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13. ABSTRACT ***(Maximum 200 Words)***

The transforming growth factor (TGF) βs are multifunctional polypeptide growth factors which potently inhibit the proliferation of mammary epithelial cells. There is evidence that autocrine TGFβ signaling promotes mammary carcinogenesis. There is also evidence that tumor cell TGFβs are involved in tumor maintenance and progression by promoting epithelial to mesenchymal transition of epithelial cancer cells, and by modulating tumor-host interactions that are critical for cancer cell survival. The objective of this proposal is to generate a transgenic breast tumor model in which the mammary epithelial expression of TGFβ1 can be temporally regulated to understand the role of this molecule in progressive sequential stages of mammary neoplasia. In addition, by expressing a dominant negative type II TGFβ receptor (dnTβRII) in MDA-231 human breast cancer cells, we will determine whether blocking autocrine TGFβ in these cells affects their invasive metastatic phenotype *in vitro* and *in vivo*. Results to date indicate that the basal migratory potential of dnTβRII expressing cells is impaired, suggesting that their invasive phenotype may indeed be affected by blocking autocrine TGFβ signaling.

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Introduction

The overall goal of this research is to elucidate the complex role of transforming growth factor βs (TGFβs) in sequential stages of mammary epithelial transformation, and to determine whether blocking autocrine TGFβ signaling in breast cancer cells affects their metastatic phenotype *in vitro* and *in vivo*. This knowledge is of potential importance for the development of rational therapeutic approaches in human breast carcinoma.

Body

Specific Aim 1

The purpose of this Aim was to generate MMTV/tTA + tet-op/TGFβ1$^{S223/225}$ mice. As stated in our 1999 Annual report, although MMTV/tTA mRNA was expressed in the mammary glands of the F1 bigenic animals we generated, expression levels were very low, and tet-op/TGFβ1 mRNA was not detectable. Since this may have been due to the low level of expression of the MMTV/tTA, we have obtained other MMTV/rtTA founder lines with more robust MMTV expression in the mammary gland from Dr. Lewis Chodosh (University of Pennsylvania, Philadelphia, PA; D'Cruz *et al.*, 2000). These mice are currently being quarantined in our mouse facility. In the presence of doxycycline, which can be administered to mice in the drinking water, the rtTA transactivates the tet-op minimal promoter upstream the active TGF 1 sequence. We anticipate being able to start mating these mice with six new tet-op/TGFβ1 homozygous founder lines, which we have generated in the same mouse strain (FVB) as Dr. Chodosh's MMTV/rtTA mice, in January 2001.

Specific Aim 2

This Aim initially proposed to study the effect of mammary TGFβ1 overexpression on different stages of breast transformation in MMTV/neu + TGFα bigenic mice. Due to the complexity of generating a mouse between these two bigenics bearing four different transgenes, these studies will be replaced by studies in which we induce tumors with the carcinogen 7, 12-dimethylbenzanthracene (DMBA) in MMTV/rtTA + tet-op/TGFβ1 bigenic mice of <8 weeks of age supplemented with estradiol/progesterone subcutaneous pellets. Following challenge with the initiator (DMBA) and the promoter (estradiol/progesterone), mice will be supplemented with doxycycline in the water to transactivate TGF 1 transcription at different times of mammary transformation and tumor progression. These studies will be initiated as soon as an appropriate MMTV/rtTA + tet-op/TGFβ1 bigenic mouse is generated.

Specific Aim 3

This Aim initially proposed to test the effect of antisense TGFβ1 and antisense TGFβ2 on MDA-231 human breast cancer cells. However, since we were unable to generate stable transfectants with sustained expression of antisense, we chose to disrupt autocrine TGFβ signaling in these cells by stably expressing a dominant negative type II TGFβ receptor in which the Lysine at position 277 within the kinase domain has been mutated to Arginine, rendering the receptor kinase dead. Expression vectors for this mutant (TβRII K277R) and the control vector were obtained from Dr. Martin Oft (University of California, San Francisco, CA). Results to date can be summarized as follows:

1. The TβRII K277R protein is expressed on the surface of MDA-231 cells, as evidenced by immunoprecipitation of ^{125}I-TGFβ1-labeled HA-tagged TβRIIK277R.
2. The TβRII K277R is functional in that it impairs various aspects of TGFβ signaling including:
    i) TGFβ-mediated Smad2 phosphorylation
    ii) TGFβ-mediated translocation of Smad2 to the nucleus
    iii) TGFβ-mediated transcription of TGFβ responsive reporter contructs (p3TP-luciferase and p(CAGA)$_{12}$-luciferase)

3. Expression of TβRII K277R in MDA-231 cells impairs their motility.

Biochemical experiments are currently underway to confirm that the impairment in motility observed upon expression of TβRII K277R in MDA-231 cells is indeed due to impaired TGFβ signaling. We have data suggesting that in addition to blocking TGFβ signaling, expression of TβRII K277R may also block BMP signaling in MDA-231 cells. We have obtained adenoviruses encoding constitutively active mutants of TGFβ and BMP type I receptors in order to assess the relative contribution of each pathway in motility.

Tumor studies in nude mice are also underway to evaluate the effect of TβRII K277R expression on tumor formation and metastases *in vivo*. Similar transfections have been done in EMT-6 and 4T1 Balb/C mouse mammary tumor cells. Characterization of these pools and clones is underway.

Key Research Accomplishments

Generation and characterization of MDA-231 pools and clones stably expressing TβRII K277R or control vector.

Reportable Outcomes

Dumont N, and Arteaga CL. Autocrine transforming growth factor-β signaling in mammary tumor cell invasiveness. Submitted to the AACR Pathobiology of Cancer Workshop, July 2000.

Dumont N, and Arteaga CL. Tumor promoting effects of transforming growth factor . *Breast Cancer Res.* 2:125-132, 2000.

McEarchern JA, Kobie JJ, Mack V, Wu RS, Meade-Tollin L, Arteaga CL, Dumont N, Besselsen D, Seftor E, Hendrix MJC, and Akporiaye ET. Invasion and metastasis of a mammary tumor involves TGF-β signaling. *Int. J. Cancer* (in press, 2001)

Conclusions

TβRII K277R is expressed, functional, and impairs motility in MDA-231 cells.

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Autocrine Transforming Growth Factor-β Signaling in Mammary Tumor Cell Invasiveness. Nancy Dumont and Carlos L. Arteaga, Departments of Cell Biology and Medicine, Vanderbilt University School of Medicine and Vanderbilt-Ingram Cancer Center.

Transforming growth factor-β (TGFβ) is a pleiotropic growth factor, which plays a critical role in modulating cell growth, differentiation and plasticity. There is increasing evidence that after cells lose their sensitivity to TGFβ-mediated growth inhibition, autocrine TGFβ signaling may potentially promote tumor progression. Studies have shown that introduction of a dominant negative TGFβ type II receptor (TβRII) in a variety of tumor cells can delay tumor formation and prevent the conversion of cells from an epithelial to a more invasive and metastatic mesenchymal phenotype. The purpose of this study is to identify the biochemical pathways perturbed by a dominant negative TβRII that are causally associated with the metastatic phenotype of tumors. In order to do that, the MDA-MB-231 human breast cancer cell line was stably transfected with either a kinase-dead TβRII-K277R (dnTβRII) construct or the vector control. Affinity labeling of cell surface receptors with ^{125}I-TGFβ1 revealed an increase in the labeling of TβRII on the surface of cells transfected with dnTβRII compared to the vector control or parental cells, suggesting that the dnTβRII was indeed expressed. This was confirmed by immunoprecipitating the affinity-labeled exogenous receptor via its HA tag. The function of the transgene was evaluated by i) examining phosphorylation and nuclear translocation of Smad2, a TGFβ signal transducer, and ii) measuring transcription utilizing the TGFβ responsive promoters, p3TP-lux and p(CAGA)$_{12}$-lux. These assays revealed that TGFβ-mediated Smad2 phosphorylation, Smad2 nuclear translocation, and transcriptional responses were reduced in cells stably transfected with dnTβRII compared to vector control. In addition, wound closure assays indicated that the basal migratory potential of dnTβRII expressing cells was impaired. Biochemical experiments are currently underway to identify the signal transducers perturbed by the dnTβRII expression which impair motility, and which may, in turn, be relevant to TGFβ-mediated invasion and metastases.

Review

Transforming growth factor-β and breast cancer
Tumor promoting effects of transforming growth factor-β

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Abstract

The transforming growth factor (TGF)-βs are potent growth inhibitors of normal epithelial cells. In established tumor cell systems, however, the preponderant experimental evidence suggests that TGF-βs can foster tumor–host interactions that indirectly support the viability and/or progression of cancer cells. The timing of this 'TGF-β switch' during the progressive transformation of epithelial cells is not clear. More recent evidence also suggests that autocrine TGF-β signaling is operative in some tumor cells, and can also contribute to tumor invasiveness and metastases independent of an effect on nontumor cells. The dissociation of antiproliferative and matrix associated effects of autocrine TGF-β signaling at a transcriptional level provides for a mechanism(s) by which cancer cells can selectively use this signaling pathway for tumor progression. Data in support of the cellular and molecular mechanisms by which TGF-β signaling can accelerate the natural history of tumors will be reviewed in this section.

Keywords: transforming growth factor (TGF)-β, TGF-β receptors, epithelial-to-mesenchymal transition, angiogenesis

Introduction

Although the transforming growth factor (TGF)-βs can be tumor suppressive [1], there is increasing evidence that TGF-β secretion by tumor cells and/or stromal cells within the peritumoral microenvironment can contribute to tumor maintenance and progression. How, then, can TGF-βs be both tumor suppressive and tumor promoting? This apparent paradox is reconciled by a study showing that, in a mouse skin model of chemical carcinogenesis, targeted expression of TGF-β1 in suprabasal keratinocytes appears to have dual effects. It suppresses the formation of benign skin tumors, but once tumors develop, it enhances their progression to a highly invasive spindle cell phenotype [2••]. These results suggest that the effects of TGF-β1 are biphasic: TGF-β1 acts early as a tumor suppressor, probably by inhibiting the proliferation of nontransformed cells, and it acts later as a tumor promoter by eliciting an epithelial-to-mesenchymal transition (EMT). Additional experiments have suggested that upregulation of TGF-β3 in the spindle carcinomas was responsible for maintenance of this invasive phenotype [2••]. This is consistent with TGF-β3 expression at sites in mouse embryos where epithelial–mesenchymal interactions are important, like the lung and palatal shelves [3,4], and also the abnormal lung development and cleft palate observed in TGF-β3 null mice [5]. Also consistent with an early tumor suppressive effect is the recent observation that *tgf-β1−/−* mice develop an accelerated progression of epithelial hyperplasia to

CTL = cytotoxic T lymphocyte; EMT = epithelial-to-mesenchymal transition; JNK = c-Jun *N*-terminal kinase; MMP = matrix metalloproteases; PAI-1 = plasminogen activator inhibitor; PTHrP = parathyroid hormone-related protein; TβRI = TGF-β receptor type I; TβRII = TGF-β receptor type II; TGF-β = transforming growth factor-β.

colonic adenomas and cancers [6•]. The existence of dual effects for TGF-βs in tumor progression follows the observation that TGF-β-induced growth inhibitory responses and extracellular matrix responses may represent distinct processes in certain cell types. For example, overexpression of the antagonistic Smad, *Smad7*, in pancreatic carcinoma cell lines not only suppresses TGF-β1-mediated growth inhibition, but enhances the ability of TGF-β1 to induce matrix associated transcriptional responses [7•].

The progression of epithelial tumors to an invasive metastatic state is often associated with EMT, downregulation of cellular adhesion molecules, elevated expression of metalloproteases, and increased motility and angiogenesis, all of which can be modulated by TGF-βs. It is therefore not surprising that the TGF-βs can also promote tumorigenesis by modulating these critical processes. In support of this view, elevated levels of TGF-β are often observed in advanced carcinomas, and have been correlated with disease progression in several studies [8–13]. This suggests that secreting higher levels of TGF-β may provide an advantage to tumor cells. Both autocrine and paracrine signaling may be involved in conferring this selective advantage. While mutations in various components of the TGF-β signaling pathway have been observed in some carcinomas, particularly colorectal cancers [14,15], an intact TGF-β signaling pathway is often retained in other malignancies as some tumors can exhibit increased invasiveness in response to exogenous TGF-β [16,17•,18,19,20•,21]. Moreover, in a recent study of a large cohort of human breast tumors, loss or low levels of the type II TGF-β receptor (TβRII) correlated with high tumor grade, but 60% of *in situ* and invasive breast carcinomas retained robust levels of TβRII expression by immunohistochemistry [22]. Finally, although *Smad4* is frequently inactivated in pancreatic cancers [23,24], the *Smad* genes, which encode proteins that transduce TGF-β signals, are rarely mutated in most human carcinomas [25–30]. This suggests that after cells lose their sensitivity to TGF-β growth inhibition, autocrine TGF-β signaling may potentially promote tumor progression. In addition, TGF-βs produced in excess by tumor cells may act in a paracrine fashion on the peritumoral stroma, tumor neovessels, or the immune system, indirectly fostering tumor progression.

Autocrine effects

Epithelial-to-mesenchymal transition

Similar to keratinocytes [2••], TGF-β1 can also induce a rapid and reversible EMT in melanoma cells [31], and in both nontumor [32] and Ha-Ras transformed [17•] mammary epithelial cells *in vitro*. In Ha-Ras mammary tumors, EMT appears to be initiated by TGF-β produced by peritumoral host cells and later maintained by autocrine TGF-β1 as the converted tumor cells themselves begin to secrete TGF-β1. The Ha-Ras tumor cells obtained after EMT *in vitro* or *in vivo* display loss of epithelial polarity, downregulation of E-cadherin, disruption of cell–cell adhesion, and invasive properties in several *in vitro* assays [17•]. Supporting the importance of autocrine TGF-β for the tumorigenesis of Ha-Ras mammary cells, introduction of dominant negative TβRII into these cells retarded tumor formation and prevented EMT *in vivo*; moreover, introduction of the same construct into highly invasive murine colon carcinoma cells reconstituted an epithelial phenotype *in vitro*, and inhibited both tumor outgrowth and the establishment of metastases [20•]. In colon cancer cells of low invasive potential and with naturally occurring mutations in the TβRII gene, re-expression of TβRII function restored tumor cell invasiveness [20•]. In another study, expression of dominant negative TβRII in clones derived from a metastatic squamous carcinoma cell line prevented their spontaneous progression to a spindle phenotype *in vivo* [21]. Furthermore, approximately 90% of colon cancers with microsatellite instability have inactivating mutations of TβRII [33], and this instability is significantly correlated with longer patient survival [34], suggesting that complete loss of TβRII in carcinomas may limit systemic metastases. Taken together, these results suggest that EMT, local tumor growth, and metastatic progression can be sustained by autocrine TGF-β signaling.

When tumors are grown in nude mice, TGF-βs made by host cells can induce responses in tumor cells with intact TGF-β signaling, with the net effect of these tumor–host interactions being deleterious to the host. For example, MDA-231 human breast tumor cells secrete parathyroid hormone-related protein (PTHrP) in response to exogenous TGF-β1, metastasize to bone when injected into nude mice, and induce osteolysis and hypercalcemia, resulting in host death. Transfection of these cells with dominant negative TβRII blocks TGF-β1-mediated stimulation of PTHrP production. Mice injected with these cells exhibited less osteolysis, higher body weight, lower serum calcium and PTHrP levels, and longer survival than mice injected with control MDA-231 cells [35•]. On the contrary, accelerated osteolysis and reduced host survival were observed when mice where injected with tumor cells transfected with a constitutively active TβRI, suggesting a possible role for TGF-β-mediated responses in the pathogenesis of some adverse paraneoplastic syndromes.

Several recent studies have contributed to our understanding of the biochemical mechanisms by which transformed cells can lose autocrine growth inhibition but retain TGF-β-mediated responses that contribute to tumor progression. For example, oncogenic activation of the Ras pathway, acting via MAP kinases, causes phosphorylation of Smad2 and Smad3 at specific Erk consensus sites in the linker region between their DNA binding and transcriptional activation domains. This results in loss of nuclear accumulation of Smad2/3 and silencing of TGF-β-mediated

antiproliferative responses [36••]. In nontransformed mammary cells, introduction of mutant Ras not only blocks growth inhibition by TGF-β, but also subverts this pathway into one that can stimulate epithelial-to-mesenchymal transdifferentiation [17•,20•]. In MDCK epithelial cells, transfection of the missense mutations Smad2.D450E and Smad2.P445H, reported in primary colorectal and lung carcinomas, does not abolish TGF-β-mediated growth arrest. Instead, it increases both basal and TGF-β stimulated invasiveness, neither of which is prevented by overexpression of the inhibitory Smad7 [37•]. This suggests the existence of *Smad* 'gain-of-function' mutations that enhance malignant progression by mechanisms independent of TβRI and Smad phosphorylation. Another study has shown that Smad7 mRNA levels are increased in human pancreatic cancers compared with normal pancreas [7•]. Stable transfection of COLO-357 human pancreatic cancer cells with a Smad7 expression vector results in loss of TGF-β1-mediated growth inhibition and p21/Cip1 promoter activity. However, TGF-β1-induced plasminogen activator inhibitor-1 (PAI-1) promoter activity is maintained and, more importantly, basal PAI-1 promoter activity, PAI-1 mRNA levels, anchorage independent colony growth, and tumorigenicity in nude mice, are all increased in the Smad7 transfected clones [7•]. This result suggests another potential mechanism, the overexpression of Smad7, for the segregation between antiproliferative and matrix associated TGF-β responses. In addition, overexpression of Smad4 in colon carcinoma cells does not reconstitute TGF-β-mediated antiproliferative responses [38•,39], but inhibits cell adhesion and spreading, reduces the levels of urokinase plasminogen activator and PAI-1, and prolongs tumor latency [39], suggesting an additional function for Smad4 in restraining genes involved in peritumor proteolysis and invasion. This is further supported by reports of homozygous deletion of *TβRI* or homozygous missense mutations of *TβRII* [40,41], each coexisting with deletions of *Smad4* in individual tumors. The coexistence of these mutations in the same tumors would not be expected if the function of these two gene products (TβRII and Smad4 or TβRI and Smad4) was limited to a single common signal transduction pathway. Taken together, these studies suggest, first, that the threshold for loss of TGF-β antimitogenic effects is lower than that required to lose responses associated with cell adhesion, invasion, and metastases; second, that not one but multiple biochemical mechanisms can contribute to the enhancement or unmasking of the tumor promoting effects of autocrine TGF-β; and, third, that some of these mechanisms may be independent of Smad function or TβRI phosphorylation. The identification of Smad dependent and independent genes causally involved in these TGF-β-mediated tumor promoting effects requires further research. Of note, Hocevar *et al* [42•] recently reported c-Jun *N*-terminal kinase (JNK) dependent TGF-β-induced fibronectin expression in cell lines lacking the *Smad4* gene or protein expression.

Increased motility

TGF-β can stimulate the motility of many cell types *in vitro* [43–45], therefore suggesting that TGF-β production *in vivo* may enhance migration of tumor cells and metastatic potential. Indeed, cyclosporine treatment of lung adenocarcinoma cells results in increased cell motility and anchorage independent growth *in vitro*, as well as increased metastases *in vivo*, all of which can be blocked with neutralizing TGF-β1 antibodies [46]. These results suggest that *in vivo* tumor progression by cyclosporine is dependent on autocrine TGF-β1. In prostate cancer cells, TGF-β1 stimulates motility without affecting cell proliferation, suggesting that the effects on motility and proliferation may occur via different biochemical pathways [43].

Whether blockade of the Smad pathway, critical for TGF-β-mediated antimitogenic effects [47,48], is also critical for the effects of TGF-βs on cell motility is not clear. Some evidence suggests that the latter may follow alternative signaling pathways, perhaps in cooperation with activated oncogenes. Atfi *et al* [49] reported recently that inactivating components of the JNK pathway, which regulates AP-1 activity via c-Jun, inhibits TGF-β-mediated induction of 3TP-Lux, a reporter construct that contains Smad and AP-1 binding elements. Dominant negative mutants of RhoA, Rac1, and Cdc42, GTPases that mediate cell shape, cytoskeletal organization, and motility, abolish TGF-β-mediated transcription of AP-1 [49,50], suggesting that the Rho family of GTPases and the JNK pathway are essential components of TGF-β signaling responses. TGF-β1 can also upregulate integrin linked kinase [31], a protein associated with fibronectin production and increased cell motility. In another study, TGF-β1 treatment of NMuMG mouse mammary epithelial cells increased the expression of *N*-cadherin [51], which has been shown to increase motility of squamous cancer cells [52].

Paracrine effects

Induction of metalloproteases

Matrix metalloproteases (MMPs) play a critical role in the proteolytic degradation of basement membrane that is required for tumor invasion [53]. The expression of several MMPs, including MMP-2 [54] and MMP-9 [18,31,55], can be induced by TGF-β. Moreover, TGF-β1 has been shown to selectively induce MMP-9 activity in a subset of metastatic but not primary mouse prostate tumors, implying that this TGF-β1-induced response may be an important selection step in tumor progression [18]. There is also evidence that TGF-β increases MT-MMP-1 and MMP-9 expression in metastatic melanoma [31]. Although MMPs are listed separately, recent data implicate them strongly in the process of tumor-induced neovascularization [56], thereby suggesting that their upregulation might be an integral component of the TGF-β-mediated angiogenic processes discussed next.

Tumor angiogenesis

It is generally accepted that solid tumors require an adequate blood supply in order to grow beyond a few millimeters in size. TGF-βs, particularly TGF-β1, have been shown to regulate new blood vessel formation both *in vitro* and *in vivo* by a combination of responses that include increased production and facilitation of vascular endothelial growth factor, facilitation of basic fibroblast growth factor mediated capillary sprouting, inhibition of endothelial cell migration, and increased production of extracellular matrix, among others (reviewed in [57]). In most cells, TβRI/ALK-5 is the signaling receptor for TGF-β. However, in endothelial cells, it has been suggested that ALK-1 may also function as a type I receptor for TGF-β [58]. In addition to the type I, II, and III TGF-β receptors, endoglin is another integral membrane protein that binds TGF-β1 and TGF-β3, and is highly expressed in endothelial cells [59]. Although TGF-β effects appear to be mediated mostly by the receptor specific Smad2 and Smad3 proteins [47,48], there is evidence that Smad5 is involved in TGF-β signaling in hematopoietic cells [60]. Targeted disruption of genes encoding various components of the TGF-β signaling pathway, including TGF-β1 itself [61], its receptors, TβRII [62], ALK-1 [63], and endoglin [64], and one of its signal transducers, Smad5 [65], has each revealed that these proteins play an important role in vascular development. The phenotype of the TGF-β1 and TβRII knockout mice is virtually indistinguishable and is characterized by defective endothelial differentiation resulting in abnormal capillary tube formation [61,62]. In contrast, disruption of ALK-1, endoglin, or Smad5 does not affect endothelial differentiation or vasculogenesis, but instead they each affect angiogenesis. In addition, *endoglin−/−* and *Smad5−/−* mice exhibit impaired vascular smooth muscle cell development. These results are consistent with previous reports demonstrating that TGF-β can regulate smooth muscle cell differentiation and migration *in vitro* [66•], thus contributing to pericyte recruitment and vessel stabilization. This hypothesis, as it applies to tumor angiogenesis, is somewhat challenged by the notion that the majority of intratumoral neovessels seem to lack periendothelial smooth muscle cells [67], suggesting that there may be additional roles for the TGF-βs in tumor angiogenesis. In that light, Higaki and Shimokado [68] recently reported TGF-β1-mediated stimulation of phosphatidylinositol-3 kinase activity and amino acid uptake in vascular smooth muscle cells, suggesting a direct anti-apoptotic role for TGF-β. Elucidation of the paracrine mechanisms driving TGF-β-mediated tumor angiogenesis requires further investigation.

Further supporting the role of TGF-βs in tumor angiogenesis, administration of a neutralizing TGF-β1 antibody to nude mice harboring CHO cell xenografts transfected with ectopic TGF-β1 inhibits both tumor growth and intratumor microvessel density [69]. In addition, a monoclonal antibody that blocks TGF-β1, TGF-β2, and TGF-β3 has been shown to suppress the growth of TGF-β1-overexpressing renal cancer xenografts [70]. In this study, the TGF-β blocking monoclonal-abrogated factor VIII staining in the xenografts, suggesting an antitumor mechanism that targets endothelial cells [70]. Furthermore, TGF-β1 and PAI-1 have been shown to inhibit the conversion of plasminogen to the anti-angiogenic molecule angiostatin in medium conditioned by human pancreatic cancer cells [71]. This suggests an additional pro-angiogenic mechanism for TGF-β by interfering with the production of endogenous inhibitors of endothelial cell proliferation. Finally, high levels of TGF-β1 mRNA correlate strongly with high microvessel density in breast tumors, and each of these factors is associated with poor patient outcome [72].

Host immunosuppression

TGF-β1 and TGF-β2 are potent immunosuppressants [73]. Thus, elevated levels of TGF-βs secreted by tumors could potentially inhibit immune effector cells and favor tumor progression. In support of this idea, Torre Amione *et al* [74] demonstrated that, unlike parental tumor cells, fibrosarcoma cells transfected to express 10 ng/ml TGF-β1 *in vitro* are unable to induce cytotoxic T lymphocyte (CTL) responses and can escape immune recognition. Likewise, EMT6 mammary tumor cells, which produce high levels of TGF-β1, can inhibit CTLs *in vivo*. Transfection of these cells with interleukin-2, a known T cell growth factor, can reverse this TGF-β1 effect and induce tumor rejection [75]. This result suggests that, by dampening the generation of tumor reactive T cells, TGF-β can promote tumor viability. There is also evidence that overexpression of the soluble TβRII extracellular domain in thymoma cells can prevent the progression of unmodified thymoma cells when injected near the primary tumor inoculation site [76], further suggesting that secretion of soluble TβRII by these cells is sufficient to restore tumor specific cellular immunity and mediate partial tumor rejection. Overall, these results are consistent with the phenotype of *TGF-β1* null mice that die shortly after birth as a result of widespread inflammation and multiorgan T cell infiltration and necrosis [77].

In addition to inhibiting CTL responses, TGF-βs can modulate other immune functions that may favor tumor progression. For example, CHO cells transfected with an expression vector encoding latent TGF-β1, when injected into nude mice, can decrease mouse spleen natural killer activity and rapidly form tumors [78]. Antagonizing TGF-βs by intraperitoneal injection of an antibody that neutralizes TGF-β1, TGF-β2, and TGF-β3 has the opposite effect. It prevents tumor and metastases formation by MDA-231 human breast carcinoma cells, and markedly increases natural killer activity of mouse splenocytes [79]. Consistent with this TGF-β-mediated immunosuppressive effect, reduced immune function has been observed in animals

bearing TGF-β overexpressing tumors [80] as well as in patients with glioblastoma, a common type of brain tumor that frequently overexpresses TGF-β2 [81].

The cited studies suggest that tumor cell secreted TGF-βs may block the efferent function of immune effectors at sites of tumor implantation. Other reports, however, suggest tumor cell TGF-βs may modify the afferent component of the immune response and confer antitumor immunity. Stable infection of breast and glioma tumor cells with antisense TGF-β1 and antisense TGF-β2 retroviruses, respectively, has been shown to restore the immunogenicity of these tumor cells when injected into immunocompetent animals. Furthermore, they induce a partial rejection of unmodified, less immunogenic established wild type tumor cells [82,83]. In both of these studies, *in vitro* and *in vivo* CTL activity was markedly increased in medium conditioned by antisense TGF-β-infected cells and/or in mice injected with tumor cells bearing the antisense compared with tumor cells infected with a control vector. These studies have therapeutic implications for the use of an antisense TGF-β based approach as a means of adoptive immunotherapy against TGF-β overproducing tumors.

Alternative views and conclusions

A tumor permissive role for the TGF-βs may not apply to all solid tumors. Indeed, transfection of an antisense TGF-β1 expression vector into FET and CBS well-differentiated human colon cancer cells has been shown to enhance tumor formation in nude mice [84,85], supporting the notion that, in some fully transformed cells, endogenous TGF-β1 can continue to mediate a tumor suppressor function. In a recent report, mice bearing transplanted gallbladder Mz-Cha-2 tumors showed inhibition of angiogenesis and leukocyte–endothelial cell interactions at a distant cranial site and threefold higher levels of circulating TGF-β1 compared with tumor free mice [86]. This reduction in microvessel density and leukocyte rolling were reversed by systemic administration of a TGF-β1 neutralizing antibody, suggesting a negative role for TGF-β1 in early neovascularization. Moreover, in a recent survey of 104 *in situ* and invasive primary breast carcinomas, 40/45 (89%) tumors with low invasive potential and low proliferation rate exhibited high levels of TβRII by immunohistochemistry [22]. Whether autocrine TGF-β signaling is causally associated with the observed low proliferation and invasiveness in this subset of breast tumors is a question that remains unclear.

Nonetheless, the potential tumor promoting effects of TGF-β provide novel molecular targets for interventions aimed at altering the natural history of solid tumors. The lack of an obvious physiological role for TGF-β signaling in postdevelopmental normal physiological states suggests that these interventions may in fact be tumor specific and spare the tumor host from undue toxicity. Several approaches have been proposed, including the use of blocking antibodies against TGF-β1, TGF-β2, and TGF-β3, using the soluble ectodomains of the type II and III TGF-β receptors, which would sequester TGF-β isoforms at tumor sites and prevent binding to cognate receptors [87,88], and, finally, using adenovirus encoding inhibitors of TGF-β signaling [89], to name a few. The theoretical and logistical strengths and limitations of these approaches are beyond the scope of this review. Nonetheless, these represent tools that, if effective in blocking TGF-β action, will allow us to address the net effect of autocrine/paracrine TGF-β signaling at early and late stages of transformation and cancer progression.

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Articles of particular interest have been highlighted as:
- • of special interest
- •• of outstanding interest

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Invasion and metastasis of a mammary tumor involves TGF-ß signaling

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ABSTRACT

Several studies have correlated escape from TGF-ß-mediated cell cycle arrest with the tumorigenic phenotype. Most often, this escape from growth control has been linked to dysfunctional TGF-ß receptors or defects in the TGF-ß-mediated SMAD signaling pathway. In this report, we found that highly metastatic 4T1 mammary carcinoma cells express functional TGF-ß receptors capable of initiating SMAD-mediated transcription yet are not growth inhibited by TGF-ß1. We further observed that TGF-ß directly contributes to the metastatic behavior of this cell line. Exposure to TGF-ß caused 4T1 cells to undergo morphological changes associated with the metastatic phenotype and invade more readily through collagen coated matrices. Furthermore, expression of a dominant negative truncated type II receptor diminished TGF-ß signaling and significantly restricted the ability of 4T1 cells to establish distant metastases. Our results suggest that regardless of 4T1 resistance to TGF-ß-mediated growth inhibition, TGF-ß signaling is required for tumor invasion and metastases formation.

Introduction

Tumor invasion and metastasis are complex processes in which cancer cells detach from the original tumor mass to establish metastatic foci at distant sites. Metastatic cells characteristically lose growth inhibitory responses, undergo alterations in adhesiveness and demonstrate enhanced production of enzymes that can degrade extracellular matrix components (Woodhouse *et al.*, 1997; Reiss and Barcellos-Hoff, 1997). Since it is the development of metastatic disease that is primarily responsible for cancer mortality, an understanding of the mechanisms that facilitate metastatic tumor progression is of great importance.

One cytokine that may contribute to the metastatic potential of tumor cells is transforming growth factor beta (TGF-ß). Originally identified as a positive growth factor for mesenchymal cells, TGF-ß has been identified as a potent growth inhibitor of most cell types, including cells of hematopoetic origin and epithelial lineage (Taipale *et al.*, 1998). TGF-ß inhibits the growth of normal epithelial cells by inducing an arrest in G1 phase of the cell cycle and, less commonly, by promoting apoptosis (Taipale *et al.*, 1998; Alexandrow and Moses, 1995). In contrast, most malignant cells are refractory to TGF-ß-mediated growth arrest (Fynan and Reiss, 1993). This loss of sensitivity has been linked to tumor progression, and may be due to loss or mutation of TGF-ß receptors or dysregulation of TGF-ß signal transduction pathways (Reiss and Barcellos-Hoff, 1997; Alexandrow and Moses, 1995; Yingling *et al.*, 1995).

It is widely accepted that TGF-ß promotes tumorigenicity by stimulating angiogenesis (Roberts *et al.*, 1986), inducing extracellular matrix degradation (Albo, *et al.*, 1997) and inhibiting anti-tumor immune responses (Torre-Amione *et al.*, 1990; Arteaga *et al.*, 1993; Park *et al.*, 1997; Wojtowicz-Praga *et al.*, 1997). Recent studies have shown that tumorigenicity can also arise via the action of TGF-ß on the tumor cells directly (Miettinen *et al.*, 1994, Oft *et al.*, 1996, Oft *et al.*, 1998, Portella *et al.*, 1998, Hojo *et al.*, 1999, Piek *et al.*, 1999, Yin *et al.*, 1999). In this report, we have employed a highly metastatic murine mammary cancer cell line (4T1) to examine the role of TGF-ß on invasion and metastatic potential in vivo. We found that although 4T1 mammary tumor cells are resistant to TGF-ß-mediated growth inhibition, TGF-ß signaling is critical to tumor invasion and metastases formation. Engagement of ligand initiated downstream signaling pathways that culminated in Smad2 phosphorylation and transcription of a Smad responsive reporter gene (3TP-lux). Furthermore, disruption of TGF-ß signaling by expression of a dominant negative truncated type II receptor significantly curbed tumor metastasis without affecting primary tumor growth.

Materials and Methods

Cell lines. The metastatic line 4T1 is a thioguanine-resistant variant of 410.4, a tumor subline isolated from a spontaneous mammary tumor that developed in a BALB/cfC3H mouse. The 4T1 cell line was kindly provided by Dr. Fred Miller of the Michigan Cancer Foundation (Detroit, MI). The TGF-ß sensitive Mink lung

epithelial cell line (Mv1Lu) was purchased from ATCC (CCL64; Rockville, MD). Both cell lines were maintained in vitro by passage in Iscove's Modified Dulbecco's Medium (IMDM) supplemented with 10% FBS.

Mice. Female C.B-17/IcrACC*scid* mice, 6-8 weeks old, were purchased from a colony maintained at the University of Arizona. The mice were housed in the University of Arizona animal facilities in accordance with the principles of animal care (NIH publication No. 85-23, revised 1985).

Cell cycle analysis. 4T1 tumor cells and TGF-ß sensitive Mv1Lu cells were arrested at the G1 phase of the cell cycle by seeding them at high density (10^7 cells/T75 flask) for 72h. The cells were then detached and plated at a concentration of 5 x 10^5 cells/100 mm x 15 mm tissue culture dish in the presence of increasing amounts of TGF-ß1. Forty-eight hours later, the cells were collected, fixed at a concentration of 10^6 cells/ml with cold 70% ethanol for 2h and stored at 4°C for up to 1 week. For analysis, 10^6 cells were suspended in 1 ml of Vindelov's PI buffer pH 8.0 (10mM Trizma base, 10mM NaCl, 0.1% NP-40, 50 µg/ml RNase A, 50 µg/ml propidium iodide). After a 20 min incubation at room temperature, the cells were filtered through a 30 µm nylon mesh and measured for DNA content with a FACStar^{PLUS} flow cytometer (Becton Dickinson Immunocytometry Systems; San Jose, CA) using an Innova 90-5 argon laser (Coherent; Palo Alto, CA) tuned to 488nm at 100mW. Fluorescence emission was captured through a 575/26 nm bandpass filter. Data were acquired using Lysys II software and analyzed with CELLFIT (Becton Dickinson Immunocytometry Systems).

Determination of TGF-ß receptor expression.

Reverse-transcriptase polymerase chain reaction (RT-PCR). Total RNA was isolated from 4T1 and Mv1Lu cells with TRIzol reagent (GIBCO BRL Life Technologies Inc.; Gaithersburg, MD), treated with DNase I and reverse transcribed to generate cDNA (cDNA cycle kit #K1310-02, Invitrogen; San Diego, CA). PCR amplification was performed in a 50 µl volume containing 2 µl of the cDNA reaction, 1.5mM $MgCl_2$, 200µM dNTPs, 0.1µM sense and antisense primers for TGF-ßRI or TGF-ßRII, and 2.5 units Amplitaq Gold DNA polymerase (N808-0241, PE Applied Biosystems; Foster City, CA) in 1X PCR buffer. Cycling parameters were 95°C for 10 min followed by 94°C for 10s, 65°C for 30s and 75°C for 45s for 50 cycles. TGF-ßRI- and TGF-ßR-II specific primers were designed using the OLIGO program (National Biosciences; Plymouth, MN) and were as follows: TGF-ßRI upper primer (5'-GGGGCGAAGGCATTACAGTG-3', position 76) and TGF-ßRI lower primer (5'-ATTTGGCACACGGTGGTGAA-3', position 461); TGF-ßRII upper primer (5'-TCCACGTGCGCCAACAACAT-3', position 915) and TGF-ßRII lower primer (5'-GCGCAAGGACAGCCCGAAGT-3',

position 1420). These primers amplify 405 bp and 525 bp fragments of TGF-ßRI and TGF-ßRII respectively.

^{125}I-TGF-ß1 Affinity Crosslinking studies. Subconfluent 4T1 tumor cells in 12-well plates were labeled with 100pM ^{125}I-TGF-ß1 (173µCi/µg, DuPont NEN; Boston, MA) in the presence or absence of 10nM unlabeled rhTGF-ß1 (Genentech; South San Francisco, CA) for 4h at 4°C. After washing, the specifically bound labeled ligand was cross-linked to cell surface receptors with 1mM bis(sulfosuccinimidyl) suberate (BS^3) (Pierce; Rockford,IL) as described previously (Koli and Arteaga, 1997). Equivalent amounts of protein were then resolved by 3-12% gradient SDS-PAGE and labeled receptors visualized by autoradiography.

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Cell Migration and Invasion Assays. Cells were seeded at a density of 10^5 cells per well in triplicate in the upper chamber of 12-well transwells (8µm pore, #29442-120, Corning Inc., Corning, NY) in the presence or absence of 2 ng/ml rhTGF-ß1 or anti-TGF-ß neutralizing antibody (100µg/ml) (ATCC HB-9849). Twenty hours later, the transwells were removed and the lower chambers were incubated for an additional 20h. Cells that migrated through the transwell into the lower chamber and attached were treated with trypsin and counted. The transwells were untreated (migration assay) or coated with rat tail type I collagen (invasion assay) as previously described (Keely *et al.*, 1997).

Confocal microscopy. Cells (10^4) were grown on 0.17 mm thick coverslips in a CO_2-humidified incubator for 48h in the presence or absence of 2 ng/ml rhTGF-ß1. The cells were then fixed with 4% methanol-free formaldehyde for 20 min at room temperature, permeabilized with 100% methanol at -20°C for 6 min, air-dried and stored at -20°C until time of staining. Morphological changes in tumor cells treated with TGF-ß were detected by staining the cells with 100 µl of a 1:40 dilution of bodipy-phalloidin (#B-3416, Molecular Probes; Eugene, OR), a fluorescent molecule that binds to actin filaments. The cells were visualized with a LEICA confocal microscope.

Western blotting. Cells grown overnight in serum free IMDM were untreated or treated with 0.25 ng/ml of rhTGF-ß1 for 45 minutes, washed twice with ice cold PBS and scraped into a buffer containing 50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1% NP-40, 0.25% Na-deoxycholate, 1 mM EDTA, 1 mM PMSF, 1 mM NaF, 1 mM Na_3VO_4, 1 µg/ml aprotinin, 1 µg/ml leupeptin, and 1 µg/ml pepstatin. The lysate was placed on a rocker at 4 °C for 30 min, centrifuged at 14,000 x g and protein in the supernatant recovered and quantified by BCA Protein Assay (Pierce, Rockford, IL). Proteins (25 µg) from the cell lysates were resolved by SDS-8% PAGE and electrotransferred to polyvinylidene fluoride (PVDF) membrane. Nonspecific binding sites were saturated by incubation in TBS containing 0.1% Tween-20 and 5% nonfat powdered milk. Membrane bound anti-

Smad2 and anti-phosporylated Smad2 antibodies (S66220; Transduction Laboratories, Lexington, KY and 06-829; Upstate Biotech, Lake Placid NY respectively) were visualized with horseradish peroxidase-conjugated secondary antibodies (Santa Cruz Biotech) and enhanced chemiluminescence (Amersham Pharmacia Biotech, UK).

Plasmids. A plasmid construct containing cDNA encoding a truncated TGF-ßRII lacking the serine/threonine kinase domain (DNRII)(Choi and Ballerman, 1995) was kindly provided by Dr. Barbara Ballerman of The Johns Hopkins University. For our studies, the 0.58 kb DNRII fragment was excised by a Nhe1/Xho digest and cloned into a commercially available plasmid pcDNA3.1zeo(+) (V860-20, Invitrogen). Proper insertion of this fragment into this plasmid was confirmed by restriction analysis and sequencing. The resultant construct (DNRII-pcDNAzeo) contained the truncated receptor under control of human CMV promoter and a SV40-driven zeocin resistance gene to allow for selection of stably transfected cells. The p3TPLux TGF-ß-inducible luciferase construct was a gift from Dr. Joan Massague (Howard Hughes Medical Institute, Memorial Sloan-Kettering Cancer Center, New York, NY) and has been described previously (Wrana *et al.*, 1992). The ß-galactosidase expression vector VR1412 used to normalize luciferase activities was kindly provided by Vical Inc. (San Diego, CA).

Transcriptional Response Assay. Cells were plated to semiconfluency and 24h later were transiently co-transfected with 1 µg each of p3TPLux and a control vector (VR1412) with Lipofectamine reagent (#18324-012, GIBCO BRL) according to the manufacturer's instructions. Twenty-four hours after transfection, the cells were plated in triplicate at a concentration of 5 x 10^4 cells/well of a white 96-well culture plate (#6005180, Instrument Company, Inc., Meriden, CT) in the presence or absence of 2 ng/ml rhTGF-ß1 for 18h. Extracts were then prepared and assayed for luciferase and ß-galactosidase activity using the LucLite Plus (#6016961, Packard) and Galacto-Light Plus (BL100P, Tropix, Bedford, MA) kits respectively. Light emmission was detected with a Packard Lumicount and luciferase activity was normalized on the basis of ß-galactosidase expression.

Northern Blot Analysis. Messenger RNA was isolated from subconfluent cells and subjected to Northern blot analysis using a NorthernMax-Gly kit (#1944; Ambion Inc., Austin, TX). Briefly, the mRNA was separated on a 1% agarose gel, transferred to nylon membrane, UV crosslinked and hybridized with biotin-labeled rat TGF-ß receptor II and mouse GAPDH-specific RNA probes overnight at 80°C and 65°C respectively. Hybridized bands were visualized by the addition of streptavidin/alkaline phosphatase using a BrightStar Biodetect kit (#1925; Ambion Inc.). To develop an antisense TGF-ß receptor II (TßRII) template for transcription of a TßRII-specific probe, the DNRII fragment obtained from a Nhe1/Xho1 digest was cloned into pcDNA3.1(+) that had been digested with Xho1 and Xbal. To generate the sense TßRII RNA probe, this template was

linearized by Apa1 digestion, treated with proteinase K and purified on a 4% polyacrylamide gel. The linearized plasmid was transcribed with T7 polymerase in the presence of a nucleotide triphosphate RNA-labeling mix (#1685 597; Roche Molecular Biochemicals, Indianapolis, IN) containing biotin-16-UTP according to the manufacturer's instructions. Biotin-labeled RNA probe specific for GAPDH and RNA millennium markers were similarly generated from linearized templates (#7431 and #7785, respectively; Ambion Inc.).

Spontaneous Metastasis Assay. Mice challenged orthotopically in the mammary gland with 10^5 cells were sacrificed 21 days post-injection. Lungs were removed, stained with India ink and the tumors bleached with Fekete's solution as previously described (Wexler, 1966). Primary tumors were measured in two dimensions every 3-4 days post inoculation. Tumor volume was calculated using the formula $v= (l \times w^2)/2$, where v = volume (mm^3), l = long diameter and w = short diameter (Wexler, 1966).

Results

TGF-ß does not inhibit cell cycle progression of 4T1 cells

We assessed whether exposure of 4T1 tumor cells to TGF-ß resulted in growth inhibition. For this purpose, cells were arrested at G1 by crowding, released to transit through the cell cycle in the presence or absence of increasing amounts of TGF-ß and then analyzed by flow cytometry. As shown in Figure 1, 4T1 cells were not growth arrested in G1 but instead progressed through the cell cycle in the presence of high concentrations (20ng/ml) of TGF-ß1. In contrast, indicator Mink lung epithelial cells (Mv1Lu) that are growth inhibited by TGF-ß were arrested in G1 following exposure to 2ng/ml TGF-ß1 (Figure 1). The resistance of 4T1 cells to TGF-ß growth control was additionally supported by cell counting; no differences in cell number and viability were observed between untreated ($2.3 \times 10^6 \pm 3.6 \times 10^5$) and TGF-ß-treated cells ($2.4 \times 10^6 \pm 1.5 \times 10^4$) after 48 hours of in vitro culture.

Expression of functional TGF-ß receptors in mammary tumor cells

Since 4T1 cells were resistant to TGF-ß-mediated cell cycle control, we examined them for the presence of functional TGF-ß receptors. Analysis of RNA by RT-PCR demonstrated that 4T1 cells transcribe mRNA for TGF-ß receptors I and II (Figure 2A). The PCR product obtained for TGF-ßRI was sequenced and determined to be ALK-5 (data not shown). 4T1 cells also express TGF-ßRI and TGF-ßRII as surface proteins capable of binding TGF-ß ligand. When labeled with ^{125}I-TGF-ß1 in a receptor cross-linking experiment, TGF-ß receptors I, II and III were detected in 4T1 cells (Figure 2B). Binding of ^{125}I-TGF-ß1 was

competitively inhibited by the addition of an excess of unlabeled TGF-ß1 (Figure 2B).

TGF-ß signaling in 4T1 tumor cells

Next we evaluated if TGF-ß signaling occurred in 4T1 tumor cells following TGF-ß treatment. The involvement of Smads in TGF-ß signaling was determined by Western blot to detect the presence of phosphorylated Smad-2 and by gene expression analysis of cells transiently transfected with the Smad responsive 3TP-Lux reporter construct. Smad2 phosphorylation and gene transcription in unstimulated 4T1 cells were significantly increased upon addition of exogenous TGF-ß1.

Morphological changes induced by TGF-ß treatment

The 4T1 mammary tumor cell line typically adheres to plastic or glass substratum and grows in rounded clumps (Figure 4A). When incubated with 2ng/ml rhTGF-ß1, the cells became less clumped, assumed a fibroblast-like appearance and demonstrated reorganization of the cytoskeleton characterized by prominent F-actin filaments and stress fibers (Figure 4B). In order to determine if this morphologic change involved an epithelial to mesenchymal transition (EMT), cells were stained with vimentin and keratin antibodies as previously described (Hendrix *et. al.*, 1997). 4T1 cells constitutively co-expressed vimentin and keratin intermediate filaments and TGF-ß treatment did not alter their expression (data not shown). This interconverted phenotype has been previously reported and is characteristic of highly malignant breast cancer cells (Thompson *et al.*, 1992, Sommers *et al.,* 1992, Lichtner *et al.*, 1991, Hendrix *et. al,* 1997).

TGF-ß stimulates in vitro migration and invasion of 4T1 cells

Since SMAD-mediated-TGF-ß signaling was intact in 4T1 cells and TGF-ß treatment induces morphologic changes, we speculated that TGF-ß might contribute to tumor progression by stimulating cell migration and invasiveness. To explore this possibility, the effect of TGF-ß on in vitro migration and invasion of 4T1 cells was evaluated. The data (Figure 5) demonstrated basal levels of migration but negligible invasive capacity in 4T1 cells in the absence of TGF-ß. Addition of TGF-ß resulted in a four-fold increase in in vitro migration and a seventeen-fold increase in invasiveness that was completely abrogated in the presence of TGF-ß neutralizing antibody (Figure 5).

Disruption of TGF-ß signaling inhibits metastasis formation

To directly evaluate the role of TGF-ß signaling in tumorigenicity and the development of metastasis, 4T1 cells were transfected with a truncated dominant-negative TGF-ß type II receptor (DNRII). This receptor lacks the cytoplasmic serine/threonine kinase domain and is therefore unable to transduce TGF-ß-initiated signals (Choi and Ballerman, 1995). Expression of DNRII was

demonstrated by Northern blot (Figure 6) and four of the stably transfected clones were selected for further analysis. Expression of DNRII blunted transcriptional activation by TGF-ß in the majority of clones tested (Figure 7). Additionally, the expression of DNRII resulted in a decrease of TGF-ß-induced phosphorylation of Smad2 (Figure 7B). Furthermore, when injected orthotopically into the mammary gland of immunodeficient SCID mice, three of the four clones expressing the DNRII were severely inhibited in their ability to form metastatic lung nodules (Table I). Approximately 114 ± 35 (n=4) metastatic nodules were found in the lungs of mice that were orthotopically injected with mock-transfected 4T1 cells (4T1Zeo) compared with 15.5 ± 6.5 (n=15) in mice injected with DNRII-expressing tumor cells. In the majority of mice injected with gene-modified tumor cells, the sizes of the primary tumors were not significantly different from those in control animals injected with tumor cells transfected with vector alone (Table I). Histologically, no discernible differences in the morphology or local invasiveness of the primary tumors were observed among the test and control groups. All primary tumors were well-demarcated and extended from the epidermal/dermal junction through the dermis with infiltration into the abdominal musculature. Each tumor was bordered by mild neutrophilic inflammatory infiltrates and contained extensive necrotic foci. Carcinoma cells were not observed in blood vessels adjacent to the primary tumors. In contrast to the effects on in vivo metastasis, the expression of the truncated TGF-ß type II receptor did not affect in vitro migration and invasion (data not shown).

DISCUSSION

In this study we investigated the role of TGF-ß in the metastatic phenotype of a highly metastatic murine 4T1 mammary tumor. We found that TGF-ß contributes to the ability of the tumor cells to invade and metastasize in vivo. The results also show that 4T1 cells are resistant to TGF-ß-mediated growth inhibition, yet respond to TGF-ß as demonstrated by the presence of membrane receptors that bind ligand, initiate down-stream phosphorylation of Smad2 and induce Smad-dependent transcriptional activation. Thus, unlike numerous cancer cells whose resistance to TGF-ß growth inhibition is due to inactivation of TGF-ß receptors (Laiho *et al.*, 1990; Park *et al.*, 1994; Myeroff *et al.*, 1995) or Smad family signal transducers (Zang *et al.*, 1997), 4T1 cells are capable of TGF-ß-receptor-mediated signal transduction. These findings are in concordance with those of others that have identified functional receptors in TGF-ß growth resistant cancers such as glioma (Isoe *et al.*, 1998), melanoma (Rodeck *et al.*, 1999) and cervical carcinoma (Kang *et al.*, 1998). The intactness of Smad-mediated gene transcription in the 4T1 mammary tumor cell line is similar to that reported in human melanoma cells in which Smad-mediated gene transcription was shown to be independent of cell proliferation (Rodeck *et al.*, 1999).

The TGF-ß-induced morphological changes that we observed in 4T1 mammary tumor cells are consistent with those reported by others in transformed

murine mammary epithelial cells (Oft *et al.*, 1996) , murine colon cancer cells (Oft *et al.*, 1998) and skin carcinoma cells (Portella *et al.*, 1998) and have been correlated with metastatic potential (Oft *et al.*, 1996; Oft *et al.*, 1998; Portella *et al.*, 1998). Recent work by Piek et al. (Piek *et al.*, 1999) suggests that Smad proteins may play a role in the invasive and metastatic phenotype. Using the NMuMG murine mammary epithelial cell line they demonstrated the requirement for Smad2 and Smad4 proteins in inducing transdifferentiation in cells constitutively expressing low levels of type I receptor (ALK-5). The transition from the epithelial to the mesenchymal phenotype was characterized by stress fiber formation and downregulation/relocalization of E-cadherin, changes frequently associated with the invasive and metastatic phenotype (Oft *et al.*, 1996; Oft *et al.*, 1998). Similar Smad-dependent pathways present in 4T1 tumor cells may contribute to their invasive and metastatic activities. That 4T1 cells secrete TGF-ß and exhibit basal levels of Smad2 phosphorylation suggests a cell autonomous mechanism of TGF-ß signaling which may contribute to the metastatic phenotype as has been proposed in murine models of renal, lung, mammary and colon cancers (Oft *et al.*, 1998; Yin *et al.*, 1999; Hojo *et al.*, 1999). Experiments are in progress to resolve this question.

The importance of TGF-ß in the invasion and metastasis of 4T1 tumor cells was demonstrated in studies in which expression of a truncated dominant negative receptor significantly suppressed formation of lung metastases in vivo. Whereas metastasis formation was severely curbed, tumorigenicity at the primary tumor injection site was not affected. This observation is in agreement with that of Yin et al. using the human breast cancer cell line MDA-MB-231 in which they showed that dominant-negative blockade of the type II TGF-ß receptor had no effect on the growth of local tumor yet significantly diminished metastasis to the bone (Yin *et al.*, 1999). In our study, histological analysis did not reveal any discernible differences in the morphology or local invasiveness of the primary tumors between mock-transfected (4T1-Zeo) and DNRII-expressing tumors. In addition, no histologic evidence of hematogenous metastases was observed in blood vessels adjacent to the primary tumors. These findings are not unexpected since hematogenous invasion of neoplastic cells is a relatively infrequent occurrence that is rarely documented histologically. A more reliable indicator of hematogenous invasion of carcinoma cells is the presence of metastases at distant sites such as the lung. The decreased number of lung metastases in the DNRII group is therefore considered a significant finding in our study and reflective of decreased hematogenous invasion of the carcinoma cells that express the mutant receptor. Taken together, our findings suggest that TGF-ß may preferentially exert its effect on cellular genes that control invasion and spread of tumor cells.

Our observation that the DNRII affected in vivo metastasis but not in vitro migration and invasion suggests that in vitro assays are limited in their ability to predict metastatic capacity and that characteristics other than increased motility are required for spread to occur in vivo. TGF-ß may promote metastasis in part

through its ability to regulate the expression or activity of extracellular matrix-degrading proteases like MMP-9 whose role in tumor metastasis is well established (Bernhard *et al.*, 1994; Ray and Stetler-Stevenson, 1994). Our finding that TGF-ß upregulates the secretion of MMP-9 by 4T1 cells (unpublished observation) is in agreement with this mechanism. Understanding the TGF-ß-induced downstream effectors mediating the metastatic phenotype will likely lead to the identification of molecular targets that once perturbed could override this phenotype.

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Table I. Expression of truncated TGF-ß receptor II suppresses metastases formation.

Cell Type	Tumor Volume	# Lung Metastases
4T1	783.4 ± 34.2	251 ± 36
Zeo	810.7 ± 102.8	114 ± 35
clone 5	899.4 ± 110.6	10 ± 3
clone 25	606.7 ± 53.2	1 ± 1
clone 32	952.1 ± 192.4	61 ± 13
clone 34	407.2 ± 23.9	3 ± 1

SCID mice were injected orthotopically with parental, mock transfected or gene-modified 4T1 cells. Three weeks post-injection, the mice were sacrificed and their lungs were collected and examined for the presence of surface metastases. Values represent the mean ± standard error (n=4).

Figure Legends

Figure 1. Effect of TGF-ß on the cell cycle of 4T1 cells. Mv1Lu and 4T1 cells arrested in G1 by crowding were released from growth arrest by detaching and plating at a lower cell density in the presence of different concentrations of rhTGF-ß1. After 48h, the cells were collected and evaluated for DNA content by flow cytometric analysis as described in materials and methods.

Figure 2. Expression of TGF-ß receptors by 4T1 tumor cells. A. RT-PCR was performed on total RNA isolated from 4T1 (lanes 2) and Mv1Lu cells (lanes 4) using primers specific for a 405 bp fragment of TGF-ßRI, a 525 bp fragment of TGF-ßRII or a 215bp fragment of histone. Lanes 1,3 and 5 represent PCR reactions containing no template, 4T1 RNA or MvLu RNA (no RT) respectively. **B.** Subconfluent 4T1 tumor cells were labeled with 10 pM ^{125}I-TGF-ß1 in the presence (+) or absence (-) of 10 nM competing unlabeled TGF-ß1. Bound labeled ligand was cross-linked to cell surface receptors, the proteins resolved by SDS-PAGE and labeled receptors visualized by autoradiography.

Figure 3. TGF-ß induction of Smad2 phosphorylation and transcriptional reponses in 4T1 cells. A. Cells were incubated in the presence (+) or absence (-) of 2ng/ml rhTGF-ß for 45 min. Cell lysates were separated by SDS-PAGE, transferred to membrane and incubated with pretested dilutions of antibodies specific for Smad2 or the phosphorylated form of Smad2 (55kD). **B.** 4T1 cells cotransfected with p3TP-lux reporter and VR1412 ß-galactosidase control plasmids were treated with 2ng/ml rhTGF-ß1 and/or anti-TGF-ß neutralizing antibody 18h before determination of luciferase activity. Bars represent the mean relative light units and standard deviation of triplicate samples. Luciferase activities were normalized based on ß-galactosidase expression.

Figure 4. Effect of TGF-ß on tumor cell morphology. Subconfluent 4T1 cells were grown on glass coverslips for 48h in culture medium in the absence (A) or presence (B) of rhTGF-ß1. Cells were visualized with a Lieca confocal microscope after staining actin fibers with bodipy-phalloidin. Magnification = 400X.

Figure 5. TGF-ß1 enhances migration and invasion of 4T1 cells. Tumor cells were placed in the upper chamber of 12-well transwells and incubated in the presence of anti-TGF-ß antibody, rat IgG (isotype) or rhTGF-ß1. Cells that moved through untreated transwells (migration assay) or collagen coated transwells (invasion assay) into the lower chamber were counted. Bars represent the mean cell number ± SE of duplicate wells. The data shown are representative of at least two experiments.

Figure 6. Expression of dominant negative truncated TGF-ß receptor II in transfected 4T1 cells. mRNAs isolated from mock-transfected control cells (Zeo) and 4T1 cells stably transfected with DNRII-pcDNAzeo (clones 5, 25, 32, 34) were subjected to Northern blot hybridization with TGF-ß receptor II and GAPDH-specific RNA probes.

Figure 7. Gene transcription in dominant negative TGF-ß receptor II expressing clones. A. 4T1 cells stably transfected with a mock vector (Zeo) or the dominant negative truncated TGF-ß receptor II (clones 5, 25, 32, 34) were transiently co-transfected with p3TP-lux reporter and VR1412 ß-galactosidase plasmid DNA. Cells were then treated with rhTGF-ß1 for an additional 18h before determination of luciferase activity. Bars represent fold increase and standard error of triplicate samples. Luciferase activities were normalized based on ß-galactosidase expression. The data shown are representative of three experiments. **B.** 4T1 cells stably transfected with a mock vector (Zeo) or the dominant negative truncated TGF-ß receptor II (clones 5, 25, 32, 34) were treated with rhTGF-ß1 and immunoblotting for Smad2 and the phosphorylated form of Smad2 was performed.

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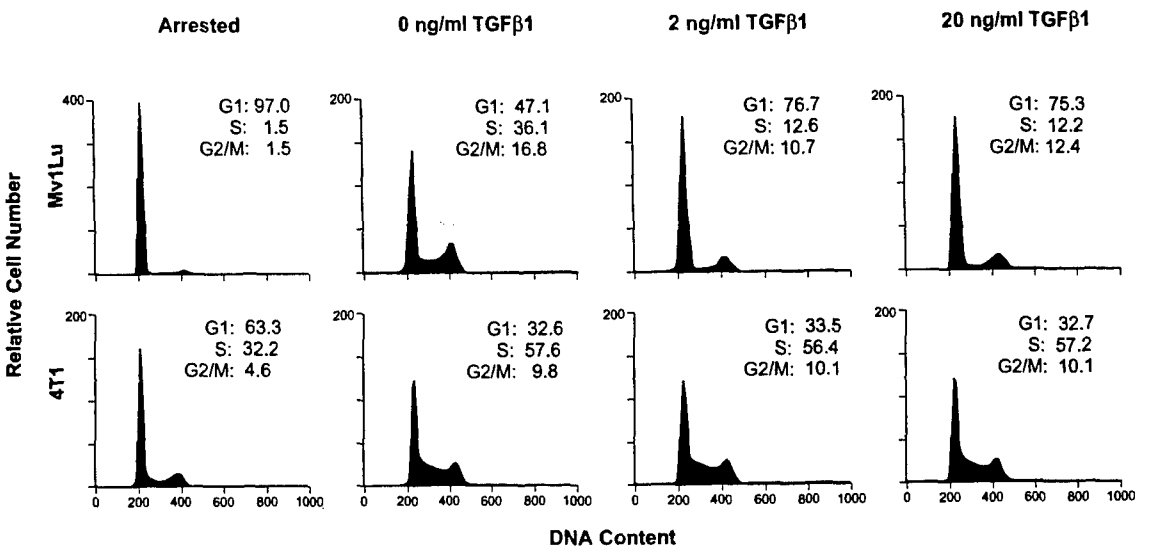
Figure 1



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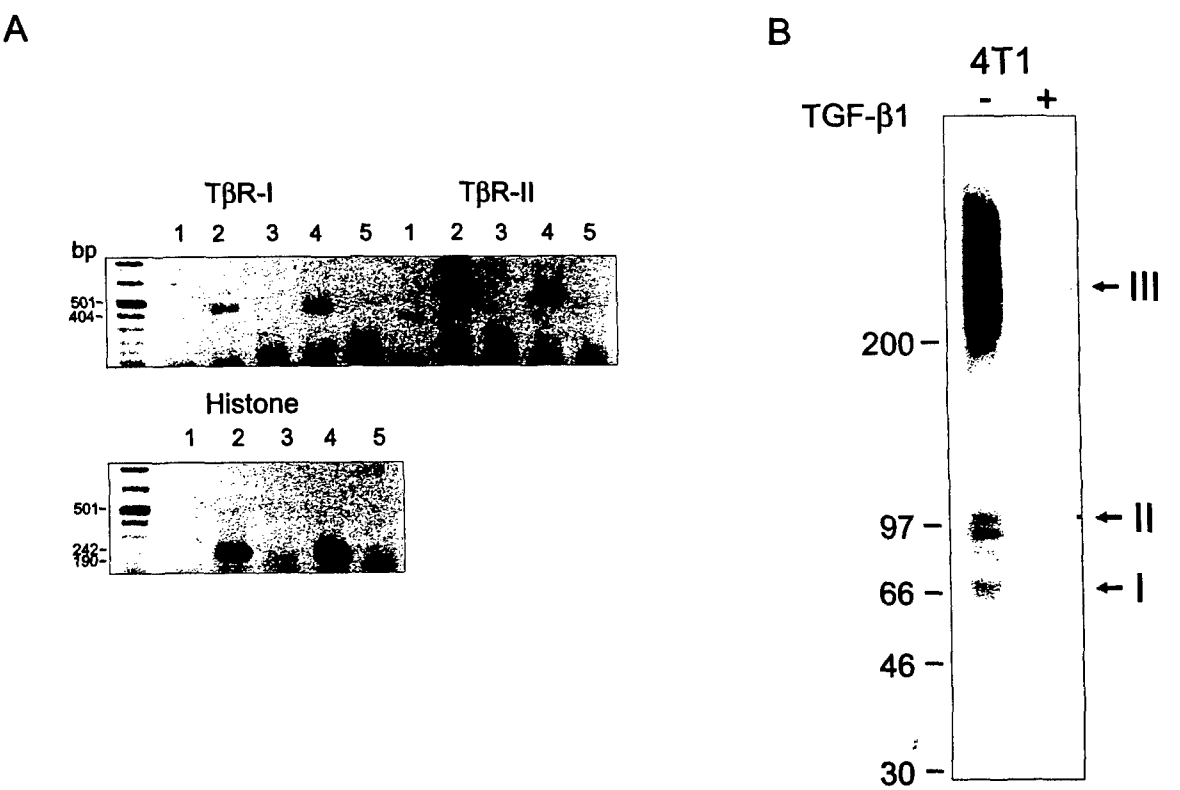

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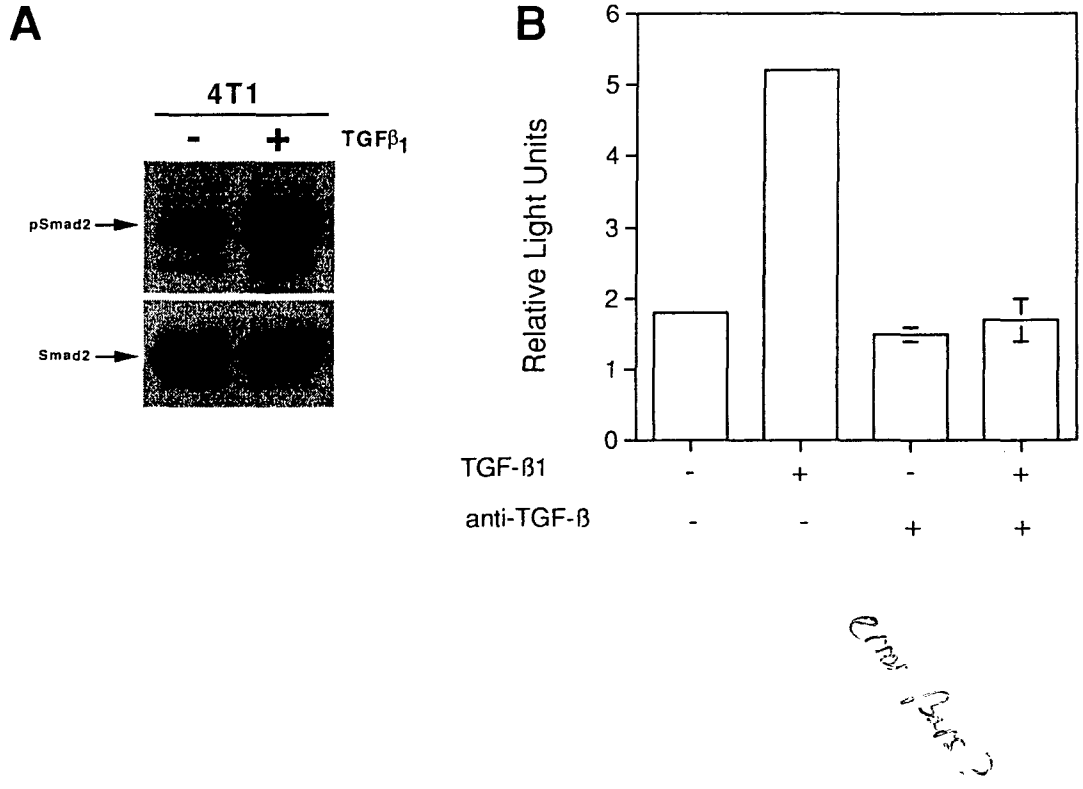
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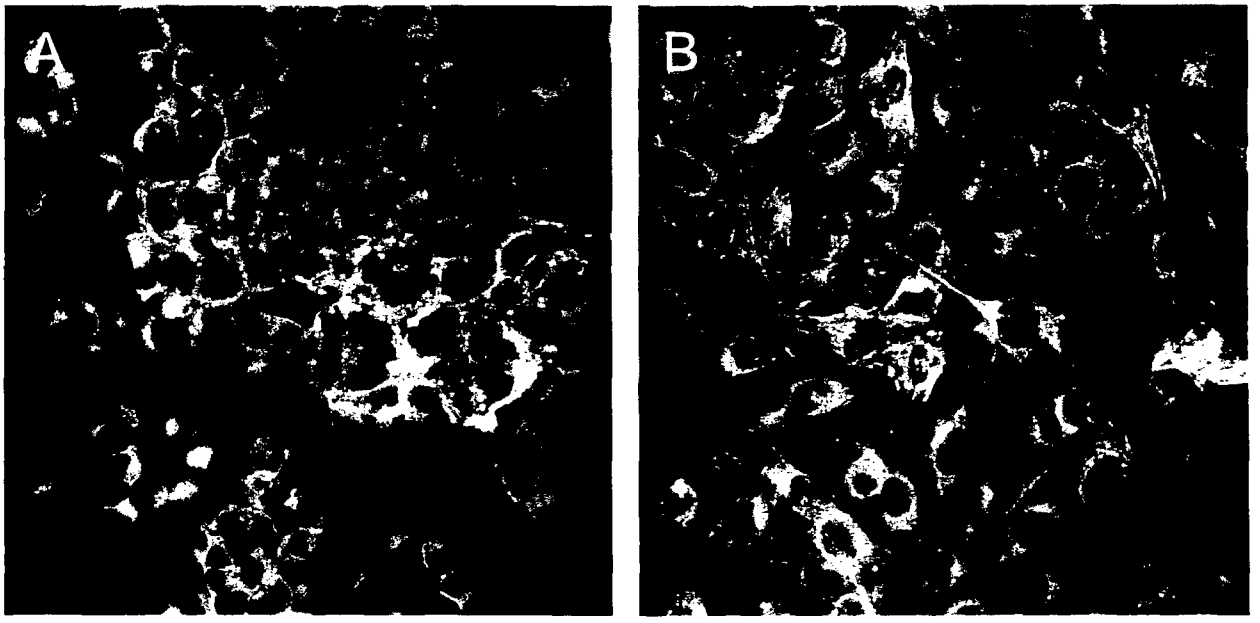
Figure 4



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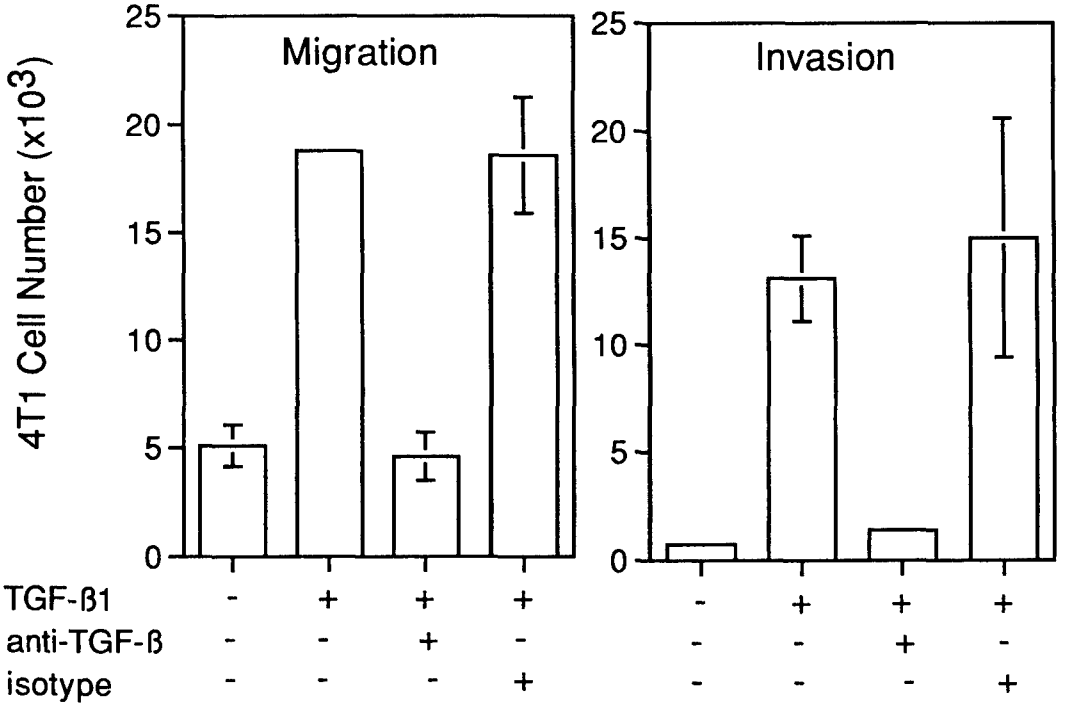
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Figure 6

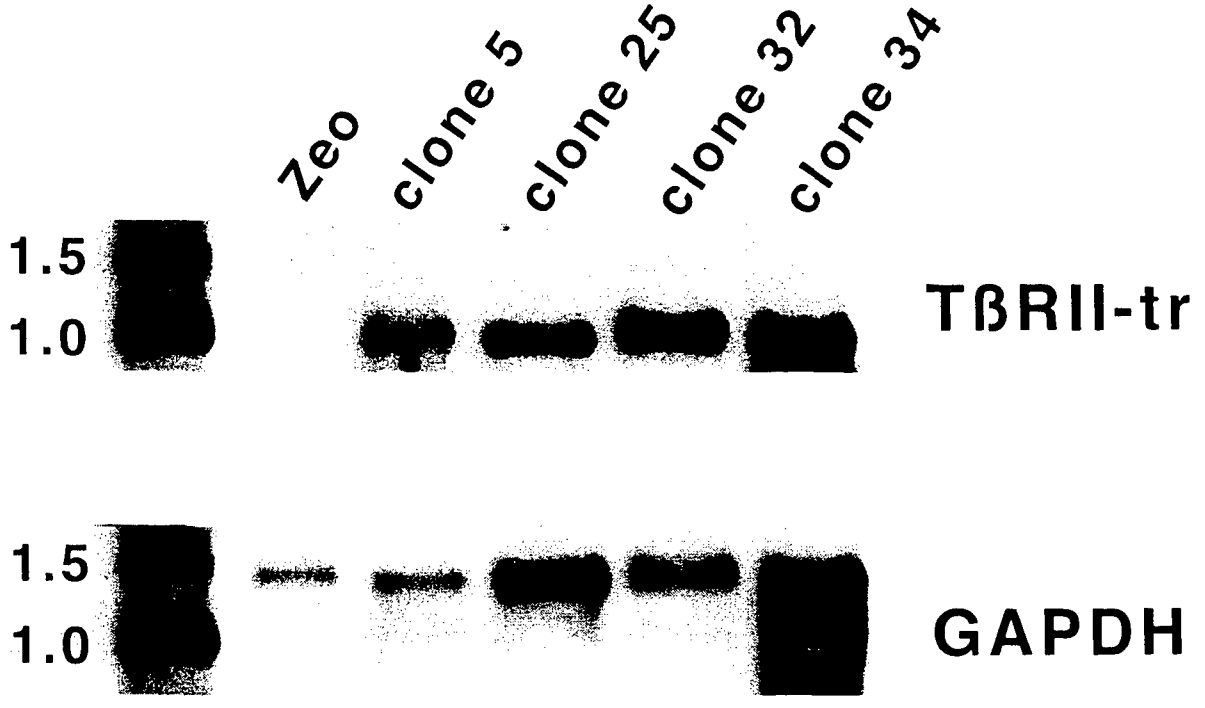
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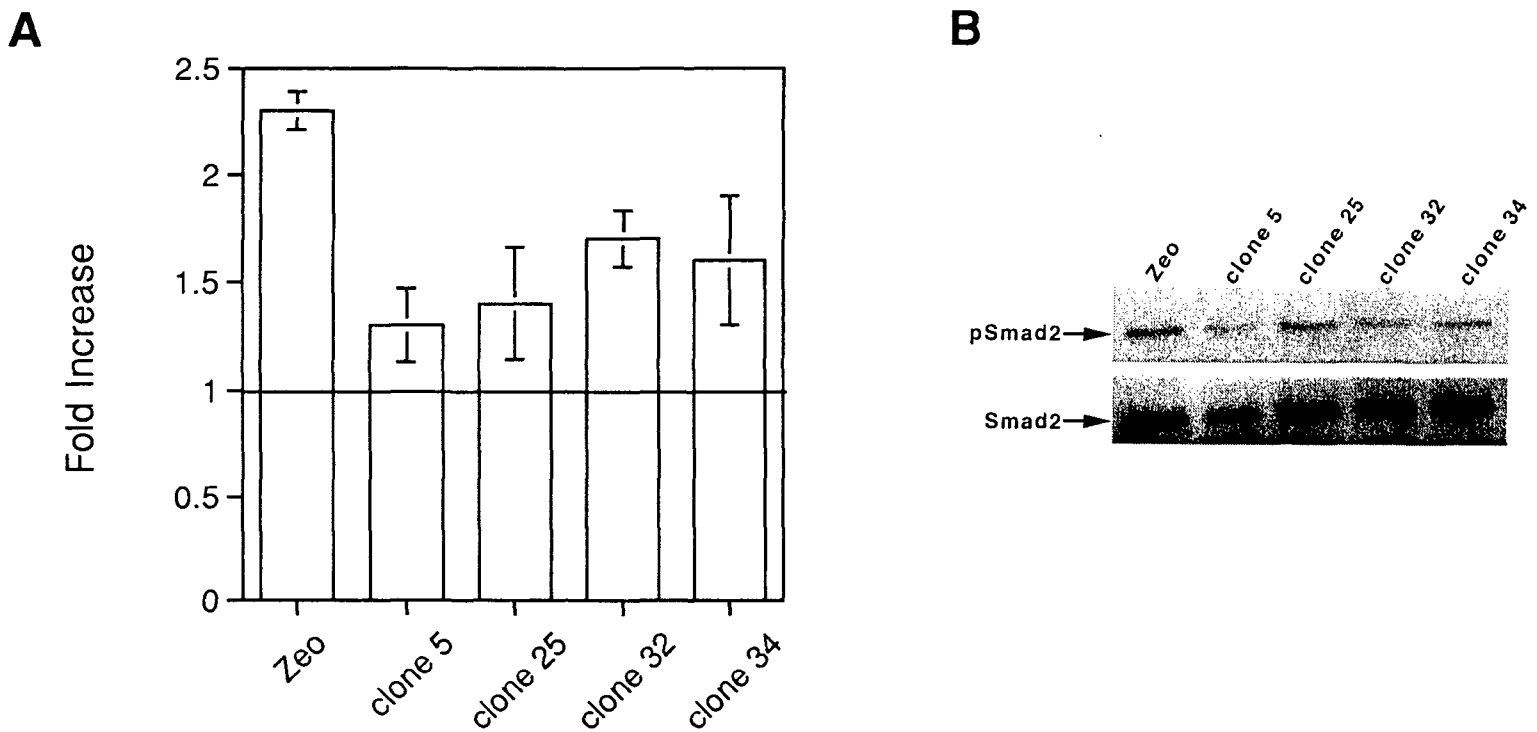
Figure 7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