mice [19–22,23•]. In the case of the TGF-β1-null mice, survival is dependent on the mouse strain [24]. The TGF-β1-null mice that do survive birth go on to die of a multifocal inflammatory syndrome soon after weaning [19,20], but their survival can be further extended by crossing onto immunodeficient or p21mutf1/cp1-null backgrounds [25] (Letterio JJ, personal communication). Smad3-null mice have immune and skeletal defects, but can survive up to approximately 8 months of age [21,22,23•].

So far there have been no reports on mammary gland development in the TGF-β1-null mice whose lifespan has been extended into and beyond puberty through the use of immunosuppressants or immunodeficient genetic backgrounds. TGF-β1+/- mice, however, which have just one functional TGF-β1 allele, show an accelerated development of the mammary ductal tree during puberty, and an increased proliferation in the mammary epithelium in response to hormonal stimulation (Barcellos-Hoff M-H, personal communication). These observations are consistent with a role for endogenous TGF-β1 in limiting proliferation of the ductal epithelium in response to ovarian hormones. The presence of a mammary phenotype in the TGF-β1+/- mice suggests that there may be pronounced dosage effects in the TGF-β system. It might therefore be instructive to examine the mammary glands of the heterozygous forms of some of the TGF-β system knockout models, such as the type II receptor knockout, that are embryonically lethal in their homozygous form.

TGF-βs are thought to signal primarily through two pathway-restricted Smad signal transduction proteins, Smad2 and Smad3, and the common mediator Smad, Smad 4 [13]. Smad2 and Smad3 are also utilized by the activins, which are closely related TGF-β family members. Our preliminary work with the Smad3-null mice indicates that these mice have underdeveloped mammary glands (Y Yang, unpublished data) This is opposite to the phenotype observed with the TGF-β1+/- mouse (see above), but is similar to that observed in activin-βB knockout mice, which show retarded ductal elongation and alveolar morphogenesis [26]. Thus, Smad3 may be more important for the mediation of activin signaling in the virgin mammary gland, with the TGF-β path utilizing a different pathway-restricted Smad, possibly Smad2.

To get around the problems of embryonic lethality seen with germline knockouts, TGF-β function has been specifically ablated in the mammary gland through targeted overexpression of a dominant-negative mutant type II transforming growth factor-β receptor (DNR). Expression of the DNR from the MMTV-LTR resulted in a phenotype of precocious lobuloalveolar development and premature production of milk proteins in the mammary glands of virgin mice [27•]. Increased lobuloalveolar formation was also observed in an independently derived MMTV-DNR model in a different strain of mice [28••]. In contrast, targeting the DNR to the mammary stroma, using a metallothionein promoter, resulted in increased ductal branching, associated with increased expression of the morphogen, hepatocyte growth factor [29•]. Taken together, these results suggest that endogenous TGF-βs have both direct and indirect actions on the mammary epithelium. TGF-βs act directly on the mammary epithelium to prevent functional differentiation in the absence of the appropriate hormonal signals, whereas they act indirectly through the stroma to limit ductal branching and maintain correct ductal spacing (Fig. 2).

Tumor suppressor activity in the mammary gland
If endogenous TGF-βs are important tumor suppressors in the mammary gland, one would predict that mice with compromised TGF-β function would show increased mammary tumorigenesis. Interestingly, both Smad3+/- and TGF-β1+/- mice show a significant incidence of spontaneous colorectal tumors, but to date no mammary tumors have been described in either model [23•,30••]. Gastrointestinal tumors in humans show a much higher incidence of mutations and deletions in the TGF-β receptors and Smads than do most other human tumors. Together the data suggest that TGF-βs may play a uniquely critical role as tumor suppressors in the gastrointestinal tract. Other organs, such as the breast, may have a more redundant system of defenses, of which the TGF-β system is just a part.

Clearly, however, endogenous TGF-βs do have tumor suppressor activity in the mammary gland. The evidence in support of this comes from work with the MMTV-DNR transgenic mice. The dominant-negative receptor approach
is expected to diminish responsiveness to all three TGF-β isoforms, and to reduce or eliminate downstream signaling through all Smad transduction components. It should therefore overcome issues of functional redundancy within the TGF-β system. In one model, the carcinogen 7,12-dimethylbenz-[a]-anthracene caused a significantly increased incidence of mammary tumors in MMTV-DNR mice compared with wild-type controls [28••]. In another model, aged multiparous MMTV-DNR mice showed an increased incidence of spontaneous mammary tumorigenesis (Moses H, personal communication). Tumors in both models arise relatively late, suggesting that multiple cooperating oncogenic events are required for a tumor to develop. Together, these data provide evidence that endogenous TGF-βs have tumor suppressor activity in the mammary gland, and that this role is compromised by decreasing the TGF-β responsiveness of the mammary epithelium.

The future: conditional expression systems and molecular mechanisms

We have only begun to address the complex role that the TGF-β system may play in mammary tumorigenesis. Because it is likely that TGF-βs play different roles at different stages of the process, a critical advance will be the use of transgenic systems that can be regulated or conditional knockout technologies to allow local TGF-β expression or response to be manipulated in a time-dependent manner. With these approaches we can determine whether upregulating TGF-β expression after the mammary gland is fully developed will still result in tumor suppression, or whether the suppressive effect is entirely due to the diminished target size for carcinogenic events presented by the hypoplastic epithelium. Similarly, upregulating TGF-β expression after a mammary tumor has become established will allow one to ask whether the oncogenic rather than suppressor activities of TGF-β dominate during the late stages of tumorigenesis.

The importance of dosage, both of the ligand and the response system, can also be addressed. One very intriguing question is the issue of whether the oncogenic and tumor suppressor activities of TGF-β might occur at different threshold levels of receptor activation. Reduced signaling secondary to reduced receptor activation or expression might modulate the nature of Smad signaling and the cross-talk with other mitogenic pathways [13]. It might also qualitatively affect the nature of the genes induced or repressed by TGF-βs. For example, activin, a closely related TGF-β family member, has been shown to induce different genes at different levels of receptor occupancy [31•]. It is interesting that, although TGF-β receptor expression is decreased in early breast lesions and some breast cancer cell lines, complete genetic inactivation of receptors or Smads is very rare [32,33]. Because TGF-β has some pro-oncogenic activities with an epithelial target (eg induction of parathyroid hormone-related peptide, and increased invasion and metastasis [33,35]), it is tempting to speculate that these activities may occur at lower levels of receptor activation than are required for growth inhibition. Thus, a decrease in TGF-β responsiveness to a level that permits oncogenic activities, but prevents suppressor activities, might actually be more oncogenic than a total loss of response.

Another particularly key area for further exploration is the question of the mechanisms that underlie the suppressor and oncogenic activities of TGF-βs in vivo. This issue has not been addressed in any of the mammary models to date. Candidate mechanisms are shown in Figure 1. It is interesting that for the colon cancers that arise spontaneously in the TGF-β1 knockout mice, there is no evidence for a change in rates of cell proliferation or apoptosis [30••]. Rather, it was suggested that TGF-β may normally play a role in the maintenance of the overall tissue architecture, and that disruption of this may predispose to malignancy. Genetically engineered mouse models are uniquely well suited for analysis of effects on this type of higher order organizational process.

Conclusion

The data from mouse models suggest that TGF-βs play roles in regulating both morphogenesis and functional differentiation of the mammary gland, with mammary stem cells or lineage-restricted progenitor cells being important targets for some of these activities. Experimental overexpression of TGF-β inhibits tumorigenesis in the mammary gland, whereas loss of TGF-β responsiveness promotes it. This suggests that the endogenous TGF-β system has tumor suppressor activity, and provides a mechanistic underpinning for the recent observations that decreased expression of TGF-β receptors in early stage human breast hyperplasias correlates with increased probability of subsequently developing invasive breast cancer. The relatively poor penetrance of the tumorigenic pheno-
types and the late age of tumor onset in the mouse models, however, suggest that many other events must cooperate with loss of TGF-β response for a breast tumor to develop. Alternatively, they may reflect the complex dual role of TGF-βs as both oncogenes and suppressors. This will be a fruitful area for further investigation.

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