

Non-redundant tumour suppressor functions of transforming growth factor beta in breast cancer

Transforming growth factor betas (TGF β s) are multifunctional peptides thought to be involved in growth regulation, development, differentiation and immune modulation of virtually all tissues in organisms, from fruitflies to humans. Together, these ligands account for a large number of intercellular signals governing cell fate. There are three mammalian isoforms (TGF β 1-3) which are the product of different genes. Appropriate levels of TGF β activity are essential for the well being of the organism. Lack of sufficient TGF β can result in severe inflammatory disease, deficient wound healing, congenital malformations including that of heart and palate and increased tumourigenesis. Conversely, excessive TGF β activity leads to scarring, development of fibrotic diseases in multiple organ systems, immune suppression, and possibly, enhancement of later stages of tumour progression. Over the last few years it has become clear that TGF β pathways are significantly involved in many processes related to carcinogenesis. Mutations in multiple TGF β signal transduction pathway components have been implicated in human colon and breast cancer.

The TGF β s 1, 2 and 3 exhibit both distinct and overlapping spatial and temporal patterns of expression and localization in tissues throughout mouse embryonic development and in the adult, with pronounced embryonic expression in areas undergoing morphogenesis (Pelton *et al* 1991). Most cells secrete TGF β s as large latent polypeptides that require activation – mostly proteolytic activation – before becoming biologically functional. These secreted latent polypeptides are always bound to alpha 2-macroglobulin (alpha 2M), which scavenges excess TGF β s and limits its local action (O'Connor-McCourt and Wakefield 1987). TGF β ligands signal through serine/threonine kinase receptors TGF β R1 and 2 to the intracellular mediators known as SMAD proteins and they differentially regulate gene expression (Massague 1998) (figure 1). TGF β R3 can also associate with the ligand and present it to TGF β R2. The functional specificities of the various receptor and SMAD protein combinations are not yet clear. The complexity found at all levels of TGF β function makes TGF β 1, 2 and 3 ideal molecules for playing significant roles in morphogenetic processes during development and in homeostatic processes in the adult. *In vivo* studies in mouse models with targeted null mutations in the three *Tgfb* genes are consistent with TGF β involvement in all of these areas (Shull *et al* 1992; Sanford *et al* 1997; Proetzel *et al* 1995).

Between the three strains of TGF β gene knockout (KO) mice, over three dozen different phenotypes have been characterized (table 1). All but those marked with an asterisk have been characterized by Doetschman's group at the University of Cincinnati Medical Center, Ohio, or through its collaborative efforts. There is clearly an overlap in the developmental localization patterns of the three TGF β s (Pelton *et al* 1991), and they all signal through one common receptor, TGF β R2, and one SMAD4 signalling molecule (Massague 1998). The three TGF β s usually, though not always, have similar functions *in vitro* (Roberts and Sporn 1990). However, the fact that the 3 *Tgfb* knockout mice have between them over 30 knockout phenotypes, none of which are redundant or overlapping, indicates that *in vivo* each TGF β ligand has unique functions.

The role of TGF β ligands and the corresponding receptor (TGF β R2) in breast cancer is very complex. A biphasic role for TGF β has been proposed in breast cancer. In normal breast and in early stages of breast cancer development TGF β acts as a suppressor, but later it facilitates tumour progression. A decreased expression of TGF β R2 in human breast tissue contributes to breast cancer progression and is related to a more aggressive phenotype in both *in situ* and invasive carcinomas (Walker 2000). Also, dominant negative interference of TGF β R2 shows defective alveolar epithelial differentiation and

Table 1. Non-overlapping phenotypes of *Tgfb* KO mice.

<i>Tgfb1</i>	<i>Tgfb2</i>	<i>Tgfb3</i>
Pre-morula lethality	Perinatal lethality	Skeletal defects
Yolk sac lethality*	Heart defects	Occipital bone
Reproductive defects	Ventricular septum defects	Parietal bone
Multifocal autoimmunity	Dual outlet right ventricle	Squamous bone
Platelet defect	Dual inlet left ventricle	Palatine bone (cleft palate)
Colon cancer	Inner ear defect – missing limbus	Alisphenoid bone
Skin cancer*	Urogenital defects	Mandibular defects
Failing heart	Dilated renal pelvis	Shortened radius and ulna
Reduced FABP activity	Agenesis (females only)	Missing deltoid tuberosity
Delayed wound healing	Uterine hornectopia	Missing third trochanter
	Testicular ectopia	Sternum malformations
	Testis hypoplasia and vas deferens dysgenesis	Rib barreling
	Lung-prenatal	Rib fusions
	Dilated conducting airways	Spina bifida
	Collapsed bronchioles	Ocular defects
	Hair follicle development	Post. chamber hypercellularity
		Partial fusion of cornea to lens
		reduced corneal stroma
		Prenatal scarring*
		Postnatal lethality: cleft palate
		Dilated conducting airways*
		Collapsed bron- chioles*
		Adult: Skin homeostasis

branching morphogenesis of the mammary gland (Joseph et al 1999). Many investigators now believe that development of resistance to TGF β by tumour cells represents a key event in the progression of malignancy. For these reasons there has been considerable interest in the signal transduction pathways utilized by these different TGF β family members. Genes that cause growth abnormalities related to differentiating alveolar epithelium in pregnant mice are most involved in breast cancer development. In pregnant mice, *Tgfb1* is moderately expressed. And, *Tgfb2* has highly specific and significant expression levels in differentiating alveolar epithelium; *Tgfb3* is found in ductal stroma and epithelium (Robinson et al 1991).

Many studies that describe the roles of TGF β in breast cancer development, whether they involve breast cancer cell lines or transgenic mouse models, suggest that TGF β 1 is the breast cancer tumour suppressor (Walker 2000). *Tgfb1* knockout (KO) mice develop an autoimmune-like inflammatory disease (Shull et al 1992) and colon cancer (Engle et al 1999), but not breast cancer (Boivin et al 1996). KO mice for *Smad3*, through which all TGF β s can signal, develop metastatic colon cancer (Zhu et al 1998); whereas, KO mice for *Tgfb1* develop non-metastatic colon cancer, suggesting that either TGF β 2 or 3 can inhibit metastasis in the colon. However, no other cancers spontaneously develop in *Tgfb1* KO mice. These data strongly suggest that TGF β 1 is not a tumour suppressor gene in mouse breast tissue. Consequently, one of the other TGF β s is likely to be a tumour suppressor in breast. The major phenotype of *Tgfb3* KO mice is a cell adhesion defect resulting in cleft palate, which in turn leads to a perinatal lethality (Proetzel et al 1995). A recent study confirms the role of TGF β 3 as a local factor that causes cell death of alveolar epithelium postpartum in post-lactational involution and remodelling of mammary gland to the virgin-like state (Nguyen and Pollard 2000). A spectrum of developmental defects in different tissues undergoing remodelling and epithelial-mesenchymal interactions are seen in *Tgfb2* KO mice, and they die within 2 h of birth from cardiopulmonary abnormalities (Sanford et al 1997).

Most of the available data on TGF β 2 and its functions in breast cancer research is highly correlative, and most research on TGF β ligands in breast cancer has involved addition of TGF β 1 or inhibition of all 3 TGF β s through inhibition of a common receptor, TGF β R2. Since growth factors and their signalling pathways are major targets for therapeutic approaches, and since TGF β signalling pathways are very likely involved in breast cancer development, it is imperative to determine which TGF β could be a therapeutic target. It would be unwise to assume that targeting any one of the three TGF β s will give the same result. At the time of lobular cell differentiation during pregnancy, the predominant isoform that expressed in the differentiating alveolar epithelium is TGF β 2 (Robinson et al 1991). Furthermore, women who are at lower risk for breast cancer have higher levels of TGF β 2 in the breast

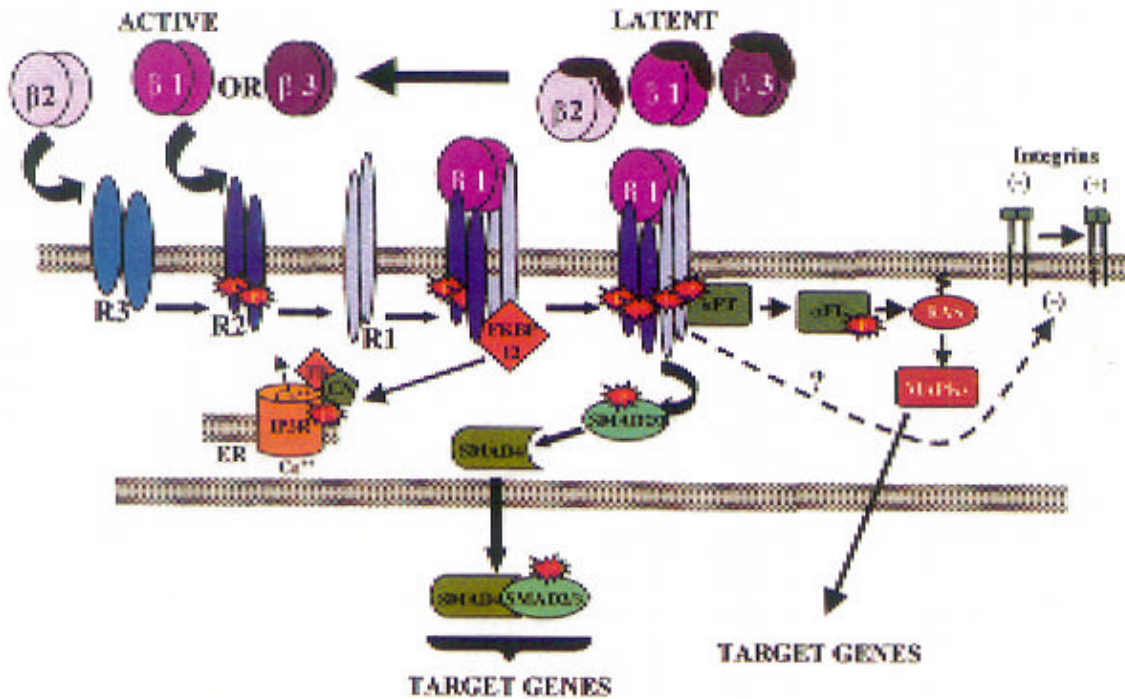


Figure 1. TGF β signalling via Smads: Upon local activation of TGF β ligands, all three TGF β s assemble a heteromeric receptor complex with one common receptor, TGF β R2. Curiously, TGF β 2 associates with TGF β R3 before interacting with TGF β R2 and TGF β R1 receptors. The receptor-ligand complex signals to the nucleus through threonine/serine phosphorylation of a series of Smad proteins which then in conjunction with other transcriptional activators and repressors regulate transcription of several target genes. The TGF β ligand-receptor complex also modulates several non-genomic signalling pathways such as calcium signalling via FKBP-12.

cystic fluid (Erbas *et al* 1999). Even in culture, TGF β 1 and TGF β 2 have been found to have opposite effects on estrogen metabolism in MCF-7 cells, and this is the case even though they both signal through the same receptor, TGF β R2 (Ee *et al* 1999; Erbas and Lai 2000). Since TGF β 2 inhibits (Erbas and Lai 2000) and TGF β 1 stimulates (Ee *et al* 1999) estrogen production, it is likely that TGF β 2 is playing the tumour suppressor role. Therefore, targeting the wrong TGF β for therapy could lead to either ineffective treatment; worse, it could have the opposite effect of inducing cancer or other undesirable effects. Consequently, it is imperative that it be determined which TGF β might be the important target for therapy, and whether that therapy should be designed to stimulate or inhibit TGF β function.

Ablation of tumour suppressor genes in the mouse has led to many mouse models for human cancers. However, there are also cases in which a traditional gene knockout results in a lethality that precludes one's ability to investigate the tumour suppressor activities in the desired tissue. To circumvent this and other similar problems, a Cre/LoxP-based conditional KO system has been developed and successfully used (Torres and Kühn 1997). It is relevant to note that TGF β 2 is hardly detectable in plasma or serum in systemic circulation and that it acts locally in specific tissues in an autocrine/paracrine fashion (Wakefield *et al* 1995). Since *Tgfb2* KO mice die within 2 h of birth, it is necessary to utilize this powerful technology for conditionally ablating the gene only in differentiating alveolar epithelial cells of mammary gland in the adult pregnant mouse. Such an innovative approach will provide a definitive answer to the question whether *Tgfb2* is a tumour suppressor gene for breast cancer in mice. If this turns out to be the case, the new strain of mouse will be a useful animal model for breast cancer and can be used for testing therapeutic approaches.

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Glutamate synthase: An archaeal horizontal gene transfer?

The availability of several new genome sequences from a variety of species of different evolutionary levels has brought interesting information about the possible existence of horizontal gene transfer between taxons. An interesting example of such a transfer may well be the case of glutamate synthase (GOGAT) which is a key enzyme in ammonia assimilation in bacteria, algae and plants. It catalyzes the reductive transamidation of amido nitrogen from glutamine to 2-oxoglutarate to form two molecules of glutamate (Temple *et al* 1998)

Glutamate synthases differ according to their molecular weights, subunit compositions, immunological characteristics, and electron donor specificities. They contain iron-sulphur cluster(s), FAD and/or FMN as prosthetic groups (Knaff *et al* 1991). Several glutamate synthases from a variety of taxonomic groups such as eubacteria, cyanobacteria, algae, yeast and plants have been cloned and sequenced (Nalbantoglu *et al* 1994). A cyanobacterial (Navarro *et al* 2000) and a bacterial glutamate synthase have been expressed in *Escherichia coli* (Vanoni *et al* 1996) and site-directed mutagenesis experiments performed to determine the NADPH binding region which is the electron donor of some glutamate synthases (Morandi *et al* 2000).

Bacterial glutamate synthases consist of two non-identical subunits and use NADPH as an electron donor (Pelanda *et al* 1993). The large subunits of bacterial glutamate synthases are highly homologous to the monomeric ferredoxin-dependent glutamate synthases of 160–180 kDa (Nalbantoglu *et al* 1994; Suzuki and Rothstein 1997). Some plants such as alfalfa also have a single subunit NADH-dependent glutamate synthase of a relatively bigger size, approximately 235 kDa (Gregerson *et al* 1996). The N-terminal region of this enzyme shows homology to the large subunit of bacterial GOGATs while the C-terminal region is homologous to the small subunit of the bacterial enzymes. The analysis of enzyme activity of *Azospirillum brasiliense* GOGAT showed that the large subunit has the *PurF*-type of glutamine amido transferase site (Pelanda *et al* 1993) while the NADPH reduction site was shown to be located in the small subunit (Morandi *et al* 2000).

The elucidation of several archaeal genomes in the recent years brought new insights to the gene organization of glutamate synthases in archaea (Bult *et al* 1996; Kawarabayasi *et al* 1998). It has been observed that the methanogenic archaeon *Methanococcus jannaschii* genome has a putative GOGAT of one third of the length of the large bacterial subunits: This is a 1.5 kb open reading frame which does not have a small subunit coding gene upstream or downstream. A small subunit-like region has not been found in any other part of the 1.7 megabase pair genome. This putative GOGAT shows high homology to the other glutamate synthases in its C-terminal region including the strictly conserved cysteines which are the ligands for the Fe-S cluster.

The ferredoxin-dependent GOGATs are functional enzymes without a small subunit although no bacterial GOGAT has been shown to be active as a single subunit. As archaea have been shown to have a more recent common ancestor with eukaryotes than eubacteria in evolution according to 16S rRNA data, a single subunit archaeal GOGAT may be considered a possibility which requires further experimental analysis for conclusion.

Interestingly, two other archaeal species, *Pyrococcus horikoshii* and *Pyrococcus abyssi* have two open reading frames which are distantly located on their genomes and they show high homology to small subunit-like GOGAT region and none of the four open reading frames has a large subunit-like region either upstream or downstream (Dincturk and Knaff 2000). The presence of two different small subunit-like regions in the genome with high homology to one another suggests a possible gene duplication. Another single small subunit-like GOGAT gene has been cloned from another *Pyrococcus* species and expressed in *E. coli*. It has been claimed that the purified recombinant protein is a functional enzyme as a tetramer (Jongsareejit *et al* 1997). This is the only report for GOGAT activity of the small subunit without the presence of a big subunit. This is rather interesting as this subunit has been attributed to play a role in NADPH-binding but not glutamine amidotransferase activity.

Could it be that the two subunits were two separate enzymes earlier in evolution and were later joined via horizontal gene transfer to provide an NADH-binding region to some higher plant GOGATs? Or as they are two separate subunit enzyme in bacteria, were they separated in archaea when another electron donor became available or more abundant? Which of the electron donors could

be earlier in evolution? Can a tetramer structure of the small subunit bring the essential amino acids in such a three-dimensional structure which allows glutamine amidotransferase activity which is shown to be located at the large subunit? Which electron donors do the archaeal enzymes use?

In short, glutamate synthase is an excellent candidate to be a model enzyme to study horizontal gene transfer in archaea.

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