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Role of TGF- \uparrow in Heart Development: Analysis of the Type II TGF- \uparrow Receptor

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LOYOLA UNIVERSITY CHICAGO

ROLE OF TGF- β IN HEART DEVELOPMENT:
ANALYSIS OF THE TYPE II TGF- β RECEPTOR

A DISSERTATION SUBMITTED TO
THE FACULTY OF THE GRADUATE SCHOOL
IN CANDIDACY FOR THE DEGREE OF
DOCTOR OF PHILOSOPHY
DEPARTMENT OF MOLECULAR BIOLOGY

BY

PATRICIA S. GRUTKOSKI

CHICAGO, ILLINOIS

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COMMONLY USED ABBREVIATIONS

bp	base pairs
β RI:	Type I TGF- β Receptor
β RII:	Type II TGF- β Receptor
β RII-DN:	Kinase Deficient, Dominant Negative Mutant of β RII
dpc:	days post coital
kb	kilobase pairs
kD	kilo-Daltons
mL	milli-liter
mRNA:	messenger ribonucleic acid
ng	nanogram
PAI-1:	Plasminogen Activator Inhibitor-1
SPARC:	Secreted Protein, Acidic and Rich in Cysteines
TGF- β :	Transforming Growth Factor- β
TNC:	Troponin C
TNI:	Troponin I
TNT:	Troponin T

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CHAPTER 1

INTRODUCTION

Embryonic and fetal development of the heart is a unique process when compared to the development of other muscle tissue in that the dividing muscle cells, or cardiomyocytes, are highly differentiated and exhibit coordinated, contractile activity. However, these cardiomyocytes lose their capacity to divide, and therefore, embryonic and fetal heart development are the primary periods of active myocyte proliferation. During the perinatal period of rat heart development, a rapid "transition" occurs in the ventricle which involves the cessation of proliferation of the cardiomyocytes. In this same perinatal transition period, the cardiomyocyte undergoes a maturation process in which a change in expression of individual isoforms of several contractile proteins occurs that affects its contractile activity. Additionally, remodeling of the ventricle occurs during the late fetal and early neonatal periods of development involving both cardiomyocyte and non-cardiomyocyte populations. We have hypothesized that the transforming growth factor- β (TGF- β) family of receptors and ligands plays an integral role in these different processes of the perinatal "transition" due to their characteristic anti-proliferative activities as well as their ability to influence the expression of numerous genes associated with ventricular remodeling.

TGF- β ligands (TGF- $\beta_{1,3}$) transduce their actions through a heterodimeric receptor complex containing the Type I and Type II TGF- β serine/threonine kinase receptors (β RI and β RII, respectively). While β RI appears to be constitutively expressed, we have shown by Northern blot analyses that TGF- β_1 , TGF- β_3 , and β RII expression in the rat ventricle is low-to-undetectable during fetal development and increase dramatically at-or-around birth. These patterns are temporally coordinated with the cellular and extracellular changes that occur during the perinatal transition period. These changes include the cessation of cardiomyocyte proliferation, the isoform switch of several members of the contractile machinery within the cardiomyocyte, the increase in extracellular matrix accumulation, and an increase in angiogenesis, or blood vessel formation, in the ventricle. Therefore, we would like to determine the direct roles TGF- β may have in these developmental processes.

It is widely accepted that both TGF- β receptors are necessary for most, if not all, actions of TGF- β signal transduction. β RII is the primary ligand binding receptor and initiator of the TGF- β signaling cascade. It has been shown that ablation of β RII kinase function is an effective way to study the effects TGF- β has on cellular functions, and several of these types of studies have been utilized to study the effects of TGF- β on cardiomyocyte growth and maturation. These studies have utilized a dominant negative methodology in which an inactive β RII, either by eliminating the kinase domain or through specific inactivating point mutations, was introduced into the cell. When overexpressed, this mutant receptor interferes with the

endogenous wild type β RII's function by limiting or eliminating its access to bioactive ligand as well as available β RI, resulting in a blunted and/or ablated response to TGF- β as the signal transduction pathway cannot be activated by the truncated receptor.

This dominant-negative methodology was chosen to study the effects of TGF- β in cardiomyocyte and ventricular development and provide a system in which the roles of each receptor in cardiomyocyte development can be elucidated. While the use of primary cardiomyocytes would be the ideal cell type for such studies as they would be expected to closely reflect events which occur *in vivo*, their lack of proliferative activity *in vitro* and low receptivity to experimental manipulation have made them a non-tenable methodology. Therefore, the model system chosen to study these effects of TGF- β on a cardiomyocyte-like cell type was the use of two rat, fetal ventricular myocyte-derived cell lines, BWEM and CLEM. To enable us to evaluate the direct effects TGF- β may have on the cardiomyocyte, we utilized the dominant negative methodology to eliminate the TGF- β response in these cell lines. To do this, truncated, kinase-deficient human β RII was stably expressed in the BWEM and CLEM rat myocyte-like cell lines to create two clonal mutant cell lines, BW-H1 and CL-B5, with reduced or absent β RII function.

Each of these cell lines, BWEM, CLEM, BW-H1, and CL-B5, as well as primary cultures of 18 day fetal cardiomyocytes were then used to study the direct effects TGF- β may have on the myocyte population during ventricular development. The goal of this dissertation project was to

establish the role(s) TGF- β plays in modifying myocyte proliferation and differentiation during the perinatal transition period. In addition to the creation and characterization of the β RII dominant negative cell lines, the data collected for this dissertation address the following questions:

(1) Does TGF- β inhibit cardiomyocyte proliferation?

(2) Does TGF- β play a direct role in cardiomyocyte differentiation by inducing the “adult” isoforms of three contractile proteins, Troponin C, Troponin I, and Troponin T?

(3) Does TGF- β promote ECM accumulation and angiogenesis through the upregulation of cardiomyocyte produced PAI-1 and SPARC peptides?

CHAPTER 2

REVIEW OF THE LITERATURE

One of the most effective means of controlling tissue formation and cellular function is the production of stimuli from cells which either act on the cells themselves in a feedback mechanism (autocrine stimuli) or on neighboring cells (paracrine stimuli). Growth factors are one type of stimuli, and therefore, determining the role of growth factors in embryonic and fetal development is an area of ongoing study. Changes in proliferative activity, differentiation, cell shape, migration, cell adhesion, and cell death are examples of the effects growth factors may elicit (Simmen and Simmen, 1991; Adamson 1993). Growth factors are important in such activities as embryonic implantation (Simmen and Simmen, 1991), determining dorsal/ventral patterning in the early embryo (Slack 1990; Christian and Moon, 1993; Nusslein-Volhard 1991; de Vries et al., 1996), regulating homeobox gene expression (Dawid et al., 1992), influencing proper limb development (Fallon et al., 1994), and regulating cell growth and/or differentiation (Adamson 1993; Murphy and Barron, 1993). The use of gene ablations has provided examples of the importance of growth factors such as the transforming growth factor-betas (Proetzel et al., 1995; Boivin et al.,

1995; Kaartinen et al., 1995) and fibroblast growth factors (Amaya et al., 1991; Ueno et al., 1992; Feldman et al., 1995) in development.

Development of the heart, like many other organs and systems, appears to be under partial control by several growth factors which work in an autocrine or paracrine manner. The presence of growth factors and their receptors in the developing heart appears to be differentially regulated in that they are expressed in both temporally and spatially discrete times and sites. These growth factors are believed to be involved in several separate, yet inter-related, processes: 1) cardiomyocyte proliferation and differentiation, 2) the formation of biomechanical structures such as the valves, 3) vasculogenesis; capillary angiogenesis and neovascularization, and 4) non-muscle cell-produced extracellular matrix (ECM) formation.

Review of TGF- β s

Identification of Ligands and Receptors

Among an expanding list of growth factors that appear to have an active part in heart development are members of the transforming growth factor-beta (TGF- β) superfamily. TGF- β is the prototypic member of this large peptide growth factor family that includes the TGF- β s, activins, inhibins, bone morphogenetic proteins, mullerian inhibiting substance, and products of the *decapentaplegic* (*Drosophila*) and *Vgl* (*Xenopus*) genes (Massague 1990; Kingsley 1994). To date, five isoforms of TGF- β have been identified: TGF- β_{1-3} (mammalian), TGF- β_4 (avian), and TGF- β_5

(amphibian) (Massague 1990). Although originally established as a novel isoform, TGF- β_4 has been shown to be the avian TGF- β_1 (Burt and Paton, 1992). Each of the three mammalian isoforms have nearly perfect amino acid identity across species, and all five TGF- β isoforms have been highly conserved throughout evolution, suggesting specific roles for each. In addition, each TGF- β isoform shares a high degree of amino acid sequence homology with each other in their mature, bioactive domain. In contrast, each TGF- β isoform is associated and secreted with a large latency associated protein (LAP) as well as a latent TGF- β binding protein (Miyazono et al., 1988; Miyazono et al., 1990; Miller et al., 1992; MacKay et al., 1992). These proteins are unique to each TGF- β isoform. In order for “latent” TGF- β to be activated, these latency proteins must be released from the bioactive TGF- β homodimer by processes that remain to be identified *in vivo*, yet *in vitro* acidification, heat, or proteolytic cleavage has been shown to be effective (Massague 1990; Ghosh and Brauer, 1996). Lastly, multiple members of the TGF- β family are often co-expressed and co-localized, and they generally elicit similar mechanisms of action on cells in culture. While almost all TGF- β peptides identified exist as homodimers, limited examples of TGF- $\beta_{1/2}$ heterodimers have also been identified in platelets and bone (Cheifetz et al., 1987; Ogawa et al., 1992).

As with most, if not all, growth factors and cytokines, TGF- β s exert their effects through cell surface receptors. To date, nine distinct proteins have been identified on the cell surfaces of a diverse array of cell types which bind the TGF- β s (Massague 1987; Sporn and Roberts, 1992). Three of

these proteins in mammalian cells are classified as functional TGF- β receptors: Type I (β RI), Type II (β RII), and Type III (β RIII). β RIII, otherwise known as betaglycan, has been cloned from rat, chicken, human, and porcine cells (Lopez-Casillas et al., 1991; Wang et al., 1991; Moren et al., 1992; Barnett et al., 1994), and varies in size from 200-400 kD, dependent on the level of glycosylation. However, the core protein is approximately 110 kD in size and is moderately conserved across species, having a 70-80% identity at the amino acid level. This receptor appears to bind each of the different TGF- β isoforms with a high affinity (K_d of 50-200 pM for β_{1-3} , Miyazono et al., 1994) and is expressed in and found on the surfaces of virtually all cell types. However, this receptor is not a "classical" receptor in that it does not have an intracellular signaling domain, and as such, its exact role in TGF- β signal transduction has yet to be fully elucidated (see below).

β RI and β RII have also been cloned from a variety of species and are the prototypes of an ever growing family of serine/threonine (S/T) kinase receptors. β RII has been cloned recently from cDNA libraries derived from human, porcine, mink, rat, chicken, and mouse cell lines (Lin et al., 1992; Wrana et al., 1992; Tsuchida et al., 1993; Lawler et al., 1994). Immediately following the cloning of β RII, Type I receptor genes were cloned through cDNA library screens utilizing sequences obtained from the kinase domain of β RII. These screens have identified numerous genes which fall into the Type I "family", but their exact identities were harder to determine due to their promiscuous ligand binding abilities in concert with β RII (example:

Ebner et al., 1993). However, a physiologically relevant, true β RI has been identified from human (ALK-5), rat (R4), and murine (ESK2/mTFR40) systems (Franzen et al., 1993; He et al., 1993; Suzuki et al., 1994b; Tomoda et al., 1994).

The bulk of β R_{II} can be divided into two domains: the extracellular (EC) domain and the intracellular (IC) domain (Figure 1). While the TGF- β ligands are almost perfectly conserved across species, the ligand-binding, EC domain of β R_{II} has only 80-85% amino acid sequence identity across species, with the divergent areas considered not to be involved in ligand binding activity. In contrast, the IC domain, of which the bulk is the S/T kinase domain, is 96-100% identical across species at the amino acid level. While the general structure of β R_{II} is highly conserved, two variants have been identified to date in human and murine cells. One contains a 25 amino acid insert in the EC domain (Suzuki et al., 1994a; Hirai and Fujita, 1996), while the other has an extended IC tail of ~400 amino acids (compared to 30 a.a. for its “normal” human counterpart; Kawabata et al., 1995a).

Like β R_{II}, β R_I receptors are highly conserved with 100% amino acid identity within the IC-S/T kinase domain and 90-97% overall sequence identity (Figure 1). Contrasting with this high level of interspecies conservation seen in the domains of β R_I, the kinase domain of different members of the type I family of receptors within the TGF- β superfamily (aka type I activin receptors, type I BMP receptors, etc.) are less conserved at the amino acid level (~65%), and interestingly, have relatively low amino

acid homology to the β RII kinase domain (~40%) (Tomoda et al., 1994).

These low homologies imply that the type I and type II receptors have distinct functions within the signaling cascade, and that the different type I receptors activate distinct pathways / downstream effectors.

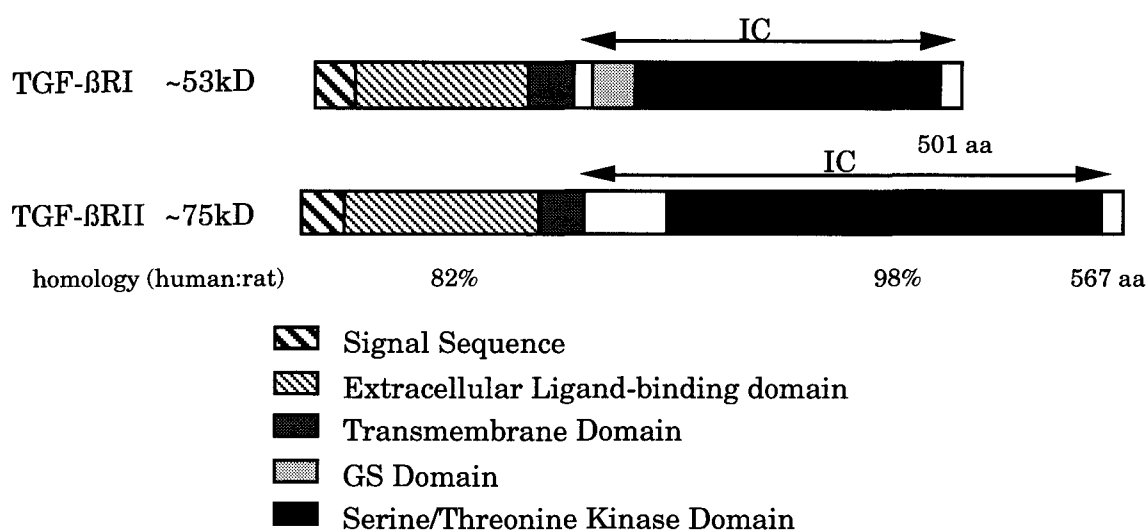


Figure 1: Schematic Diagrams of β RI and β RII

Diagrams of each TGF- β receptor and the major functional domains are depicted. Arrows indicate full intracellular (IC) domain, a majority of which is the kinase domain, but also including activation domains such as the well conserved GS domain in the type I receptors. Amino acid homologies between the extracellular domain and kinase domains are given for β RII.

General Functions of TGF- β

The general biological functions of TGF- β can be broadly grouped into three major, albeit overlapping, categories: 1) actions which affect cellular proliferation and migration; 2) actions involved in cell adhesion and ECM formation; and 3) actions that affect a cell's phenotype (Massague 1990; Roberts and Sporn, 1992a). In general, all of these activities can be mediated by any of the five TGF- β isoforms known to date.

Cellular Proliferation

While TGF- β is able to stimulate cell division in a distinct and limited subset of cell types (Anchan and Reh, 1995, and references therein; Zhao and Young, 1996), the TGF- β 's have significant anti-proliferative activities on almost all cell types in culture (Massague 1990; Sporn and Roberts, 1992). While such studies continue, the effects of TGF- β on cellular proliferation appear to involve the regulation of the synthesis and/or activities of proteins involved in the normal progression through the cell cycle. For example, the retinoblastoma (Rb) gene is a critical cell cycle regulatory protein which is inactivated by phosphorylation in late G_1 , and this inactivation is necessary for entry into S phase and DNA replication (Laiho et al., 1990). TGF- β_1 has been found to maintain Rb in its unphosphorylated state leading to G_1 arrest (Laiho et al., 1990). Addition of TGF- β_1 at the mid-to-late G_1 stage of the cell cycle did not have any effect on the Rb which was already phosphorylated, but inhibited further

phosphorylation, thus regulating the phosphorylation and not the dephosphorylation of Rb (Laiho et al., 1990).

Of growing importance in the study of TGF- β 's anti-proliferative effects is the understanding of the multifaceted regulatory role TGF- β has in regards to the many proteins which are involved in the cell cycle. TGF- β_1 has been found to regulate cyclin/cdk activities, one of which mediates Rb phosphorylation (Lafon et al., 1995), which is needed during G₁ to S phase transitions. While cyclin D mRNA levels appear unchanged in response to TGF- β_1 , the mRNA levels of two other G₁ cyclins, cyclin E and cyclin A, are reduced to almost undetectable levels in cells treated with TGF- β_1 (Geng and Weinberg, 1993). The lower levels of cyclin A can be attributed to a direct downregulation of mRNA synthesis by TGF- β (Feng et al., 1995). In addition to cyclin synthesis, synthesis of the cyclin E/D co-activator, cyclin dependent kinase (cdk) 4, is suppressed by TGF- β_1 in a post-transcriptional manner (Ewen et al., 1993; Ravitz et al., 1995). This suppression appears to be critical in preventing the formation of active cyclin E/cdk4 complexes in cells treated with TGF- β_1 . Inhibitors of cdk2 (cyclin A-associated) and cdk4 such as p27/Kip1 and p21/Cip1/WAF-1 were identified as proteins which were in excess in contact-inhibited or TGF- β_1 treated cells (Polyak et al., 1994a; Polyak et al., 1994b; Toyoshima and Hunter, 1994). In mink lung cells, WAF-1 synthesis was induced by TGF- β_1 (Raynal and Lawrence, 1995). However, excess Kip1 was not caused by increased production of the protein itself, but by decreased competition for binding from cyclin D/cdk4 complexes which resulted from down-regulated cdk4 synthesis in response

to TGF- β_1 . Additionally, another inhibitor of cdk4, p15^{INK4B}, was identified which inhibits cdk4 and cdk6 (Hannon and Beach, 1994). p15^{INK4B} is induced approximately 30-fold in response to TGF- β_1 , and while it may not result in the release of Kip1 from cyclin D/cdk4 complexes, it inhibits cyclin activity needed for progression through G₁. Additionally, it has been shown that TGF- β_1 induction of Cip1 and p15^{INK4B} relies on Sp1 consensus binding sites within their respective promoters, indicating that the activity of the Sp family of transcription factors are also regulated by TGF- β_1 (Li et al., 1995; Datto et al., 1995).

Cellular Differentiation

As cellular differentiation is usually coupled to the cell's withdrawal from the cell cycle, TGF- β is a prime candidate when studying differentiation factors. While inhibition of proliferation is not sufficient for bronchial epithelial cells to undergo squamous differentiation, TGF- β was identified as the serum factor that induced this differentiation, which included decreased proliferation accompanied by morphological changes (Masui et al., 1986). Similarly, TGF- β induces the differentiation of endothelial cells *in vitro* into cells that appeared indistinguishable from smooth muscle cells and expressed α -smooth muscle actin, a state which was irreversible after prolonged exposure to TGF- β_1 (Arciniegas et al., 1992). As TGF- β_1 can induce myocyte-like characteristics in endothelial cells (Arciniegas et al., 1992), pericytes (Verbeek et al., 1994), epithelial cells (Kurodaka et al., 1995), and fibroblasts (Ronnov-Jessen and Petersen, 1993),

it is no surprise that TGF- β has been postulated to play a role in muscle development.

In skeletal muscle development, progenitor cells proliferate as non-differentiated myoblasts which do not express muscle-specific genes and have no or limited contractile ability. Upon withdrawal from the cell cycle, these cells terminally differentiate and fuse to form contractile myofibers. This differentiation process can be triggered *in vitro* by maintaining the cells in a low serum media. TGF- β_1 appears to be necessary for differentiation as mutant myoblasts which are unable to respond to TGF- β_1 do not undergo differentiation under low serum conditions (Filvaroff et al., 1994). This is in contrast to most of the previous studies relating TGF- β_1 activity to myoblast differentiation. In myoblasts treated with TGF- β_1 under low serum conditions, the muscle-specific transcription factors MyoD1 and myogenin were inhibited or not expressed, which blocked contractile protein expression and myoblast fusion into myotubes (Massague et al., 1986; Olson et al., 1986; Florini et al., 1986; Vaidya et al., 1989; Brennan et al., 1991). However, *in vivo*, differentiation occurs where mitogens, or positive stimuli, are potentially high. Therefore, addition of TGF- β_1 to myoblasts maintained in high serum (or mitogen enriched) media is also able to induce myoblast differentiation (Zentella and Massague, 1992). This mechanism(s) involved the inhibition of proliferation (one aspect being downregulation of *c-myc*) and decreasing the levels of the myogenic inhibitor of differentiation, Id. While the anti-proliferative activity of TGF- β_1 aides in the induction of differentiation, overexpression of MyoD alone

releases the cells from their dependence on TGF- β_1 to differentiate (Filvaroff et al., 1994).

ECM Formation, Cell Adhesion, and Angiogenesis

One of the classic effects of TGF- β stimulation is the regulation of ECM deposition. This regulation involves the stimulation of ECM protein production and secretion, inhibition of protease synthesis and increasing synthesis of protease inhibitors, and stimulation of integrin receptor production which enhances the cell's ability to interact with the ECM and surrounding cells (Roberts and Sporn, 1992a). Two classic ECM promoting factors which are responsive to TGF- β actions are an ECM protein, fibronectin, and a protease inhibitor, plasminogen activator inhibitor-1 (PAI-1). Increases in expression or promoter activity of these two genes, in addition to growth inhibition, are standards used to determine a cell's ability to respond to TGF- β (e.g. Massague 1990; Chen et al., 1993; Brand et al., 1993; Attisano et al., 1994; Reed et al., 1994). Other ECM genes/proteins or ECM promoting factors which are induced by TGF- β include the pro α 1 and pro α 2 chains of type I collagen, and tissue inhibitor of metalloproteinases (TIMP) (Laiho et al., 1986; Ignatz et al., 1987; Wrana et al., 1988; Overall et al., 1989; Reed et al., 1994). In addition to promoting expression of "positive" factors of ECM biosynthesis, TGF- β inhibits or decreases expression of factors associated with ECM degradation such as collagenase, plasminogen activator, and stromelysin/transin (Laiho et al., 1986; Edwards et al., 1987; Kerr et al., 1988).

While most effects of the TGF- β_1 are transcriptional, post-transcriptional regulation such as increasing mRNA stability of collagen and fibronectin has also been demonstrated (Wrana et al., 1991). Specifically, regulation of Type I collagen expression demonstrates the ability of TGF- β_1 to regulate both transcription and translation (Fine and Goldstein, 1993). Type I collagen is made up of two subunits, $\alpha 1(I)$ and $\alpha 2(I)$. Upon TGF- β_1 treatment, $\alpha 1(I)$ mRNA levels increase resulting in increased collagen protein production. This increase in $\alpha 1(I)$ is attributed, at least in part, to increased protein binding to a TGF- β_1 activating element within the $\alpha 1(I)$ promoter (Ritzenthaler et al., 1993). In contrast, $\alpha 2(I)$ mRNA levels are unchanged; by an as yet unknown mechanism, TGF- β_1 induces and/or increases translation from the "basal level" of this specific mRNA resulting in an equal increase in both Type I collagen protein subunits (Fine and Goldstein, 1993).

In addition to promoting ECM accumulation, TGF- β also promotes cell adhesion to the ECM through the induction of several members of a family of receptors called integrins which function as ECM receptors (Ignatz and Massague, 1987; Heino and Massague, 1989; Wahl et al., 1993). These ECM-binding integrins are responsible for transmitting signals from the ECM into the cell and are thought to be expressed before the ECM proteins are laid down, especially in fetal development (Borg et al., 1990). Integrins are composed of one α and one β subunit, with the α subunit specifying the particular ECM protein to which the receptor binds. Depending on cell type, TGF- β can change a cell's affinity for different ECM

proteins (e.g. increase α_2 , α_5 , and β_1 while decreasing α_3 in osteosarcoma cells) (Heino and Massague, 1989), or increase a cell's affinity for ECM in general (monocytes and thymocytes) (Ignatz and Massague, 1987; Wahl et al., 1993). Similar to the regulation of collagen synthesis, this upregulation of mRNA and protein levels of the integrin subunits has a dual level of control: increasing transcription rates and increasing the rate at which the protein is processed and expressed at the cell surface (Ignatz and Massague, 1987).

In contrast to these ECM promoting factors, in processes such as tissue remodeling and wound repair, TGF- β exerts opposing effects. While TGF- β_1 usually induces ECM accumulation, it can also transiently induce degradation of ECM components by increasing the production of proteinases such as type IV collagenase (Overall et al., 1991; Wahl et al., 1993). This degradation allows cell detachment from and migration through the ECM (Overall et al., 1991; Salo et al., 1991). The actions of type IV collagenase are also limited by TGF- β_1 as it co-induces its inhibitor, TIMP, to a greater degree, but at a slower rate, thus allowing detachment and migration of cells and then promoting their reattachment and ECM accumulation (Overall et al., 1991). TGF- β 's ability to promote cell migration is also one method in which TGF- β controls angiogenesis, or formation of blood vessels. TGF- β_1 is able to stimulate an angiogenic response when injected into chicken chorioallantoic membrane or subcutaneously, with the new capillary cords growing toward the site of TGF- β_1 injection (Roberts et al., 1986; Yang and Moses, 1990). This activity

is similar to wound healing in that TGF- β_1 induces cell migration (possibly having a chemotactic role) and changes in ECM composition. Similar to the negative control demonstrated with TIMP, TGF- β also limits angiogenesis after promoting it by upregulating PAI-1, which inhibits plasmin, an important enzyme in angiogenesis (MacGregor et al., 1995).

In addition to its ability to control angiogenesis through ECM turnover, it has been proposed that TGF- β may also promote angiogenesis by inducing cells to produce angiogenic factors (Roberts et al., 1986). One of these factors is SPARC (secreted protein that is acidic and rich in cysteine), osteonectin, or BM-40. SPARC has been associated with tumor metastasis and with tissues undergoing morphogenetic processes, particularly cells undergoing proliferation, migration, and detachment from the ECM (Mason et al., 1986; Sage et al., 1989; Wrana et al., 1991; Iruela-Arispe et al., 1991a; Reed and Sage, 1996; Sage 1997). The effects SPARC is able to elicit in these processes are broad, encompassing cellular proliferation (negative), cytoskeletal rearrangements, cell adhesion, cell migration, and ECM turnover (Sage 1997). As most of these processes are involved in the formation of blood vessels, it is not surprising that SPARC has been found to be actively synthesized by endothelial cells undergoing angiogenesis (Iruela-Arispe et al., 1991b; Lane et al., 1992). Therefore, TGF- β may promote morphogenesis and angiogenesis through its ability to induce the expression of SPARC in both fibroblasts and endothelial cells (Wrana et al., 1988; Wrana et al., 1991; Reed et al., 1994), and this induction may be at the transcriptional and/or post-transcriptional level (Wrana et al., 1991; Iruela-

Arispe et al., 1995). Interestingly, intact SPARC has been found to have no angiogenic activity on its own, but a cleavage product of SPARC as a result of digestion with plasmin induces angiogenesis *in vivo* (Iruela-Arispe et al., 1995). Therefore SPARC, as well as TGF- β , also limits its own angiogenic activity as it has been found to increase PAI-1, an inhibitor of plasmin activation (Reed and Sage, 1996).

TGF- β Signal Transduction Pathway

As previously mentioned, three receptors for TGF- β have been identified to date, β RI, β RII, and β RIII. The cloning of these receptors has provided a molecular and cellular means by which to study the interactions and specific roles each plays in TGF- β signaling. While it appears that all three mammalian isoforms of TGF- β ligands mediate their responses through their interactions with β RI and β RII, the exact function of β RIII in these processes is unclear. β RIII is able to bind all three isoforms of TGF- β as well as members of another family of growth factors, fibroblast growth factors (Andres et al., 1992), but as it does not have an intracellular signaling domain, its actions remain unclear. Membrane bound β RIII has been postulated to be involved in TGF- β ligand presentation to β RII (particularly for TGF- β_2) or ligand sequestration (Lopez-Casillas et al., 1993; Lopez-Casillas et al., 1994). To support its role in ligand presentation, β RIII has been shown to form hetero-oligomeric complexes with β RII when TGF- β_1 is present (Moustakas et al., 1993; Henis et al., 1994), and

increases cells' responsiveness to TGF- β_1 (Lopez-Casillas et al., 1993). This hetero-oligomeric complex may be disrupted upon ligand binding to β RII, with β RIII being displaced by β RI. (Henis et al., 1994). Additionally, a non-membrane bound form of β RIII has been found to be an antagonist to TGF- β_1 signaling as it binds extracellular ligand and does not allow subsequent binding to cell surface β RII (Lopez-Casillas et al., 1994).

Nevertheless, most, if not all, actions of TGF- β are mediated through β RI and β RII, which are the receptors containing the functional S/T kinase domain. While β RI cannot bind TGF- β on its own, β RII binds TGF- β ligands with varying affinities (K_d of 5-50 pM for β_1 and β_3 , K_d of 500 nM for β_2 , Miyazono et al., 1994). Therefore, β RII binds TGF- β and complexes with β RI to initiate the signaling cascade (Wrana et al., 1992). This is clearly demonstrated in cells resistant to TGF- β actions in that many do not express one of these two receptors, and can be rescued by transfection with expression vectors encoding for the missing receptor (Laiho et al., 1991; Wrana et al., 1992; Markowitz et al., 1995). In addition, the kinase domain of β RII is constitutively active and autophosphorylates its IC domain in a ligand independent manner (Wrana et al., 1994a). This autophosphorylating activity is independent of its ability to phosphorylate β RI as mutants have been identified which can phosphorylate themselves but not β RI (Carcamo et al., 1995). Additionally, β RII is also phosphorylated by cellular kinases as a kinase defective β RII has also been found to be phosphorylated, yet to a lesser degree than its active form (Wrana et al., 1994a). Because β RII exists at the cell surface as homomeric

complexes in a ligand independent manner (Chen and Derynck, 1994), cross-phosphorylation of β RII may also occur.

It appears that the sole purpose of TGF- β_1 binding to β RII is the recruitment of β RI. Upon ligand binding to β RII, β RI is recruited to the complex, and is directly phosphorylated by β RII in the GS domain (Wrana et al., 1994a; Ventura et al., 1994; Vivien and Wrana, 1995). This domain is a highly conserved, 30 amino acid domain found in most type I TGF- β receptors (Wrana et al., 1994b). It contains seven serine and threonine residues, of which deletion or mutation of more than one results in an inactive receptor (Wieser et al., 1995). As β RII exists in the cells as homomeric complexes, it is no surprise that hetero-oligomeric complexes containing two or more of each, β RI and β RII, have been found and are thought to mediate TGF- β signaling (Bassing et al., 1994; Yamashita et al., 1994). It is interesting to note that this complex formation and subsequent phosphorylation of β RI is not dependent on ligand binding to β RI itself (Vivien and Wrana, 1995). It has been shown that the intracellular domains of β RI and β RII directly interact upon ligand binding to β RII, and that this interaction is sufficient for β RI phosphorylation by β RII (Ventura et al., 1994; Chen et al., 1995a; Feng and Derynck, 1996). The functionality of the interaction between the intracellular domains is complex as an activation-defective β RI and a kinase-defective β RI are able to complement/rescue each other's defect when co-expressed and assembled into a single complex with active β RII (Weis-Garcia and Massague, 1996).

A simple, multi-step mechanism of TGF- β receptor activation has been proposed by Wrana et al. (1994a). In this four step process, any of the TGF- β ligands binds to a pre-phosphorylated β R_{II}, β R_I is recruited into this complex, β R_I is phosphorylated by β R_{II} and thus activated, and then β R_I is able to phosphorylate downstream effectors which mediate the different responses to TGF- β (Figure 2).

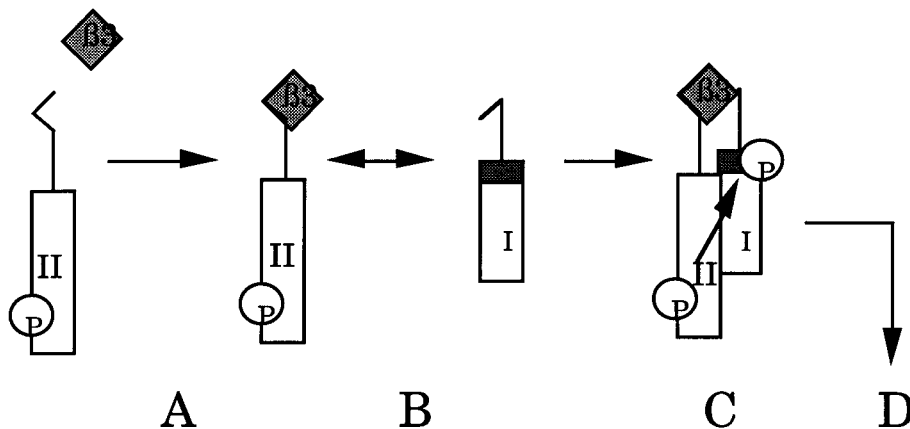


Figure 2: Postulated TGF- β Receptor Activation Sequence

TGF- β_3 binds to β R_{II} (A), resulting in the recruitment of β R_I (B). Once the β R_{II}- β R_I complex is formed, β R_{II} phosphorylates β R_I in the GS domain (C), which activates β R_I resulting in activation of downstream effectors (D).

In support of the 4-step hypothesis for TGF- β receptor activation diagrammed in figure 2, a point mutation in the kinase domain of β R_{II} has been identified which results in β R_{II} being unable to phosphorylate β R_I, while being able to autophosphorylate itself (Carcamo et al., 1995). The inability of β R_{II} to phosphorylate β R_I results in a complete loss of TGF- β_1

responsiveness and indicates that transphosphorylation of β RI by β RRI is essential for signal transduction. However, as illustrated above and described previously, this mechanism of receptor activation is not as simple as first anticipated. In addition to the complex interactions of the intercellular domains of the two receptors, it is becoming apparent that the different responses that can be elicited by TGF- β are determined by β RI and the signal transduction pathway it activates. Deletion analyses have determined that the juxtamembrane domain preceding the GS domain in β RI is responsible for growth inhibition, but not the induction of ECM-promoting genes, PAI-1 and fibronectin (Saitoh et al., 1996), while deletion of the juxtamembrane region preceding the kinase domain in β RRI is essential for all functions (Feng et al., 1995). Additionally, uncoupling of growth inhibition and gene transcription can be demonstrated in cell lines which have undetectable β RRI or which express a kinase deficient β RRI (Chen et al., 1993; Lafon et al., 1995). In these cells, addition of TGF- β_1 does not inhibit cellular proliferation, but does induce genes such as PAI-1, c-jun, and TGF- β_1 . These results suggest that β RI is able to activate some pathways without phosphorylation by β RRI, but this issue remains controversial.

While the "beginning" or activation of the TGF- β signal transduction pathway has been examined extensively, the events occurring between ligand binding to the cell surface receptors and the cellular response are only beginning to be understood through the discovery of proteins that act downstream of or are physically associated with β RI or β RRI. Two proteins

have been identified by their association with the receptors themselves: Farnesyl-protein transferase- α (FT- α) and TRIP-1 (Chen et al., 1995b; Ventura et al., 1996). FT- α associates with β RI, is phosphorylated by β RI *in vitro* (Kawabata et al., 1995b), yet appears unnecessary for TGF- β activity (Ventura et al., 1996). Therefore, the role for FT- α and β RI interaction remains unknown. Similarly, data has been collected which supports the hypothesis that TRIP-1 may be involved in the TGF- β_1 signaling pathway (Chen et al., 1995b). The TRIP-1 protein associates with β RII, but not other type II S/T kinase receptors, in a ligand-independent fashion, and is phosphorylated on threonine and serine residues, potentially by either, or both, β RII or β RI. However, while the available TRIP-1 clues to intracellular pathways, no mutants or knockout studies have been performed to directly demonstrate its involvement in a TGF- β signaling pathway.

In the world of tyrosine kinase receptors, the Ras-MAP (mitogen-activated protein) kinase signaling cascades are integral components of the cell's intracellular responses to extracellular signals. Similarly, members of these families of kinases are being identified as components of the "downstream" TGF- β signal transduction pathway(s). Rac, a small GTP-binding protein assumed to be near the beginning of the cascade, exhibited a 3.7-fold increase in activity after exposure to TGF- β , which led to increased transcription from the PAI-1 promoter (Mucsi et al., 1996). Additionally, activity of extracellular signal-regulated kinase-1 (ERK1) was also increased 4.5-fold after TGF- β_1 treatment, whereas other proteins such

as Ras, Rho, and MAP kinases such as Jun kinase (JNK) are not involved (Mucsi et al., 1996). While Rac and ERK1 are activated by a wide variety of signals, other members of the MAP kinase cascade have been identified which appear to be more closely associated with signal transduction from the receptors of the TGF- β superfamily. TAK1 (TGF- β -activated kinase) is a MAP kinase kinase kinase whose activity was stimulated in response to TGF- β_1 and another TGF- β family member, BMP-4, resulting in increased PAI-1 promoter activity (Yamaguchi et al., 1995). Additionally, another protein, TAB1 (TAK1 binding protein) was subsequently identified which interacts with TAK1 and may activate it by binding to its catalytic domain (Shibuya et al., 1996). The importance of TAB1 in the TGF- β signaling cascade was demonstrated by the creation of a dominant negative TAB1 mutant which was able to inhibit PAI-1 promoter activity in response to TGF- β_1 (Shibuya et al., 1996).

While MAP kinase signal transduction cascades are a rapidly expanding area of study in growth factor and cytokine actions, other signal transduction pathways appear to be involved in some manner in TGF- β 's activity. The G-protein $G_{i\alpha 1}$ was found to be necessary for cytoskeletal reorganization in fibroblasts which occurs in response to TGF- β_1 , although the exact mechanism of activation remains unknown (Kataoka et al., 1993). Two other common signaling molecules have also been associated with TGF- β 's activation of gene expression in human lung carcinoma cells: phosphatidylcholine-phospholipase C (PC-PLC) and protein kinase C (PKC) (Halstead et al., 1995). When these proteins are inhibited by specific toxins,

steady state mRNA levels of PAI-1 and fibronectin were not increased in response to TGF- β_1 as was seen in untreated cells. In support of the involvement of PKC in the increased mRNA levels of PAI-1 in response to TGF- β_1 , inhibition of PKC also eliminated the increase in PAI-1 promoter activity in response to TGF- β_1 which was detected in control cells (Halstead et al., 1995). While it appears that TGF- β_1 can activate transcription from the PAI-1 promoter through several pathways, caution must be used in these interpretations as it has been strongly implicated that PKC may act upstream of MAP kinase (Blumer et al., 1994).

Most significantly, nuclear proteins which may mediate TGF- β 's ability to alter gene expression have also been discovered. Among the transcription factors which appear to be regulated by TGF- β_1 are the oncogenes *c-jun*, *junB*, *c-fos*, and *c-myc* (Coffey et al., 1988; Pertovaara et al., 1989; Li et al., 1990; de Groot and Kruijer, 1990; Pietenpol et al., 1990). While *junB* and *c-fos* are significantly induced within 1-2 hours of TGF- β_1 treatment in all cell lines tested, changes in *c-jun* expression in response to TGF- β_1 is cell type dependent (Pertovaara et al., 1989; Li et al., 1990; de Groot and Kruijer, 1990). In contrast, *c-myc* expression is downregulated in response to TGF- β_1 at the transcriptional level, a process, unlike *jun* and *fos* induction, which requires protein synthesis (Coffey et al., 1988; Pietenpol et al., 1990). This suggests that TGF- β_1 triggers the synthesis or modification of a transcriptional inhibitor for the *c-myc* gene. It is of interest to note that induction of *jun* and *fos* and the inhibition of *c-myc* expression reflect TGF- β 's ability to activate ECM gene transcription and

inhibit cell proliferation. Additionally, the induction of these immediate early genes appears to occur through different pathways, as depletion of PKC activity within a breast carcinoma cell line eliminates TGF- β_1 induction of *c-fos*, but not *c-jun* (Lafon et al., 1995).

In addition to these "traditional" transcription factors, a family of proteins is beginning to be defined which are transcriptional activators specific to the TGF- β superfamily signaling pathway(s): Dwarfins. Members of this family of cytoplasmic proteins have been identified from *Drosophila*, *C. elegans*, human, and murine cells and appear to be involved in the signaling pathways of the different members of the TGF- β superfamily (Liu et al., 1996; Massague 1996; Savage et al., 1996; Yingling et al., 1996; Hahn et al., 1996; Zhang et al., 1996; Lechleider et al., 1996). This family of proteins contain DH1 and DH2 (dwarfin homology 1 and 2) regions which are homologous in function to SH2 and SH3 domains (Savage et al., 1996; Yingling et al., 1996). These SH2 / SH3 are common in effector proteins associated with tyrosine kinase-mediated signal transduction pathways, and these domains along with the DH1 / DH2 domains are believed to be involved in protein-protein interactions that are often regulated by phosphorylation. Different members within the dwarfin family appear to mediate the effects from the different ligands and receptors within the TGF- β superfamily. As the "prototype" member of this family, Mad (Mothers against decapentaplegic) proteins and Mad-related (MADR) proteins have been found to be important in BMP / dpp signaling from mammals to *drosophila* (Liu et al., 1996; Newfeld et al., 1996;

Wiersdorff et al., 1996; Hoodless et al., 1996). Similarly, human and murine Mad and MADR homologues have been identified which appear to play direct roles in TGF- β signaling pathways (Yingling et al., 1996; Lechleider et al., 1996; Zhang et al., 1996; Eppert et al., 1996). In murine cells, Dwarfins A and C are phosphorylated in response to TGF- β_1 , but neither is phosphorylated directly by β RI or β RII, thus appearing to act further downstream in the pathway (Yingling et al., 1996). Similarly, human Mad proteins, Bsp-1, DPC4, and h-MAD4, are also phosphorylated in response to TGF- β_1 , but are not associated with TGF- β receptors (Lechleider et al., 1996; Zhang et al., 1996). However, h-MAD3 and MADR2 have been found to associate with β RI and be phosphorylated by β RI *in vitro* (Zhang et al., 1996; Macias-Silva et al., 1996). To further support their roles in TGF- β signaling, overexpression of Dwarfins A or C in L6 rat skeletal muscle fibroblasts causes growth arrest and induction of PAI-1 (Yingling et al., 1996), and deletion of Dwarfins A or mutation of MADR2 activity have been demonstrated to block TGF- β_1 signaling (Zhang et al., 1996; Yingling et al., 1996; Eppert et al., 1996).

Clearly, a significant amount of progress has been made in the identification of several components of the TGF- β signal transduction pathway, from the receptors on the cell surface to proteins within the nucleus. In concert with their identification, examination of the function of each protein in the pathway and the direct effects TGF- β exhibits in a variety of cell types has been an area of ongoing study. While many of these functions, such as its antiproliferative activity, are common to many cell

types, several examples have been cited here where these effects are cell type specific. Therefore, the role of TGF- β in processes such as organ development is now being examined *in vitro* through the use of specific cell types found within that organ as well as *in vivo* utilizing transgenic mice which overexpress or do not express members of the TGF- β signal transduction pathway. One organ system in which these types of experiments are being utilized is the development of the heart.

TGF- β and Heart Development

Expression Patterns

TGF- β Ligands in the Heart

While most of what is known about the TGF- β ligands has been ascertained from their genetic organization derived from their cloning, biosynthesis, and pleiotrophic actions *in vitro*, determination of their spatial and temporal patterns of expression *in vivo* is critical to elucidate their functions in development. Expression patterns for the TGF- β ligands during heart development have been best studied in rodent and avian systems. These expression patterns, in general, are similar, but some significant differences between species have been found. Expression patterns of the three TGF- β ligands are very distinct, both temporally and spatially, suggesting that while they have similar effects *in vitro*, they may play very specific roles *in vivo*.

TGF- β_1 expression was originally undetected in the chicken embryonic heart or cultured myocytes obtained from embryonic hearts (Jakowlew et al., 1991; Runyan et al., 1992), but these results are misleading given the fact that TGF- β_4 is the chicken isoform of TGF- β_1 (Burt and Paton, 1992). TGF- β_4 can be localized to myocytes within the atria and ventricles of the developing avian heart from as early as stage 22 (day 4) through adulthood, with ligand expression increasing as the chick matures (Jakowlew et al., 1991; Jakowlew et al., 1992). TGF- β_1 expression during rodent heart development is better defined. The earliest expression of TGF- β_1 mRNA is in cardiac precursor cells within the splanchnic mesoderm (Schneider and Parker, 1991). However, defined expression patterns of TGF- β_1 later in heart development have also been documented (Schneider and Parker, 1991; Roberts and Sporn, 1992a; Engelmann et al., 1992). At 9 days post-coitum (dpc), TGF- β_1 is strongly localized to those tissues which are undergoing epithelial to mesenchymal (e-m) transformation, a phenomenon which occurs during the formation of the heart valves (Schneider and Parker, 1991; Roberts and Sporn, 1992a). At 11-12 dpc, TGF- β_1 can be detected in the ventricular myocardium in a uniform manner with TGF- β_1 being localized to the myocytes themselves (Schneider and Parker, 1991; Roberts and Sporn, 1992a; Engelmann et al., 1992). The expression levels of TGF- β_1 increase significantly in the late fetal (18 dpc), early neonate, and remain elevated in the mature rat heart (Engelmann et al., 1992).

TGF- β_2 expression patterns suggest that it may play an important role in heart development in chickens as well as rodents. However, these expression patterns deviate markedly from one another. In the murine heart, TGF- β_2 is expressed at 9.5-10.5 dpc in both the atrioventricular (AV) region and in the pre-valvular myocardium (Schneider and Parker, 1991; Akhurst et al., 1992). However, by 12.5 dpc, TGF- β_2 expression cannot be detected in the myocardium, yet is still highly expressed in the AV region, particularly in the valves (Schneider and Parker, 1991; Akhurst et al., 1992). In contrast to the distinct temporal and regional changes in TGF- β_2 expression in the mouse, TGF- β_2 expression in the developing chicken heart appears to be in both heart regions at Hamilton-Hamburger stages 15-20 (this corresponds to the time in which differences were seen in mice) (Jakowlew et al., 1991; Runyan et al., 1992) and decreases with age after stage 29 (Jakowlew et al., 1994).

TGF- β_3 expression appears to be the most limited of the three primary TGF- β isoforms. In the chicken, TGF- β_3 has a distinct localization to the AV canal and has been implicated to be critical in the epithelial-mesenchymal (e-m) transition associated with valve formation (see below) (Runyan et al., 1992). Interestingly, TGF- β_3 cannot be detected in the early mouse heart at similar stages (Runyan et al., 1992; Akhurst et al., 1992) suggesting a different isoform or family member may be involved in the e-m transition / valve formation. TGF- β_1 and TGF- β_2 expression patterns suggest they may "substitute" or compensate for TGF- β_3 in the mouse. Later in heart development (11.5-16.5 dpc) TGF- β_3 can be detected in the

ECM and mesenchyme of the valves as well as the atrial and ventricular myocardium (Schneider and Parker, 1991; Burton et al., 1993) and continues to be expressed at relatively high levels in the ventricular myocardium in the immediate postnatal period and through weaning where the expression levels drop off markedly (Engelmann 1993).

TGF- β Receptors in the Heart

While expression patterns of the TGF- β ligands indicate where they may be important for various developmental processes, an understanding of the expression patterns of the TGF- β receptors is also necessary to fully appreciate the role(s) TGF- β plays in development. The expression of the three primary TGF- β receptors has been studied to varying degrees in chicken (Barnett et al., 1994), murine (Lawler et al., 1994; Roelen et al., 1994), and rat (Engelmann and Grutkoski, 1994) systems. In general, the expression patterns observed in these three systems are similar within the heart. While data are limited for early heart development, neither β RI nor β RII could be detected by PCR analysis at 6.5-7.5 dpc. in the three mouse embryo germ layers, but could be detected by 14 dpc through adulthood (Lawler et al., 1994; Roelen et al., 1994). Similarly, expression of β RII and β RIII can be detected in the chicken heart at low levels in the Stage 26 embryo in atrial and ventricular cells (Barnett et al., 1994). This expression significantly increases between stage 26, day 6, and day 10, and greatly increases upon hatching. It is interesting to note that in the fetal avian heart, the protein levels of all three receptors (determined by receptor

crosslinking) is approximately 10-fold higher in the atria than in the ventricles, suggesting that TGF- β plays a significantly different or more prominent role in the development of the atria than the ventricles in the avian heart (Barnett et al., 1994). Expression of the three receptors was studied extensively in the rat from the late fetal stage through adulthood, and these results support and extend those obtained in the mouse and chicken (Engelmann and Grutkoski, 1994). β RI and β RIII mRNA levels were readily detected in total RNA from ventricular tissue of 18 dpc through 9-week postnatal animals, with only a modest age-related increase in transcript abundance. However, β RII expression was age-restricted with low-to-undetectable levels in the late fetal periods, but transcript levels increased markedly at-or-around birth and increased through the postnatal-to-mature stages of development (Engelmann and Grutkoski, 1994). Immunolocalization of β RII within the ventricle was primarily in the cardiomyocytes, but surrounding non-myocytes also expressed the receptor (Engelmann and Grutkoski, 1994). Additionally, there were no significant differences in steady state transcript abundance between the different regions of the mature heart (right ventricle, left ventricle, and septum) (Engelmann and Grutkoski, 1994). Studies in the rat did not address the expression of the TGF- β receptors in the atria, however it is expected that mRNA patterns for the three isoforms would resemble those seen in other rodents such as the mouse.

TGF- β Effector Protein - Cardiac Expression *in vivo*

As important as it is for the ligands and receptors to be present in order for a cell to respond to TGF- β , the factors that act downstream of the receptors must also be present. To date, limited developmental or *in vivo* expression data are available for the recently identified downstream effectors of TGF- β signal transduction. Nonetheless, it has been found that TRIP-1 is expressed in the heart, and that TRIP-1 expression in mouse embryos and human fetal tissue samples correlates with β RII expression (Chen et al., 1995b). This supports the postulated functional role it may have in the TGF- β signaling pathway as well as the ability of the cells in developing tissues to respond to TGF- β_1 . Developmental data on many of the other postulated TGF- β effector proteins are not yet available. However, of interest to our studies, it has been demonstrated that TAK1 and TAB1 transcripts are found in adult mouse and human heart RNA samples, although not yet localized to the cardiomyocyte population (Yamaguchi et al., 1995; Shibuya et al., 1996). Transcripts for the MAD proteins, in general, are expressed ubiquitously in adult rat and human tissues, including the heart (Yingling et al., 1996; Lechleider et al., 1996), and steady-state Bsp-1 transcript levels in adult human tissues are highest in heart and skeletal muscle, two non-mitotic, fully differentiated tissues (Lechleider et al., 1996). Developmental expression data for the MAD family of proteins appears to be limited to studies in *Xenopus* where Xmad is uniformly expressed from fertilization through the tadpole stage,

suggesting that it is not the limiting factor for BMP-2/4 mesoderm-inducing activity in the developing embryo (Graff et al., 1996).

Regulation of TGF- β ligand and receptor expression

The distinct temporal and spatial expression patterns observed for both TGF- β ligands and receptors suggest that each is regulated in an independent manner that may occasionally overlap. Regulation of both ligands and receptors may occur at both the transcriptional and post-transcriptional levels through the use of different promoters, regulatory transcription factors, untranslated regions (UTRs), as well as post-translational processing and protein turnover or "latency" of the ligands. These regulatory mechanisms may respond to humoral, neurohumoral and mechanical stimuli differently, supporting the hypothesis that each TGF- β ligand and receptor may have distinct physiological and development functions.

The production of mRNAs and proteins for TGF- $\beta_{1,3}$ and their receptors is influenced by many diverse factors. One of these factors may be the TGF- β ligand itself. In cardiac myocytes isolated from 10 day chicken embryos, addition of TGF- $\beta_{1,3}$ results in an increase in steady state expression levels of TGF- $\beta_{3/4}$, but not of TGF- β_2 (Jakowlew et al, 1992). Autoinduction of TGF- β_1 is also seen in primary cultures of rat neonatal ventricular myocytes after treatment with exogenous TGF- β_1 (MacLellan et al., 1993; Flanders et al., 1995). Similarly, expression of β RI has also been shown to be induced by TGF- β_1 in human lung fibroblasts and smooth

muscle cells (Bloom et al., 1996). However, while several factors have been identified that can downregulate β RII, the effects of TGF- β on expression of β RII has yet to be determined. Autoregulation of TGF- β_1 activity through the regulation of TGF- β_1 ligand and TGF- β receptor expression is just one level of control. Another regulatory feedback loop can be demonstrated with ECM proteins. Mammary epithelial cells plated on ECM have a much lower level of TGF- β_1 transcripts than those plated on plastic, while TGF- β_2 expression appears unaffected (Streuli et al., 1993). These data suggest a mechanistic, positive feedback loop wherein low levels of ECM stimulate TGF- β_1 expression which in return may stimulate ECM production. Once ECM concentrations reach a critical level, they may downregulate TGF- β_1 gene expression; thereby downregulating ECM production and completing the regulatory loop. Other factors that have been found to regulate TGF- β_1 gene expression in myocytes are retinoic acid, calcium, oestradiol, phorbol esters, cAMP, and steroids (Burt et al., 1991; Roberts and Sporn, 1992b). The role of these inducers, whether direct or indirect, and the signaling pathways relevant to cardiomyocytes and heart development are still under investigation.

Roles for TGF- β in Heart Development

The roles that the TGF- β s are thought to play in the development of the heart, as mentioned above, involve three important aspects: 1) cellular differentiation, 2) ECM deposition and tissue remodeling, and 3) cellular proliferation. Limited studies have been performed on ventricular cardiomyocytes *in vitro* which address each of these. However, a significant amount of data have been collected on the role of TGF- β in valve formation in the chicken, and gross anatomical effects of TGF- β on development inferred through the use of knockout mice.

Epithelial-Mesenchymal (e-m) Transformation

The formation of mesenchymal tissue from an epithelial sheet is a developmentally significant event and often is mediated by ECM proteins (Huang et al., 1995). This phenomenon of the transformation of epithelial cells to mesenchymal cells (e-m transformation) is especially important in heart development since the valves and membranous portions of the atrial and ventricular septum are derived from cells which are endothelial in origin. This type of transformation is regionally specific within the heart and occurs only in the atrioventricular (AV) canal. This process of e-m transformation in the heart, as well as similar transformations earlier in embryonic development, have been recently reviewed (Huang et al., 1995). In order to study the e-m transformation in the heart, a collagen gel culture system is employed in which explants (AV canal explants which contain

myocardium and endothelium) from the embryonic heart can be cultured and allowed to grow and differentiate in a 3-D system (Runyan et al., 1992). In these cultures, the myocardium aggregates into a beating myoball, the endothelium expands across the gel surface, and the mesenchymal cells can migrate into the matrix of collagen. It is these migrating cells which are the products of the e-m transformation.

As mentioned above, TGF- β_1 is expressed in the AV canal in the chicken embryo at the time of the e-m transformation. A series of experiments have determined that TGF- β_1 plays a role in this transformation. In the first set of experiments, stage 14 AV canal explants were cultured in the presence of TGF- β_1 and TGF- β_2 +/- either b-FGF or EGF (Potts and Runyan, 1989). However, when the "AV myoball" was replaced with ventricular myocardium, e-m transitions occurred, while the myocardium itself could not induce the transformation. Additionally, an antibody immunoreactive against all three forms of TGF- β was added to the cultures and was able to block the transformation (Potts and Runyan, 1989). This suggested that a TGF- β family member along with a ventricularly produced co-factor could induce the e-m transformation.

Since exogenous TGF- β s are able to elicit similar functions when added to cells *in vitro*, an alternative method was used to determine which TGF- β isoform(s) is important for the e-m transformation. In this approach, oligonucleotides complementary to the non-conserved 5'-ends of the TGF- β ligand mRNA transcripts were used to block production of endogenous TGF- β s (Potts et al., 1991). These oligonucleotides were

topically applied to the explants twelve hours after cultures were started, after which the explants were allowed to "grow" for 12-18 hours when e-m transformations normally occur. It was demonstrated that only the oligonucleotides specific for TGF- β_3 were able to inhibit transformation by 80%, whereas oligonucleotides to TGF- β_1 and TGF- β_2 mRNA had no effect. These data suggested that "only" TGF- β_3 is essential for the e-m transformation in the chicken heart as judged by the appearance and migration of mesenchymal cells into the collagen matrix. The data further demonstrated that even though an added substance can elicit a certain function *in vitro*, it may not truly reflect what actually occurs *in vivo*.

To further support the importance of TGF- β 's in chick heart valve formation, similar studies were performed using antibodies to β RII (Brown et al, 1996). Through the use of antibodies in the same explant system, it was shown that TGF- β plays two distinct roles in valve formation: inducing e-m transformation and controlling the migration of the newly transformed mesenchymal cells. β RII antibodies added to explants from different staged embryos (14-18) inhibited e-m transformation at least 50%. Those that underwent transformation were thought to have been activated prior to explant, and these transformed cells, while expressing mesenchymal markers, migrate at a slower rate (approximately 50% of control cells). Therefore, the role of TGF- β in valve formation in the chicken is two-fold: 1) to induce transformation, and 2) to promote migration of the newly formed cells. It would be of interest to use this system on mammalian models as

the specific roles for each TGF- β isoform in heart development do not completely coincide across species.

TGF- β Ligand and Receptor Knockouts and Developing Hearts

As the expression pattern for each TGF- β isoform is distinct, each ligand appears to have a distinct role in heart development. Due to the ability of each TGF- β isoform to mediate similar effects *in vitro*, data collected from *in vitro* models or systems can give an unrealistic picture of what may actually occur during *in vivo* heart development. Therefore, *in vivo* studies are necessary to elucidate the distinct role each TGF- β isoform may have on heart development, as well as those that may overlap with other isoforms or TGF- β family members. Targeted overexpression of TGF- β_1 in transgenic mice has allowed the examination of the role TGF- β_1 has in mammary development (Arteaga et al., 1996), lung morphogenesis (Zhou et al., 1996), renal disease (Kopp et al., 1996), skin tumor development (Cui et al., 1996), and central nervous system development (Galbreath et al., 1995), but none of these studies has addressed cardiac development.

Another method in which to study *in vivo* roles of TGF- β is through the use of knockout mice. These *in vivo* gene knockout models have been reported for TGF- β_1 and TGF- β_3 . In these murine models, TGF- β_1 and - β_3 deficient mice were created by knocking out a TGF- β allele by homologous recombination in embryonic stem cells (Shull et al., 1992; Kulkarni et al., 1993; Kulkarni et al., 1995; Kaartinen et al., 1995; Proetzel et al., 1995). For

both isoforms, heterozygous animals were phenotypically normal and fertile, and thus were crossed to obtain homozygous null mutants (-/-).

While not all of the TGF- β_1 (-/-) mice were born (only 40-50%), those that made it to term were indistinguishable from littermates and had no gross developmental abnormalities upon external anatomical examination. However, these animals developed a wasting syndrome and ultimately died within the first three weeks due to cardiopulmonary failure. Upon more detailed histological examination, it was noted that the primary cause of death was a dysfunctional immune response system which is normally held in check, at least in part, by TGF- β . Between 7-20 days after birth, mononuclear inflammatory cells infiltrated the lungs and heart, both the myocardium and pericardium, resulting in lesions, myocyte lysis, and tissue necrosis.

While it appeared that TGF- β_1 was not essential for embryonic or fetal heart development, this conclusion is premature as TGF- β_1 has been proposed to be supplied by the mother through the placenta during fetal development and breast milk during neonatal development (Letterio et al., 1994). However, one observation was made in the myocytes of the -/- pups which reflected one of the characteristic effects of TGF- β in general: inhibition of proliferation. Several myocytes from a 5-day old "TGF- β_1 null" mouse were found to have mitochondrial changes which were characterized as being enlarged and having a decreased number of cristae, suggesting extended proliferation in the absence of TGF- β_1 (Boivin et al., 1995). Additionally, in animals in which the inflammatory response was

pharmacologically suppressed, nuclear labeling (by bromodeoxyuridine) indicated hyperplasia in cardiac tissue isolated from 5 week old animals (Diebold et al., 1995). However, in TGF- β_1 null mice devoid of lymphocytes (backcrossed onto a SCID background), any evidence of postnatal myocyte proliferation was not seen (Diebold et al., 1995). This suggests that it is not the absence of TGF- β_1 but the absence of lymphocytes, by a mechanism as yet unknown, that has an effect on the extended proliferation of postnatal cardiac myocytes (Diebold et al., 1995).

Like the TGF- β_1 null mice, TGF- β_3 -null animals exhibited no gross abnormalities in the heart (Proetzel et al., 1995; Kaartinen et al., 1995). Nevertheless, these animals died within 24 hours after birth due to their inability to breathe properly or suckle as a result of improper formation of their cleft palate and abnormal development of their lungs. Therefore, possible roles of TGF- β_3 in early neonatal and adult development could not be analyzed.

The importance of either TGF- β isoform in heart development appears to be minimal according to the data from these transgenic murine models, which is rather unexpected given the importance of TGF- β in valve formation in the chicken. While it does not appear that the expression patterns of the non-ablated TGF- β ligands change in the knockout models (Proetzel et al., 1995), we can not rule out the apparent “rescue” effect other TGF- β isoforms or TGF- β family members may have in compensating for the missing isoform. Ideally, TGF- β receptor knockout models would resolve this possible rescue by other isoforms. However, β R_{II} knockout

mice have been created, but provide no additional information about the roles of TGF- β in heart development as this knockout is embryonic lethal at 10.5 dpc (Oshima et al., 1996). This embryonic lethality is not due, however, to cardiac malfunction, which is consistent with the fact that β RII is not expressed in the heart at these early timepoints. Conditional, tissue-specific TGF- β receptor knockout or dominant negative studies have recently been initiated. Targeted expression of a truncated β RII to epidermal cells have demonstrated the need for TGF- β to maintain the proper proliferation rates important for skin development (Wang et al., 1997). Similar studies using targeted expression of a dominant negative β RII in the heart may better address the role TGF- β plays as a whole in heart development, as well as cardiac structure-function relationships, but would not address the importance of each individual ligand.

Coordinate Changes in Ventricular Development

The onset of TGF- β_1 , TGF- β_3 , and β RII expression in the ventricle during the late fetal period of development and their subsequent increase in steady-state transcript levels in the neonatal period temporally correlates with several developmental phenomena critical to the formation of the heart: 1) regulation of cardiomyocyte proliferation and differentiation, 2) the formation of biomechanical structures such as the valves, 3) vasculogenesis; capillary angiogenesis and neovascularization, and 4) non-muscle cell-produced extracellular matrix (ECM) formation. While it has

been determined that TGF- β_1 may regulate cardiomyocyte gene expression through a signaling pathway that involves the activation of *ras* and immediate early genes such as *c-fos* and *c-jun* (MacLellan et al., 1993), other pathways also involved and their regulatory roles, if any, remain to be fully elucidated. However, no matter what the pathway or mechanism of action, the TGF- β s are able to mediate changes in cardiomyocyte gene expression, mRNA translation, and protein production.

Cellular Proliferation

It is a well established fact that the increase in ventricular size during fetal development is due primarily to cellular proliferation (hyperplasia), while the increase in size seen throughout the rest of the organism's life is through the increase in mass of each individual cardiomyocyte (hypertrophy) in concert with the proliferation and migration of non-myocyte populations as ventricular remodeling occurs. While ventricular myocytes divide during fetal development, the rate of division falls off dramatically during the late fetal period, resulting in only 1-2% of the myocytes still dividing at birth (Zak 1973; Bugaisky et al., 1992). While nuclear division occurs, as each myocyte in the rat heart generally becomes binuclear and/or polyploid, the mitotic division rate continues to decrease until all myocyte division ceases by 3-4 weeks postnatal (Zak 1973; Bugaisky et al., 1992). This rapid decline in myocyte division in the late fetal and early neonate temporally correlates well with the heightened expression of the TGF- β ligands and β RII.

As discussed previously, TGF- β has significant anti-proliferative activities on most cell types in culture. It is important to note that studies on cell cycle times and specific cell cycle components have not been rigorously done in cardiomyocytes. Therefore, the role that TGF- β may play in the regulation of cardiomyocyte proliferation at the level of specific cell cycle-related proteins is speculative. However, TGF- β_1 has been shown to have antiproliferative activities in neonatal cardiomyocytes (Kardami 1990; Engelmann et al., 1992; Nair et al., 1995). These antiproliferative activities utilize an alternative/complementary method than that described previously involving specific components of the cell cycle: TGF- β is able to interrupt the stimulation of proliferation by other growth promoting stimuli.

Epidermal growth factor (EGF), acidic and basic fibroblast growth factor (a-/b-FGF), and the insulin-like growth factors (IGF) -I and -II are five "mitogenic" growth factors which are found within the heart at various developmental stages, particularly during embryonic and fetal development when most of the proliferative expansion of the cardiomyocyte population occurs. Co-stimulation of embryonic chicken cardiomyocytes with TGF- β_1 and the mitogens b-FGF or IGF-II decreased or canceled out, respectively, their stimulatory effects on subsequent DNA synthesis and cell division (Kardami 1990). Downregulation of the expression of the receptors for b-FGF by TGF- β_1 has been hypothesized to play a role in this inhibition. This is supported by the finding that the FGF receptor, Flg, is expressed fetally, but decreases to low/undetectable levels just after birth (Engelmann et al.,

1993a), an expression pattern that correlates well with the increases in TGF- β ligand and β RII expression. Results similar to those observed for FGF in culture were seen in rat cardiomyocytes, both fetal and neonatal, treated with IGF-I or IGF-II and TGF- β_1 (Engelmann et al., 1992). These results demonstrate that one of the antiproliferative activities of TGF- β_1 involves interfering with other proliferative stimuli commonly found in the developing heart. Another example of TGF- β modulating the activity of another growth factor is that seen in co-treatment of neonatal rat cardiomyocytes with TGF- β_1 and EGF (Nair et al., 1995). TGF- β_1 has the ability to decrease the cAMP elevation resulting from EGF stimulation in a time and dose-dependent manner. This decrease is bi-functional with TGF- β_1 decreasing the EGF receptor's kinase function as well as altering the activity of the catalytic subunit of adenylyl cyclase (Nair et al., 1995).

Differentiation

In rodents, many muscle-specific, contractile proteins undergo an isoform switch during the fetal-to-neonatal-to-adult transition period, and for several of these proteins, this switch involves the replacement of a skeletal muscle isoform with a cardiac form of the protein. Among the proteins that have been shown to undergo isoform switching are myosin heavy chain (MHC), actin, tropomyosin, troponin I (TNI), and troponin T (TNT), and creatine kinase (Table 1).

Table 1: Protein Isoform Switch in the Rodent Ventricle

Fetal Isoform		Adult Isoform	
β -Myosin Heavy Chain	(β -MHC)	α -Myosin Heavy Chain	(α -MHC)
α -Skeletal Actin	(α Sk-actin)	α -Cardiac Actin	(α Ca-actin)
Skeletal Troponin I	(sTNI)	Cardiac Troponin I	(cTNI)
Fetal Cardiac Troponin T	(fTNT)	Adult Cardiac Troponin T	(aTNT)
Muscle Creatine Kinase- Brain	(MCK-B)	Muscle Creatine Kinase - Muscle	(MCK-M)

MHC has two isoforms in the rat heart: the fetal isoform, β -MHC, and the adult isoform, α -MHC. β -MHC is the predominant isoform in the embryonic and fetal heart, with α -MHC appearing later in gestation (Lompre et al., 1991; Shwartz et al., 1992). Just after birth, α -MHC becomes the predominant isoform with β -MHC not being detected by three weeks of age. Similarly, two isoforms of actin are found developmentally in the heart: α -skeletal actin (α Sk-actin) and α -cardiac actin (α Ca-actin). Both isoforms are expressed in the fetal heart, but just after birth, α Sk-actin is downregulated so that by one week of age, it comprises only 30-40% of the total actin in the ventricle, and continues to decrease until it represents <5% of the total actin mRNA by two months of age (Zak et al., 1990; Lompre et al., 1991; Shwartz et al., 1992).

TNC is the thin filament subunit which confers Ca^{++} sensitivity to the myofibrils (Lompre et al., 1991). Two isoforms have been identified to date:

fast skeletal TNC (sTNC) and cardiac/slow skeletal TNC (cTNC) (Parmacek and Leiden, 1989; Parmacek et al., 1990). Similar to other contractile proteins, this family undergoes an isoform switch as skeletal muscle matures (Dhoot and Perry, 1979). Originally it was thought that fast skeletal TNC was present in the embryonic heart (Gahlmann et al., 1988), however low stringency washes led to the questioning of these results. Similarly another skeletal isoform was identified in the heart (Dhoot and Perry, 1979), but the cloning of TNC from cardiac muscle indicated that the skeletal isoform detected was identical to cardiac TNC (Wilkinson 1980). Subsequent studies have failed to locate sTNC in the heart at any age in murine, avian, and rabbit systems, being expressed exclusively in skeletal muscle (Toyota and Shimada, 1981; Gahlmann et al., 1988; Parmacek et al., 1990). Not unexpectedly, cTNC has been found to be expressed at a low level in the embryonic heart in chicken and mice (Toyota and Shimada, 1981; Parmacek and Leiden, 1989; Parmacek et al., 1992). While a fetal isoform has not yet been identified for cTNC, its expression appears to be regulated developmentally as cTNC is upregulated within the heart during the perinatal period.

TNI is the thin filament subunit which is a specific inhibitor of Mg^{++} -stimulated ATPase of actomyosin (Lompre et al., 1991). TNI exists in three isoforms, fast skeletal (fTNI), slow skeletal (sTNI), and cardiac (cTNI). In mice, fTNI is expressed very early in heart development and is completely absent by 16dpc (Zhu et al., 1995). However in the rat, the expression patterns of sTNI and cTNI are more defined. sTNI appears to be the only

isoform detected at 10dpc (Gorza et al., 1993). By 11dpc mRNA for cTNI can be detected, however cTNI protein could not be detected until 16-18dpc, suggesting transcriptional and translational regulation of TNI isoform expression (Ausoni et al., 1991; Gorza et al., 1993). After birth, cTNI increases dramatically, being easily detected in 1-2 day neonatal hearts, and by the third week of life, is the only isoform detected (Saggini et al., 1989; L'Ecuyer et al., 1991; Ausoni et al., 1991; Murphy et al., 1991). Profiles of TNI expression in human hearts are similar to those of rat (Bhavsar et al., 1991; Hunkeler et al., 1991; Sasse et al., 1993).

Cardiac TNT is the thin filament subunit which binds directly to tropomyosin (Lompre et al., 1991). It has two isoforms simply labeled as the fetal cTNT (fTNT) and adult cTNT (aTNT). Unlike its thin filament counterparts, the TNT isoform switch does not occur through the differential expression of two separate genes. Cloning of each isoform mRNA from rat, chicken, rabbit, and bovine cells reveal that the two isoforms differed only in a 30bp insert present in the embryonic form (Cooper and Ordahl, 1985; Jin and Lin, 1989). Genomic cloning confirmed that the two isoforms were the result of a developmentally regulated alternative splicing event of a single exon, therefore each isoform comes from the same primary transcript (Cooper and Ordahl, 1985; Jin et al., 1992). While the mechanism by which this occurs is still unknown, the developmental profile of the isoform switch in the rat has been well established. The profile is similar to that for MHC and actin: fTNT being expressed in the fetal heart with trace levels of aTNT, aTNT increasing

dramatically after birth with a concomitant decrease in β TNT, and α TNT as the sole isoform by 2-3 weeks of age (Saggin et al., 1988; Jin et al., 1990; L'Ecuyer et al., 1991).

These changes in isoform expression coordinate well with the onset of TGF- $\beta_{1/3}$ and β RII, but any direct correlation has yet to be made. However, it has been shown that several additional factors can influence the expression of a few of these genes. Thyroid hormone has been found to affect the expression of cardiac genes such as MHC and the TNIs, with the isoform switching of the latter being delayed in hypothyroid rats (Lompre et al., 1991; Bugaisky et al., 1992; Avery-Fullard et al., 1994). The mitotic growth factors aFGF and bFGF are able to induce the fetal isoform of MHC as well as sarcoplasmic reticulum calcium ATPase (Sr-Ca⁺⁺-ATPase), while having opposing effects of α Sk-actin and α Ca-actin in cultured neonatal rat cardiomyocytes (Parker et al., 1990a). Similarly, TGF- β_1 treatment of neonatal rat cardiomyocytes decreases the expression of α -MHC and SR Ca⁺⁺-ATPase, increases β -MHC and α Sk-actin, while having no effect on α Ca-actin (Parker et al., 1990b; MacLellan et al., 1993). Whether TGF- β_1 has these same effects on fetal myocytes has yet to be completely determined.

Angiogenesis and ECM Biosynthesis

The fetal to neonatal to adult transition period is a time when the ventricle undergoes significant remodeling, a process which involves the migration of non-muscle cells into the ventricular wall, an increase in

ECM formation and reorganization, and angiogenesis (Engelmann 1993). As described previously, TGF- β , directly or indirectly, can influence each of these processes. It has been shown that TGF- β receptor and ligand expression coordinate well, both temporally and spatially, with changes in ECM production (e.g. the collagens) and expression of the ECM receptors, the integrins, during several morphological changes (Borg et al., 1990; Engelmann 1993; Carver et al., 1994). Similarly, the increase in formation of new blood vessels, as well as expanding those vessels which already exist, are temporally correlated with TGF- β_1 expression (Sage et al., 1989). As noted earlier, SPARC may play an integral role in each of these processes (migration, ECM reorganization, and angiogenesis). To support this hypothesis, SPARC, both protein and transcripts, have been localized to organs or tissues undergoing morphogenesis, including the fetal and neonatal (but not adult) heart (Sage et al., 1989; Reed and Sage, 1994). Ventricular expression of SPARC has been determined developmentally, and increases dramatically around the same time in which TGF- $\beta_{1/3}$ and β RII appear (Engelmann 1993). However, whether these correlative expression patterns have functional significance has not been determined.

It is important to note that the cellular migration, ECM formation, and angiogenesis which occurs in ventricular development does not directly involve the cardiomyocyte population. Most of the effects TGF- β may have in this aspect of ventricular remodeling involve the migration of fibroblasts and endothelial cells and induction of ECM protein expression in fibroblasts and other non-myocyte cell types. However, TGF- β may play a

role in cardiomyocyte regulation of these processes. While exogenous SPARC has been shown to affect ECM interactions as well as induce endothelial cells to undergo angiogenesis, it is probable that increases in cardiomyocyte-produced SPARC in response to cardiomyocyte-produced TGF- β would promote angiogenesis and other morphogenetic processes. Similarly, it has been proposed that cardiomyocytes may be able to regulate angiogenesis and cellular migration through the production of inhibitors of ECM degradation enzymes, such as PAI-1, which are necessary for migration and angiogenesis (MacGregor et al., 1995). Therefore, the autocrine actions of TGF- β induced PAI-1 from cardiomyocytes may play a dual paracrine role in the surrounding non-myocyte cell types: 1) by promoting ECM accumulation by inhibiting its degradation, and 2) by regulating cellular migration and capillary angiogenesis.

Introduction of Cell Lines and Dominant Negative Mutants

Dominant negative (DN) mutants are defined as mutant polypeptides which when overexpressed disrupt the activity of the wild-type (wt) gene product (Herskowitz 1987). These DN mutants can disrupt function, in general, in one of three ways: 1) DN can compete with wt for a rate-limiting substrate, 2) DN can compete with wt for regulatory molecules or transactivating molecules, and 3) DN can form inactive dimers or oligomers with wt (Sheppard 1994). While several examples can be cited in nature, DN mutants are becoming a widely used research tool which has the advantage that one can study the function, or loss of function, of a particular gene/gene product without having to inactivate each of its alleles within the genome (Herskowitz 1987). Studies examining p21^{Ras} function have utilized the DN approach in *C. elegans*, *Xenopus*, and mammals (Han and Sternberg, 1991; Whitman and Melton, 1992; Abdellatif et al., 1994). These mutants disrupted vulval formation in response to EGF in *C. elegans*, mesoderm formation in response to FGF in *Xenopus*, and gene transcription in response to TGF- β_1 in mammals. Additionally, DN models of two other cellular proteins, p53 and the estrogen receptor, have been identified and studied (Unger et al., 1993; Ince et al., 1993). These mutants are able to be activated or bind ligand, respectively, and dimerize with wt partners, yet these resulting dimers do not contain their normal transcriptional transactivating activities.

An increasing use of the DN approach can be seen in the study of tyrosine kinase-receptor activation and identification of its signal transduction pathway components. In general, tyrosine kinase receptors form homo-dimers or -oligomers upon ligand binding, auto- or trans-phosphorylate their intercellular domains, and transduce their signal(s). The most frequent type of DN receptor mutation is a receptor with its kinase domain deleted by truncation; however, point mutations which render the receptor inactive have also been produced. Truncations of the receptors for EGF, ANF, and IGF-1 have been shown to dimerize with their wt counterparts, and the resulting dimers are unable to respond to growth factor stimulation as no cross-phosphorylation and activation of the receptors occurs (Kashles et al., 1991; Chinkers and Wilson, 1992; Prager et al., 1992). Inactivation of the kinase domain in the insulin receptor was found not only to interrupt signaling by insulin, but was also able to interrupt the cells' response to IGF-1, which clearly demonstrated that some responses to IGF-1 can be mediated through hybrids of IGF-1 receptors and insulin receptors (Frattali et al., 1992). *In vivo* studies utilizing DN receptors have demonstrated the particular receptor's importance in development. One naturally occurring DN mutation in the *c-kit* gene, whose product binds Steel factor, has been identified in mice and humans which results in anemia, sterility, and hypopigmentation (Fleischman 1992). Deletion of the kinase domain of FGFR1 abolishes all FGF receptor function within the cell, and this mutant was used to demonstrate the role of FGFs in mesoderm induction in *Xenopus* embryos

(Amaya et al., 1991; Ueno et al., 1992) and keratinocyte organization and differentiation in transgenic mice (Werner et al., 1993).

Following in the tradition of using kinase deficient or inactive tyrosine kinase receptors, the DN approach is now being utilized to study the serine/threonine kinase receptors in the TGF- β superfamily. Just as a truncated FGF receptor blocked mesoderm induction in *Xenopus* embryos, a truncated, kinase deficient activin type II receptor (ActRII) was also used to determine its role in mesoderm induction. These data showed that activin is required for induction of mesoderm as well as anterior-posterior and dorsal-ventral body patterns (Hemmati-Brivanlou and Melton, 1992). In addition to ActRII, DN mutants of the type I receptors (ActRI and ActRIB) have also been used to elucidate the signaling mechanism used by activins. In these studies, a kinase deficient ActRIB receptor blocked activin-induced transcription, while a kinase deficient ActRI did not have any effect, demonstrating that these two receptors are functionally distinct (Tsuchida et al., 1995). Similarly, kinase deficient type I and type II BMP receptors have been used *in vivo* to demonstrate that BMPs are involved in the induction of axial mesoderm and hematopoietic tissue in *Xenopus* embryos (Ishikawa et al., 1995; Maeno et al., 1996) as well as mediating interdigital cell death in embryonic chicken limbs (Zou and Niswander, 1996).

While *in vivo* data on TGF- β activities utilizing the DN approach are limited to epidermal development (Wang et al., 1997), numerous studies *in vitro* have used non-functional β RI and β RII to examine the mechanism of

signaling as well as effects TGF- β has on cellular functions. In an attempt to uncouple the different effects of TGF- β signaling at the level of the receptors, overexpression of a kinase deficient, dominant negative β RRII (β RRII-DN) has been utilized. Since effects on cellular proliferation are a hallmark of TGF- β activity, overexpression of β RRII-DN in lung fibroblasts was able to block TGF- β induced proliferative activities (+ in adult, - in fetal) (Zhao and Young, 1996). In mink lung epithelial (Mv1Lu) cells, β RRII-DN expression resulted in a block in the anti-proliferative activity of TGF- β , but not transcriptional upregulation of PAI-1 and fibronectin, suggesting that there are β RI and β RRII specific responses (Chen et al., 1993). However, similar studies in primary cultures of neonatal cardiomyocytes alongside Mv1Lu cells demonstrated that β RRII-DN blocked the TGF- β induction of skeletal α -actin and PAI-1 promoter activity in addition to TGF- β 's inhibition of α -myosin heavy chain transcription, suggesting that all TGF- β effects are mediated through β RRII (Brand et al., 1993; Brand and Schneider, 1995). Finally, β RRII-DN has also been utilized to study developmental events such as capillary morphogenesis and myoblast differentiation. In each case, interference with TGF- β signaling through β RRII-DN was able to disrupt processes such as apoptosis and capillary morphogenesis in rat glomular capillary endothelial cells (Choi and Ballermann, 1995) and myoblast differentiation and fusion into myotubes (Filvaroff et al., 1994).

Although the TGF- β knockout studies indicate no gross abnormalities in the hearts of the null mutants, the distinct expression

patterns for the TGF- β ligands and receptors suggest a defined role each of the TGF- β isoforms in development. Although compensation for missing ligands in the knockout mice is possible, it is also possible that the roles the TGF- β s play in the heart are more subtle, affecting changes at the molecular and cellular level. The onset of expression of TGF- β ligands and β RII temporally correlates with the transition the mammalian heart goes through as it progresses from its fetal-to-neonatal-to-adult stages of maturation with regard to myocyte proliferation, myocyte differentiation, and ECM formation/angiogenesis. Due to TGF- β 's established anti-proliferative activity and ability to influence ECM biosynthesis and angiogenesis, we have postulated that ventricular TGF- β s participate in the three major developmental events ongoing during the neonatal ventricular transition period: (1) inhibits cardiomyocyte proliferation in an autocrine manner in the newborn, (2) influences differentiation of the cardiomyocyte in an autocrine manner in the late fetal/early neonatal stages, and (3) stimulates non-myocyte ECM biosynthesis/deposition and angiogenesis in the postnatal period in a paracrine manner.

Because β RI and β RII are needed for most, if not all, actions of the TGF- β s, receptor manipulation appears to be the method of choice to define the role(s) TGF- β may play in regulating myocyte proliferation and influencing their differentiation and paracrine mechanisms of action. Results from the DN TGF- β studies described above indicate that TGF- β may be directly involved in several processes (proliferation, muscle protein isoform expression, ECM biogenesis) which occur in or are associated with

the cardiomyocyte population during heart development. However, due to variability in responses between different cell types, only tenuous relationships can be made as to TGF- β 's direct effects on ventricular development *in vivo*.

To begin to address the role(s) of TGF- β in ventricular development, we have chosen to use the β RII-DN approach to evaluate changes that occur in myocytes in response to TGF- β stimulation. While primary cardiomyocyte cultures would be the cell culture system most applicable to the *in vivo* situation, their general lack of proliferative activity, heterogeneity, and transfection efficiency limit their *in vitro* utility. Two ventricular myocyte-derived cell lines, BWEM and CLEM, are available in which to study the aspects of TGF- β signaling in a myocyte background. These cell lines were formed from 16dpc rat ventricular myocytes by retroviral transformation with *v-myc* (Engelmann et al., 1993b). Unlike their primary counterparts, these cell lines are non-contractile, which is reflective of their lack of organized sarcomere-like structures. However, these lines have maintained the expression of several genes which are known to be expressed in embryonic and fetal cardiomyocytes: cTNC, α -cardiac actin, early growth response-1, creatine kinase-B, connexin-43, myosin light chain-2v, and several muscle-specific transcription factors (Engelmann et al., 1993b; Engelmann et al., 1996). These cell lines have been used to create mutant myocyte lines with reduced or absent β RII function in which the direct effects of TGF- β can be uncoupled and evaluated in a myocyte background. Data are presented here on the

creation and receptor expression/function in each of the parental and mutant myocyte-like cell lines. Data are then presented in which these lines were used to study the TGF- β signal transduction pathway and test these hypotheses:

- (1) TGF- β inhibits cardiomyocyte proliferation
- (2) TGF- β plays a direct role in cardiomyocyte differentiation by inducing the “adult” isoforms of three contractile proteins: TNC, TNI, and TNT.
- (3) TGF- β promotes ECM accumulation and angiogenesis through the upregulation of cardiomyocyte produced PAI-1 and SPARC peptides.

CHAPTER 3

MATERIALS AND METHODS

Culturing of Cell Lines: BWEM and CLEM are myocyte cell lines derived from 16-day fetal rat ventricular myocytes by *v-myc* transformation (Engelmann et al., 1993b). Cells were maintained in 4% Fetal Clone Serum (FCS-I, a bovine serum product, Hyclone Laboratories, Inc., Logan, UT) in Dulbecco's Modified Eagle's Medium Nutrient Mixture F-12 Ham (DME/F-12, Sigma Chemical Co., St. Louis, MO). Cells were passaged or split for subsequent use by trypsinization (Trypsin-EDTA, Gibco-BRL, Grand Island, NY).

Primary Fetal Cultures: 18 day gestation cardiomyocytes were obtained from time-pregnant Sprague-Dawley rats (Zivic-Miller, Portersville, PA) as previously described (Engelmann et al., 1990). Cells were plated on collagen coated tissue culture plates and allowed to attach overnight in a 1:1 dilution of PC-1 (Ventrex, Portland, Maine):DME/F-12. After plating, media was changed to a 1:2 dilution PC-1:DME/F-12, and timepoints and/or transfections (see below) were started.

Establishment of Stably Transformed Clonal Cell Lines: BWEM and CLEM parental cell lines were transformed with pRSV₂Neo (5 µg) (American Type Culture Collection (ATCC), Rockville, MD) and the plasmid phβRIIDN (Chen et al., 1993), which encodes a truncated, human βRII (25 µg). Both parental lines were electroporated at 280 mV with a capacitance extender option (BioRad, Hercules, CA), allowed to sit on ice 15 minutes, and plated in 4% FCS-I for 14-16 hours (overnight). Resulting transformants were then selected for with the antibiotic geneticin (400 µg/mL active G418, Gibco-BRL). After the fifth passage, transformed lines were considered "stable" and G418 was reduced to 200 µg/mL of active G418 for maintenance. After 20-25 passages, cells were collected from each set of transformants by trypsinization, pelleted, and resuspended in Hank's balanced salt solution (HBSS, Sigma Chemical Co.). Cells were stained with Hoechst dye and single, G₀ cells were sorted using pulse-processing to exclude doublets and collected into 96-well plates by fluorescence activated cell sorter analyses (FACS, FACStar Plus, Becton-Dickenson, Mountainview, CA). Each well contained 50% conditioned media from the parental line of origin to facilitate their attachment and growth. All resulting single cell clones were then expanded into full colonies. >50 of these colonies were screened by Northern and Southern blot analyses, and a select group were characterized in greater detail and contrasted to their parental sources. One BWEM-derived clone, BW-H1, and one CLEM-derived clone, CL-B5 were chosen for these more extensive and detailed studies.

Southern Blot Analysis: Genomic DNA was obtained from each myocyte cell line or clone as previously described (Laird et al., 1991). DNA was subjected to HindIII, ApaI, or BamHI digestion, extracted twice with phenol:chloroform, precipitated with isopropanol, resuspended in TE (10mM Tris-HCl, pH 8.0, 1mM EDTA), and quantitated by ultraviolet (UV) absorbance at 260 and 280 nm (U-2000 Double-Beam UV/Vis Spectrophotometer, Hitachi Instruments, Inc., Danbury, CT). Equal amounts of genomic DNA (25 µg) were loaded and electrophoresed on 0.6% agarose TBE gels. After DNA was depurinated in 0.25M HCl for 15 minutes, DNA was denatured in 0.5N NaOH/ 1.5M NaCl. The gel was then neutralized in 0.5M Tris-HCl, pH 8.0/1.5M NaCl and transferred to nylon membranes (Duralon-UV, Stratagene, La Jolla, CA) by overnight capillary transfer. DNA was UV-crosslinked to the membranes after transfer (UV Stratalinker, Stratagene) and used for hybridization analysis. Membranes were blocked for 2 hours with cDNA hybridization buffer (50% formamide, 10% dextran sulfate, 1% sodium dodecyl sulfate (SDS), 1 M NaCl, 100 µg./mL herring sperm DNA) at 42°C in a Hybridization Incubator (Robbins Scientific, Sunnyvale, CA). A cDNA probe specific for the CMV promoter was obtained from the phβRIIDN plasmid by restriction digestion with Bgl II and Hind III and isolation in low melt agarose, and labeled by random prime labeling (Decaprime II Labeling Kit, Ambion, Austin, TX). The probe was boiled in cDNA hybridization buffer for ten minutes just prior to addition to hybridization tubes. After overnight hybridization at 42°C, blots

were washed twice in 2X SSC , 0.5% SDS at room temperature for 20 minutes, followed by 10 minutes washes in 0.1X SSC, 0.1% SDS at 50-65°C in 5° increments. X-ray film (Biomar Blue Sensitive Autoradiographic film, MARSH Biomedical Products, Inc., Rochester, NY) exposures were done at -80°C for 24 hours.

TGF- β_3 Treatments: Treatments with or without TGF- β_3 were begun after suspended cells resulting from splitting and/or electroporation were allowed to settle and reattach to the cell cultures plates 14-16 hours (overnight). Plating media and dead cells were removed, and plates were washed twice with HBSS. Unless otherwise indicated, 4% FCS-I (cell lines) or 1:2 PC-1:DME/F-12 (fetal) media \pm TGF- β_3 (Ken Iwata, Oncogene Science, Uniondale, NY) was added to each plate and incubated at 37°C, 5% CO₂. Media was changed daily (mRNA/protein collection- cell lines) or spiked with additional TGF- β_3 (CAT assays, all fetal experiments) every 24 hours. Cells were then washed twice with HBSS and samples collected.

Northern Blot Analysis: Total RNA from cell cultures was obtained by scraping cell monolayers in guanidine isothiocyanate, the resulting homogenate was then phenol/chloroform extracted, precipitated with isopropanol, and the final RNA pellet was resuspended in formamide (Chomczynski and Sacchi, 1987). Samples were quantified by UV absorbance at 260 and 280 nm. Equal amounts of total RNA were loaded and electrophoresed on denaturing 1.2% agarose gels. Separated RNA was

hydrolyzed in dilute alkali, UV-crosslinked to nylon membranes after overnight capillary transfer, and used for hybridization analysis. Probes used for hybridization analyses were ^{32}P -labeled using random prime labeling of cDNA probes (Decaprime II Labeling Kit, Ambion) and terminal deoxynucleotidyl transferase (Pharmacia Biotech, Piscataway, NJ) labeling of oligonucleotide probes. Membranes were blocked in cDNA hybridization buffer (see Southern blot analysis) or oligo hybridization buffer (6X SSC, 1X Denhardt's, 0.1% SDS, 0.05% Na-pyrophosphate, and 100 $\mu\text{g}/\text{mL}$ herring sperm DNA) for 2 hours. Overnight hybridizations were performed at 42°C for cDNA probes or at 51°C for oligo probes. Blots having cDNA probes were washed as described for Southern blots. Blots hybridized with oligonucleotide probes were washed 4x 10 minutes in 6X SSC, 0.1% SDS at room temperature, and twice at 50°C in the same solution. mRNA levels were quantitated by phosphoimager analysis (*InstantImager*, Packard Instrument Co., Meridan, CT). X-ray film exposures were done at -80°C for the indicated times for each blot. Loading and RNA transfer equivalencies were assessed by hybridizations with an 18S or 28S rRNA oligonucleotide probe as previously described (Engelmann et al., 1989, Samarel and Engelmann, 1991).

cDNA Probes: inserts isolated from plasmids containing cDNAs for:

rat TGF- β R1 (R4) 3'UTR (He et al., 1993)

rat TGF- β R2 (pr β R2, extracellular domain)

(Tsuchida et al., 1993)

human h β R2IDN (extracellular domain)

cardiac Troponin C (cTNC, full length probe)

(Parmacek and Leiden, 1989)

murine skeletal Troponin C (sTNC, full length probe)

(Parmacek et al., 1990)

skeletal Troponin I (sTNI, full length probe)

(Murphy et al., 1991)

cardiac Troponin I (cTNI, full length probe)

(Murphy et al., 1991)

SPARC (full length probe, Mason et al., 1986).

Oligo Probes:

adult/fetal Troponin T, recognizes nt 942-974 (3'coding region)

(CCAR, Jin and Lin, 1989)

5' GGCAAGACCTAGAGCTGGGATTCACAGGGCAAG 3'

fetal Troponin T, recognizes exon 4 (PE4, Jin and Lin, 1989)

5' GGAAGACTGGAGCGAAGAAGAAGAAGACG 3'

18S and 28S rRNA (25 nt) (Yu et al., 1992)

18S 5' TCACCTCTAGCGGCGCAATACGAAT 3'

28S 5' ACCTTTTCTGGGGTCTGATGAGCGT 3'

Protein Collection: Full cell extracts (FCE) were obtained from tissue culture plates by scraping directly into freshly prepared 250 mM Tris-HCl, pH 7.5, containing 3% sodium dodecyl sulfate (SDS), 1 mM sodium fluoride (NaF), 1 mM sodium vanadate (NaVO_4), and 1 mM phenylmethylsulfonyl fluoride (PMSF) (Tris-SDS solution). Conditioned media were collected from tissue culture plates at indicated times and dead cells/debris were removed by centrifugation at 2000 rpm for 5 minutes. Media proteins were precipitated with 10% TCA, washed 4 times with ice cold acetone, and allowed to dry. Proteins were resuspended in the Tris-SDS solution described above. All samples (FCE and media) were boiled for six minutes and allowed to return to room temperature. Samples were quantitated by a standard BCA assay (Pierce, Rockford, IL). Samples were stored at -20°C between uses. To avoid protein degradation, large sample volumes were divided into several tubes to prevent repeated freeze-thaw cycles.

Western Blot Analyses: Samples were diluted in 250 mM Tris-HCl, pH 7.5, containing 3% SDS. 5X dye solution (20% β -mercaptoethanol, 80% glycerol, bromphenol blue) was added to each and heated at 65°C for 10 minutes. Biotinylated standards (broad range, BioRad) were used at 1 μL per lane. Samples were then loaded on a 10 or 12% SDS-PAGE gel and run for ~2 hours at 105 volts at room temperature on a Mini-PROTEIN II Slab gel apparatus (BioRad) according to manufacturer's protocol. Proteins were transferred to nitrocellulose (BioRad) in transfer buffer containing 20% methanol for 1.5 hours at 105 volts with the Mini Trans-Blot system

(BioRad) according to manufacturer's instructions. Membranes were then washed with TBST (10 mM Tris-HCl, pH 8.0, 150 mM NaCl, 0.05% Tween-20) and used for western blot analysis. Membranes were blocked in TBST containing 10% normal goat serum (NGS, Sigma Chemical Co.) and 2% bovine serum albumin (BSA, Sigma Chemical Co.) for 2 hours at room temperature (RT). Primary antisera (α -SPARC, #5944, 1:5000 dilution, Sage et al., 1989; α - β RI, 1:1000 dilution, Santa Cruz Biotechnology, Inc., Santa Cruz, CA) was added to the membranes (diluted in blocking buffer) and incubated overnight at 4°C. Membranes were then washed 3 times at RT with TBST (10 mM Tris-HCl, pH 8.0, 150 mM NaCl, 0.05% Tween-20). Standard lanes were cut off from the remaining blot for separate 2° antibody treatment. Alkaline phosphatase (AP) conjugated secondary antibody (α -rabbit or α -mouse, Santa Cruz Biotechnology, Inc.) was added to each membrane and incubated at RT for 1 hour. Standard lanes were incubated with streptavidin conjugated AP (BioRad). Membranes were then washed at RT twice (5 minutes each) with TBST and twice (5 minutes each) with deionized H₂O. Blots were developed with the 5-bromo-4-chloro-3-indolyl-phosphate/p-nitro blue tetrazolium chlorohydrate color development system (BCIP/NBT, 5 Prime --> 3 Prime, Inc., Boulder, CO) for 5-15 minutes. Development was stopped by washing the blots with deionized H₂O and blotting dry.

Receptor-Ligand Crosslinking: TGF- β_1 crosslinking was performed as previously described (Massague, 1987) with minor alterations. Cells were plated at $1-2 \times 10^5$ cells per 12-well or 35mm plate and allowed to incubate for approximately 48 hours. As a control, 3-day old neonatal cardiomyocytes were isolated by sequential collagenase digestions as previously described in detail (Engelmann et al., 1990). 100pM of ^{125}I -TGF- β_1 (Biomedical Technologies, Inc., Stoughton, MA) was added to each dish in cold binding buffer (128 mM NaCl, 5 mM KCl, 5 mM MgSO_4 , 1mM CaCl_2 , 50 mM HEPES, pH 7.4, and 0.2% BSA) and incubated at 4°C for 4 hours. Receptor bound ligand was then crosslinked with Disuccinimidyl glutarate (4.4 mg/mL in dimethyl sulfoxide) (Pierce, Rockford, IL) for 15 minutes at room temperature. The crosslinking reaction was stopped by washing 4 times with PBS (10 mM H_2NaPO_4 , pH 7.25, 150 mM NaCl).

Identification of TGF- β Binding Proteins/Receptors: Full cell extracts of ^{125}I -TGF- β crosslinked samples were collected directly into Laemmli SDS-PAGE buffer containing 1mM DTT and boiled for 5 minutes. Fractions of each sample were taken to quantify available radioactivity (COBRA II Auto-Gamma, Packard Instrument Co., Meridan, CT). Equal counts of radioactivity for each sample were loaded and run on 10-12% SDS-PAGE gels. The gels were dried and exposed to X-ray film at -80°C for 3-5 weeks. Kaleidoscope standards (BioRad) were run on each gel for size determination.

Identification of Receptors/Receptor Complexes: To more clearly observe β RII-DN and possibly provide evidence for β RII-DN: β R1 association, immunoprecipitations (IPs) were performed on ^{125}I -TGF- β crosslinked samples. Samples were collected into PBSTDS (10 mM Na_2HPO_4 , 150 mM NaCl, 1% Triton-X 100, 0.1% SDS, 1 mM NaF, 10 mM Na-deoxycholate, and 30 mM Na-azide) and immunoprecipitated (IP) with M2 α FLAG monoclonal antibody (M2) (Sigma, St. Louis) or α - β R1 and Protein G Plus/Protein A Agarose (Oncogene Sciences, Manhasset, NY). Final IP pellets were resuspended in Laemmli buffer containing 1 mM DTT and equal volumes were loaded on 10-12% SDS-PAGE gels. Gels were dried and exposed to X-ray film for 5 weeks at -80°C . Kaleidoscope standards (BioRad) were run on each gel for size determination.

Growth Assays: $2\text{-}2.5 \times 10^5$ cells were plated on 35 mm plates in 2% FCS and allowed to settle and reattach for 4-5 hours. The cells were then washed twice with HBSS and refed with media containing 0.5% FCS with 0-10 ng/mL TGF- β_3 . The number of attached cells was determined from nuclei counts of these mononuclear cells at this time (T=0) for plating efficiency (Engelmann and Gerrity, 1988). After 48 hours of continuous exposure (without additional TGF- β_3 being added at 24 hours), nuclei were isolated and counted (T=48). Duplicate plates were counted for each data point. The data are expressed as the relative percentage of cell proliferation compared to control cultures not exposed to exogenous TGF- β_3 .

Luciferase Assays: $2\text{-}2.5 \times 10^6$ cells were used for each electroporation. Cells were collected by trypsinization, counted, pelleted, and resuspended in electroporation buffer (20 mM HEPES, pH 7.05, 137 mM NaCl, 5 mM KCl, 0.7 mM Na_2HPO_4 , 6 mM glucose). 25 μg p3TP-Lux (PAI-1, Wrana et al., 1992) or BW147 (sTNI, Corin et al., 1994; Corin et al., 1995) and 5 μg pRSVZ (ATCC) plasmid DNAs were added to the cells in a total volume of 800 μl for each electroporation. Cells were electroporated at 240 mV with a capacitance extender option (BioRad), allowed to sit on ice for 15 minutes and then replated. Cells were allowed to recover/attach for 4-6 hours after which the plates were washed with HBSS and refed with fresh media containing 0-10 ng/mL TGF- β_3 . After an additional 36 hours, luciferase activity was measured from the cell lysates according to the manufacturer's instructions (Enhanced Luciferase Assay Kit, Analytical Luminescence Lab., Ann Arbor, MI). Data were normalized for electroporation efficiency by determining β -galactosidase activity from each sample and expressed as fold changes relative to untreated (0 ng/mL TGF- β_3) controls.

CAT Assays: $4.5\text{-}5 \times 10^6$ cells were used per poration. Cells were collected by trypsinization, counted, pelleted, and resuspended in 800 μL electroporation buffer. 25 μg reporter plasmid [cTNC (p-124SV0CAT, Parmacek et al., 1994), sTNC (1.7/900-CAT, Parmacek et al., 1990), SPARC (639-SPARC, McVey et al, 1988), TNT (303-CAT, Wang et al., 1994)] and 5 μg pRSVZ

(ATCC) were used for each poration. Cells were electroporated at 240 mV, allowed to sit on ice for 15 minutes and plated. Cells were allowed to recover/attach overnight after which dead cells were washed off and treatments were started. After 48-72 hours, proteins were collected, quantitated by BCA, and CAT assays were performed using 150 μ g protein (cell line) or 40-70 μ g protein (fetal) (Gorman et al., 1982). CAT activity was quantitated by phosphoimager analysis (InstantImager, Packard Instrument Co.). Data were normalized for poration efficiency by determining β -galactosidase activity for each sample utilizing equal amounts of cellular protein.

Transfection and Reporter Assays Utilizing Primary Fetal Myocytes:

Primary fetal cultures were transfected as previously described using Lipofectin (Gibco-BRL) (Engelmann et al., 1990; Ciccarone and Hawley-Nelson, 1995). Briefly: 24 μ g Lipofectin/mL final volume 1:2 dil. PC-1:DME/F-12 was used for each transfection. Lipofectin and media were combined and allowed to incubate at RT for ~1 hour. Reporter plasmid (25 μ g, BW147, 180-CAT, 1.7/900-CAT, 303-CAT, 639-SPARC) and pRSVZ (5 μ g) were mixed with lipofectin/media mixture and allowed to incubate at RT for 30 minutes. The resulting mixture was then added to plates and allowed to incubate at 37°C, 5% CO₂ overnight. Lipofectin-media-plasmid mixture was then removed, plates washed 3 times with HBSS, and TGF- β ₃ treatments started. After 36 (luciferase) or 48 (CAT) hours, samples were collected and activities were assayed as described above.

Statistics: Using InStat Software and a P value of <0.05 as an index of achieving statistical significance, Northern blot and luciferase data from each parental line and mutant clone was analyzed using one way analysis of variance (ANOVA) followed by Tukey-Kramer multiple comparisons test. Unpaired T-tests were performed on all fetal data, and on cell line data obtained from the CAT assays. Comparisons between parental cell lines and their mutant clones at each dose of TGF- β_3 were evaluated by one-way ANOVA. Comparisons of the dose curves between each parental line and its respective mutant were evaluated by two way ANOVAs using SUPERANOVA software.

CHAPTER 4

RESULTS

Creation and Initial Characterization of Cell Lines

Creation of Dominant Negative Cell Lines

As a model system for the study of the effects of TGF- β in ventricular development, the BWEM and CLEM myocyte-like cell lines not only provide a means to directly examine changes TGF- β may elicit, but also to examine the mechanisms or pathways which mediate these effects. To more clearly define the direct roles TGF- β may have in ventricular development and to examine the specific roles of the Type I and Type II TGF- β receptors, we chose to knock out β R_{II} function in each of the myocyte cell lines utilizing the dominant negative approach.

In order to create cell lines in which a response to TGF- β could be abrogated, the expression plasmid ph β R_{II}DN was introduced into the BWEM and CLEM cell lines by electroporation. This plasmid contains a cDNA encoding a truncated, kinase-deficient human β R_{II} (see Figure 3) whose expression is under the control of the cytomegalovirus (CMV) promoter. As this plasmid did not contain a eukaryotic selection marker,

pRSV₂neo expression plasmid conferring genestine (G418) resistance was co-transfected. After G418 selection was maintained through 5-7 passages, four stable, clonal lines were established; two BWEM derived, BDN and BDNT, and two CLEM derived, CDN and CDNT. Because G418 resistance alone does not guarantee stable integration of ph β RIIDN, the polyclonal nature of these lines made interpretation of the resulting data (not shown) troublesome. Therefore, single cell derived clonal sublines were established by fluorescence activated cell sorting (FACS) from each of the four polyclonal populations. To ensure each subline was derived from a single cell (clonal), each well of the 96-well plate was screened within 24 hours of FACS sorting to select/verify those wells which contained a single cell. Greater than 50 clonal cell lines were screened by Southern and Northern blot analyses for ph β RIIDN integration and β RII-DN expression, respectively (e.g. Figure 4). From these analyses, four clones were selected based on indices of high expression of the estimated 1 kb transcript for the truncated receptor and were further characterized: BDNT 96 F8, BDNT 296 H1, CDNT 296 B5, and CDNT 96 F5 (indicated by **, Figure 4). These four clonal lines (two from each parental line) are referred to as BW-F8, BW-H1, CL-B5, and CL-F5 from here on.

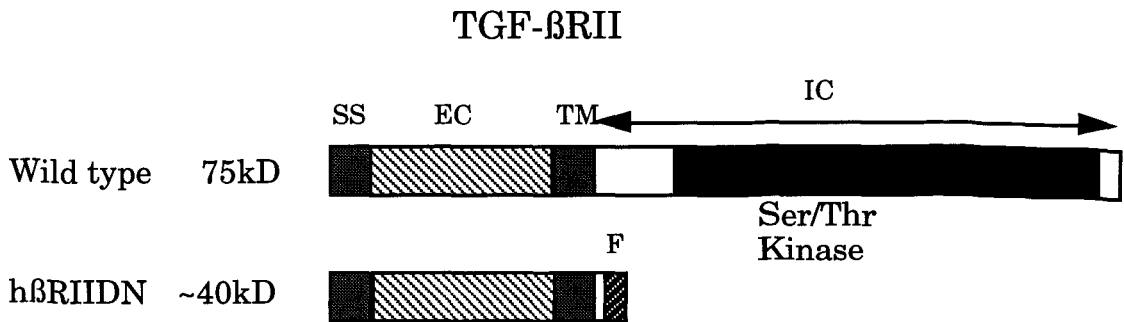


Figure 3: Diagram of Wild Type and Mutant TGF- β RII

Schematic of the protein domains found in the wild type TGF- β RII and the truncation mutant present in the ph β RIIDN expression vector used to create the DN cell lines.

SS: Signal Sequence

TM: Transmembrane domain

F: FLAG Epitope tag.

EC: Extracellular domain

IC: Intracellular domain

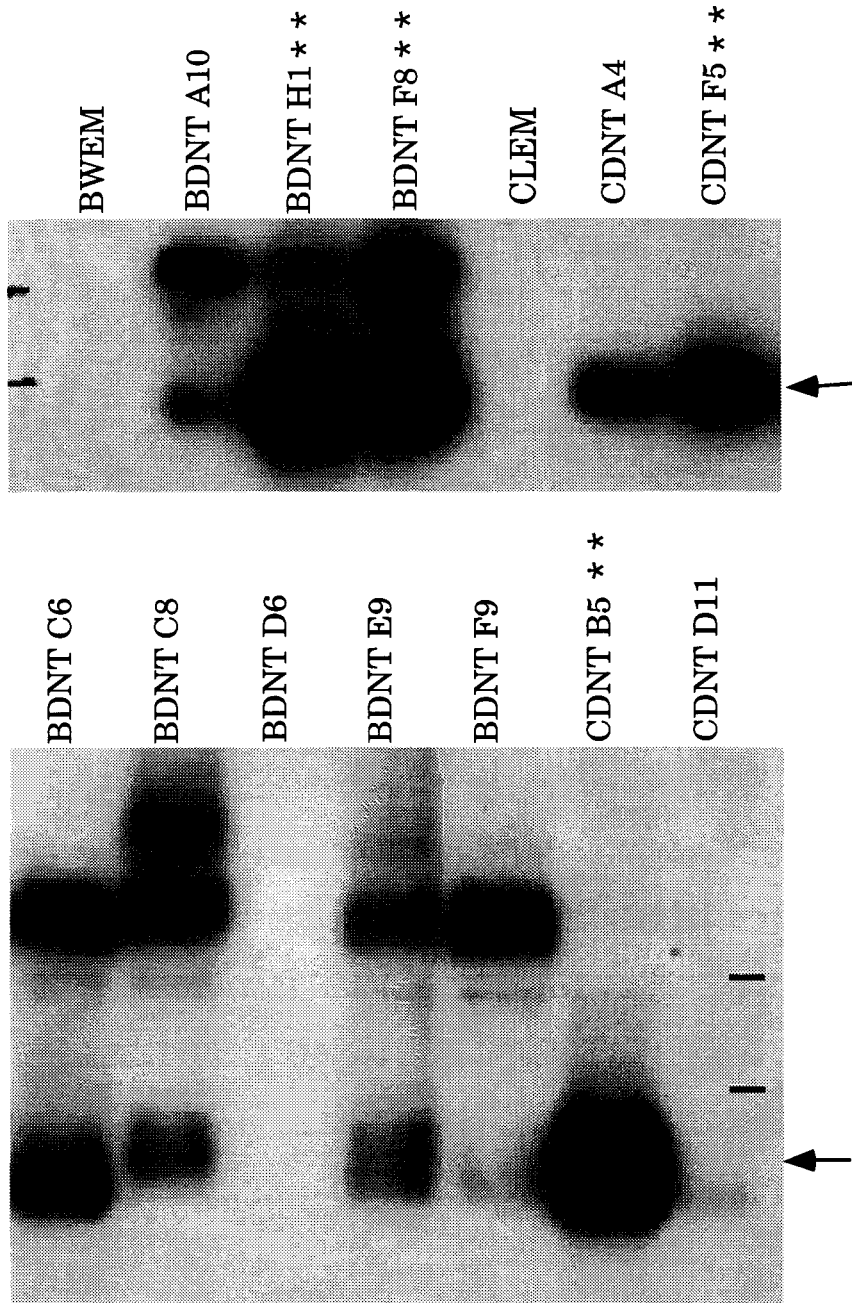


Figure 4: Screen of Clonal β RII-DN Mutants

Examples of Northern Blot analyses used to choose mutant cell lines which incorporated ph β RIIDN and expressed the β RII-DN transcript. Arrow indicates expected size (~1 kb) for the β RII-DN transcript. ** Indicates those DN lines which were chosen for further study. 25 μ g total RNA loaded for each sample. Results shown are from 24 hr. exposure to film at -80°C . Bars on right indicate migration of 28S and 18S rRNA markers.

Expression and Ligand Binding of Endogenous and Mutant Receptors

As previously described, β RI and β RRII are necessary for most, if not all, TGF- β -mediated responses. Therefore, expression and ligand-binding ability of each receptor, in all of the cell lines is of utmost importance. While the expression of the endogenous TGF- β receptors was not expected to be affected by the transformation, we chose to verify their expression in each of the mutant lines as well as their parental cell lines. Additionally, to verify the stable integration and expression of β RRII-DN after multiple passages (>20), expression and ligand-binding ability of β RRII-DN was verified in the mutant cell lines.

To verify expression of β RI in the parental and mutant cell lines, Northern blot and Western blot analyses for the endogenous β RI were performed on RNA and protein, respectively, isolated from the mutant clones as well as the parental cell lines (Figure 5 and 6). As expected, transcript levels for β RI (~6 kb) were not affected by the transformation, nor did they differ significantly between parental lines or clones (Figure 5). Similar results were seen with Western blot analyses for β RI protein, which has an estimated size of 53 kD (Figure 6). Therefore, since it appears that β RI is avidly expressed in each of the cell lines, any lack of response to TGF- β observed in the mutant cells should not be attributed to abnormal, cell surface expression of β RI.

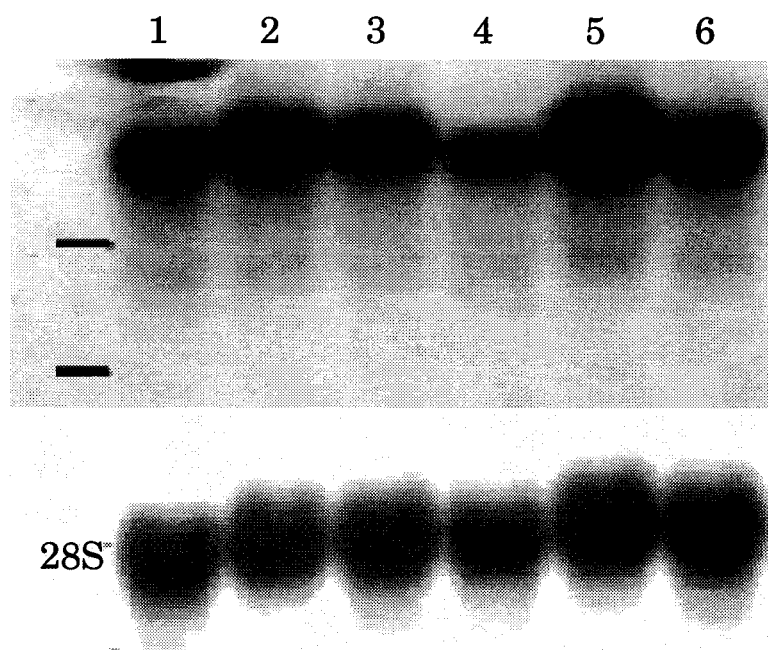


Figure 5: Analysis of Endogenous β RI Expression

Northern blot hybridization analysis was performed to verify expression of the endogenous rat Type I TGF- β receptor utilizing the rat β RI (R4) cDNA insert as the probe. Bars represent migration markers of 28S and 18S rRNA. Results shown are from 24 hour exposure to film at -80°C . 28S rRNA hybridization is shown.

Lane 1: BWEM

Lane 2: BW-F8

Lane 3: BW-H1

Lane 4: CLEM

Lane 5: CL-B5

Lane 6: CL-F5

*Each lane contained 25 μg of total RNA

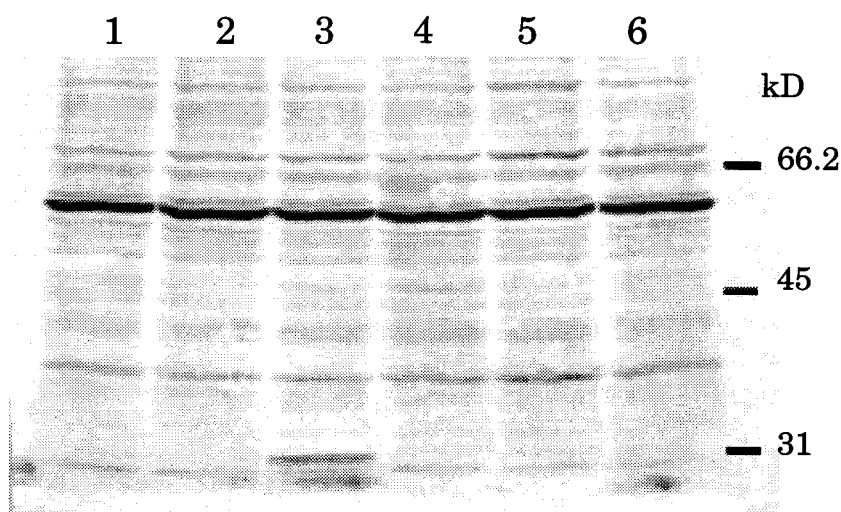


Figure 6: β RI Western Blot Analysis

Total cellular proteins from cell cultures of each cell line were run on 10% SDS-PAGE gels and used for Western blot analyses. 25 μ g of protein was loaded per lane. Markers on right indicate size in kilodaltons as determined by co-running biotinylated standards on the gel. Staining of gel indicated that protein loading was consistent (data not shown).

Lane 1: BWEM
Lane 4: CLEM

Lane 2: BW-F8
Lane 5: CL-B5

Lane 3: BW-H1
Lane 6: CL-F5

Expression of the endogenous β RII in each of the cell lines was also verified by Northern blot analyses. As mentioned previously, the extracellular domain is less conserved across species (see figure 1, Chapter 2). This reduced homology results in low cross-reactivity between human and rat. Therefore, using a probe corresponding to the extracellular domain of rat β RII, Northern blot analyses were performed to verify expression of the endogenous β RII. As expected, expression of the rat β RII transcript (~5.2 kb) was detected in each of the cell lines (Figure 7). Due to the low amino acid homology between the extracellular domains of β RII across species (e.g. 82% between human and rat), most available antisera for β RII are made against peptides found within the conserved kinase domain using the published human sequence. However, detection of the endogenous rat β RII by Western blot analyses was not successful, as these antisera identified multiple bands, none of which were prominent and at the expected size of ~75 kD (data not shown).

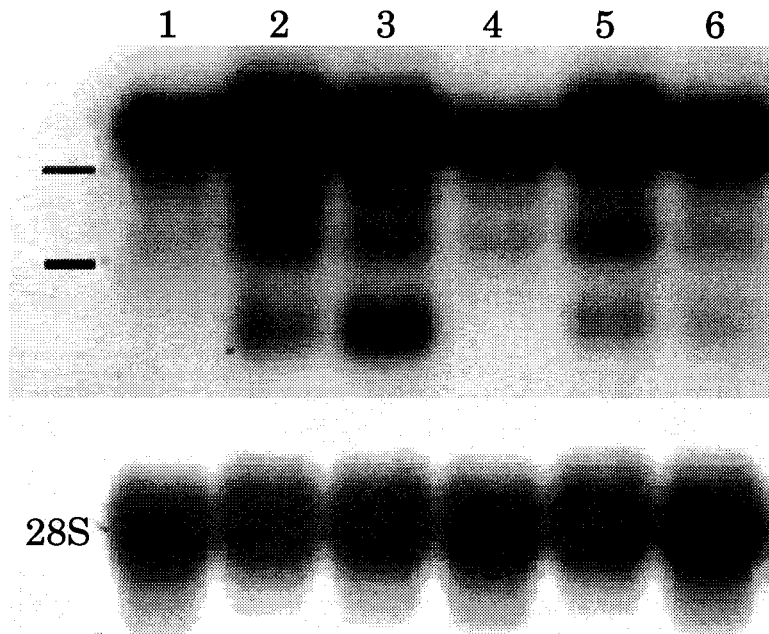


Figure 7: Analysis of Endogenous β RII Expression

Northern blot hybridization analysis was performed to verify expression of the endogenous rat Type II TGF- β receptor utilizing the rat β RII cDNA insert as the probe. Bars represent migration markers of 28S and 18S rRNA. Results shown are from 24 hour exposure to film at -80°C . 28S rRNA hybridization is shown.

Lane 1: BWEM

Lane 2: BW-F8

Lane 3: BW-H1

Lane 4: CLEM

Lane 5: CL-B5

Lane 6: CL-F5

*Each lane contained 25 μg of total RNA

While the mutant cell lines were selected because of their high transcript level for β RII-DN (Figure 4), the stable integration of and expression from the β RIIDN plasmid was verified after multiple (>20) passages in all four mutant clones by Southern (Figure 8a) and Northern (Figure 8b) blot analyses, respectively. In addition to transcript size differences between the endogenous rat (~5.2 kb) and mutant human β RII (~1 kb) gene products, the relatively low level of homology (at the cDNA level) of the extracellular domains of β RII across species allowed us to easily distinguish between the endogenous rat and mutant human receptors. As expected, results were negative for both the Southern and Northern blot analyses of the parental cell lines (CLEM Southern data not shown). Similar to the results obtained from Northern blot analyses performed on RNA obtained from the mutant lines at an early passage, the ~1 kb human β RII-DN transcript was readily detected in each of the mutant cell lines (Figure 8). A larger (~5 kb) band is consistently detected in the BWEM derived lines (both early and later passages). As these bands coincide with the size of the endogenous rat β RII, crossreactivity was considered to explain the bands. However, since this band does not appear in the parental BWEM line, this does not appear to be the case. Therefore, the larger bands have been hypothesized to be the result of improper read-through of the plasmid during transcription or tandem integration of plasmids in the genome. These hypothesis have not been tested further.

Since the available antisera for β RII are made against peptides derived from the kinase domain, detection of β RII-DN with these antisera was impossible as these peptide sequences are deleted during truncation. However, to aid in its detection, β RII-DN was constructed with an epitope tag, FLAG. Western blot analyses utilizing antisera against this epitope from several independent sources were attempted, but each epitope-directed antisera detected several protein bands in the protein samples, none of which was unique to the DN lines (data not shown). However, this antibody proved to be useful for immunoprecipitations (see receptor-ligand crosslinking).

Figure 8: Integration of and Expression from ph β RIIDN Vector**A: Integration of ph β RIIDN**

BWEM BW-F8 BW-H1 CL-B5 CL-F5

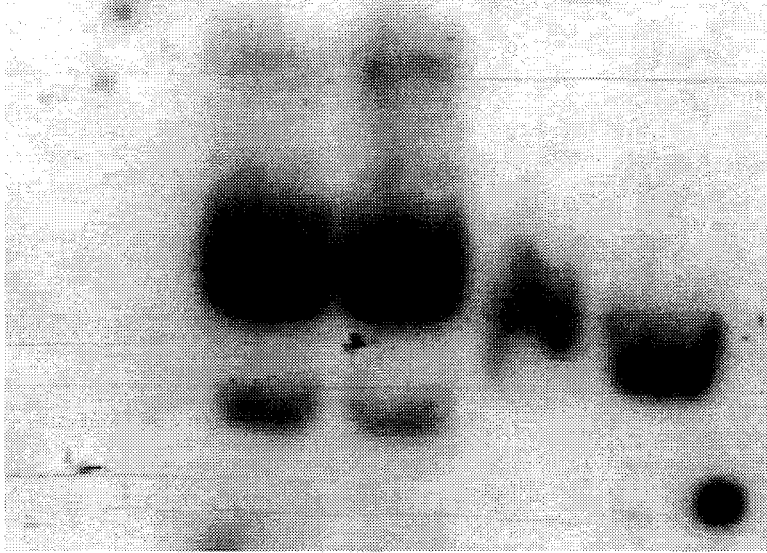
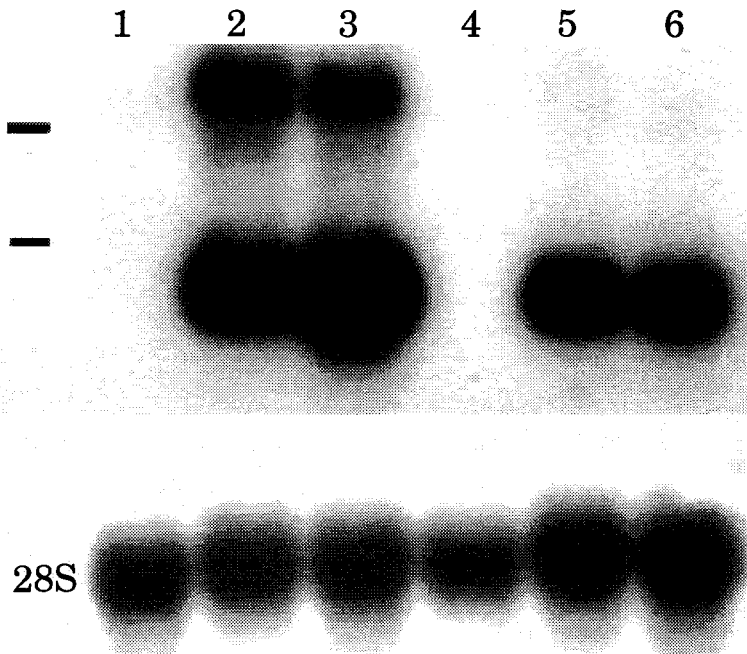
**B: Expression of β RII-DN**

Figure 8: Integration of and Expression from ph β RIIDN Vector

(A) Southern Blot hybridization analysis of genomic DNA (30 μ g/lane) after HindIII digestion. The CMV promoter fragment from the ph β RIIDN vector was utilized as a probe. Results shown are from a 24 hour exposure to film at -80°C.

(B) Northern Blot hybridization analysis for expression of the mutant human Type II receptor utilizing the entire coding region (primarily the extracellular domain) from ph β RIIDN as a probe. Results shown are from 24 hour exposure to film at -80°C. Bars represent migration markers of 28S and 18S rRNA. 28S rRNA hybridization is shown for the Northern blot.

Lane 1: BWEM

Lane 2: BW-F8

Lane 3: BW-H1

Lane 4: CLEM

Lane 5: CL-B5

Lane 6: CL-F5

*Each lane contained 25 μ g of total RNA

Cell surface expression and ligand binding abilities of the endogenous and mutant TGF- β receptor proteins were confirmed by ^{125}I -TGF- β_1 crosslinking. Although Northern and Western blot analyses detected the expression of the TGF- β receptor transcripts and proteins, these analyses did not verify ligand binding capabilities. Based on the mRNA studies, endogenous protein levels for each rat TGF- β receptor did not vary significantly between the cell lines or clones. To verify that these proteins were functional, their ligand binding capabilities were determined by standard ligand-receptor crosslinking methodologies. As shown in figure 9a, crosslinking with ^{125}I -TGF- β_1 showed the presence and ligand binding capabilities of the endogenous rat Type I (53 kD) and Type II (75 kD) TGF- β receptors as well as the truncated, human Type II (~40 kD) TGF- β receptor. In addition to the cell lines, results from a primary culture of two day neonatal cardiomyocytes was included for comparison. Duplicate, ^{125}I -TGF- β_1 crosslinked samples were used for immunoprecipitation (IP) with the M2 antibody against the FLAG epitope on $\beta\text{RII-DN}$ to more precisely evaluate expression of this receptor. In correlation with the mRNA studies, the mutant, human βRII receptor, estimated to be ~40 kD, was the only receptor-ligand band immunoprecipitated with the M2 α -FLAG antibody from lysates of the four mutant clones (overexposure of film shows weak band in CL-F5 sample), while none were detected using lysates from the parental cell line (Figure 9b).

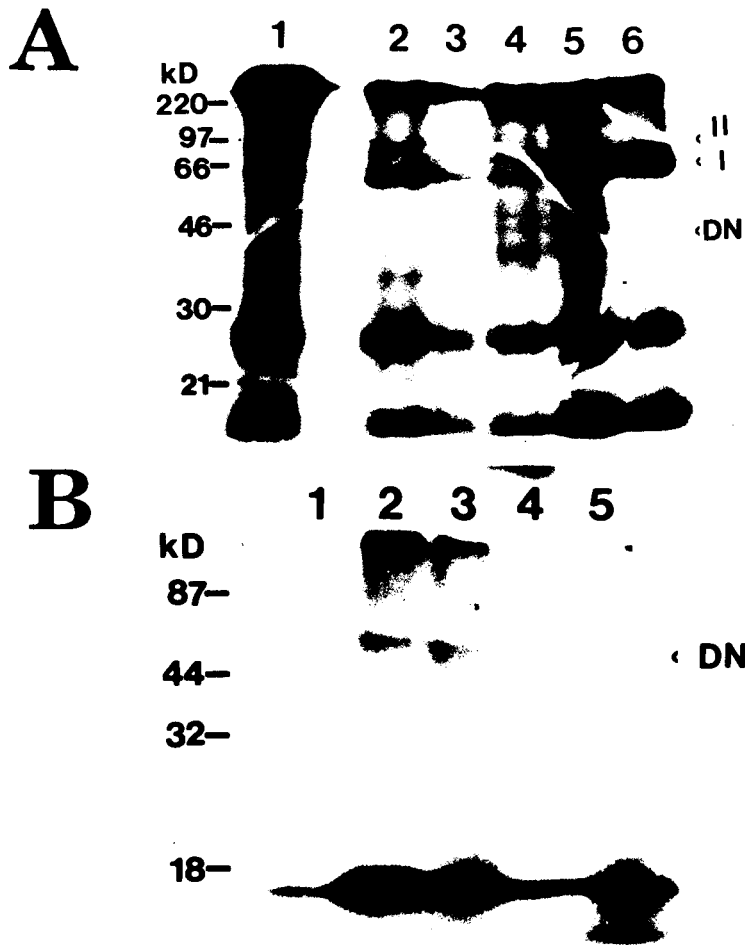


Figure 9: ^{125}I -TGF- β_1 Receptor-Ligand Crosslinking

^{125}I -TGF- β_1 (100 pM) was crosslinked to cell monolayers of each cell line to determine cell surface receptor expression and ligand binding capability.

(A) Full cell extracts were collected directly into Laemmli buffer and run on 12% SDS-PAGE gels. Results shown are from a 3-week exposure to film at -80°C .

Lane 1: Primary neonatal rat cardiomyocytes Lane 2: BWEM
Lane 3: CLEM Lane 4: BW-F8 Lane 5: BW-H1 Lane 6: CL-F5.

(B) Cell lysates were immunoprecipitated with M2 α FLAG with all of the IP-materials loaded and run on 12% SDS-PAGE gels. Results shown are from a 5-week exposure to film at -80°C .

Lane 1: BWEM Lane 2: BW-F8 Lane 3: BW-H1
Lane 4: CL-B5 Lane 5: CL-F5.

Initial Characterization of the Response to TGF- β

To initiate the characterization of each of the parental lines and clones to TGF- β responses, we analyzed two "standard" effects that have been shown to be elicited by all three mammalian isoforms of TGF- β in other well characterized cell lines (such as Mv1Lu): 1) inhibition of proliferation and 2) induction of transcription from the p3TP-Lux reporter construct derived from the promoter region of the PAI-1 gene. In addition to being standard TGF- β effects, the characterization of these responses to TGF- β_3 in each cell line also provided data which addresses two parts of our hypothesis: 1) TGF- β_3 can directly inhibit cardiomyocyte proliferation and 2) TGF- β_3 can influence ventricular remodeling by supporting ECM accumulation. As these effects are standard indicators of TGF- β responsiveness, the BWEM and CLEM cell lines are expected to exhibit significant changes in proliferation and PAI-1 induction after TGF- β_3 treatment. In contrast, the established expression of β RII-DN in the mutant cell lines should result in the elimination of TGF- β_3 responsiveness, and therefore, the elimination of any changes in proliferation or PAI-1 expression after TGF- β_3 treatment.

Growth assays were performed on each cell line using 0-10 ng/mL TGF- β_3 . The parental cell lines BWEM and CLEM each demonstrated maximum inhibition of proliferation at 1 ng/mL TGF- β_3 (Figure 10). Highlighting the individual nature of the two parental lines, the levels of TGF- β inhibition differed between these two lines. At 1 ng/mL TGF- β_3 ,

growth of the CLEM line was inhibited approximately 80% while growth of the BWEM line was inhibited only about 50%. These levels of inhibition are similar to those previously obtained in fibroblasts (~70%, Zhao and Young, 1996), capillary endothelial cells (~65%, Choi and Ballerman, 1995), and mink lung epithelial cells (~90%, Chen et al., 1993).

Similar growth assays were performed on each of the dominant negative cell clones. Two of these clones, BW-H1 and CL-B5, had a significantly reduced sensitivity to TGF- β_3 -mediated growth inhibition (Figure 10, Table 2). The BW-H1 clone's sensitivity to TGF- β_3 -mediated growth inhibition was such that the dose necessary to reach the 50% inhibition seen in its parental BWEM line at 1 ng/mL TGF- β_3 was approximately 10X higher (i.e. 10 ng/mL TGF- β_3). In contrast, growth of the CL-B5 clone was only modestly inhibited (approximately 20%) at 1-10 ng/mL TGF- β_3 . The results for CL-B5 reflect those obtained in mink lung epithelial cells where expression of the β RII-DN reduced proliferation only 10% compared to the 90% reduction observed in the parental cells (Chen et al., 1993). These results indicate that expression of the human β RII-DN is successfully competing out the endogenous rat β RII for available rat β RI and reducing or virtually eliminating the growth inhibitory activities of biologically active, recombinant TGF- β_3 .

48 Hour Growth Assays

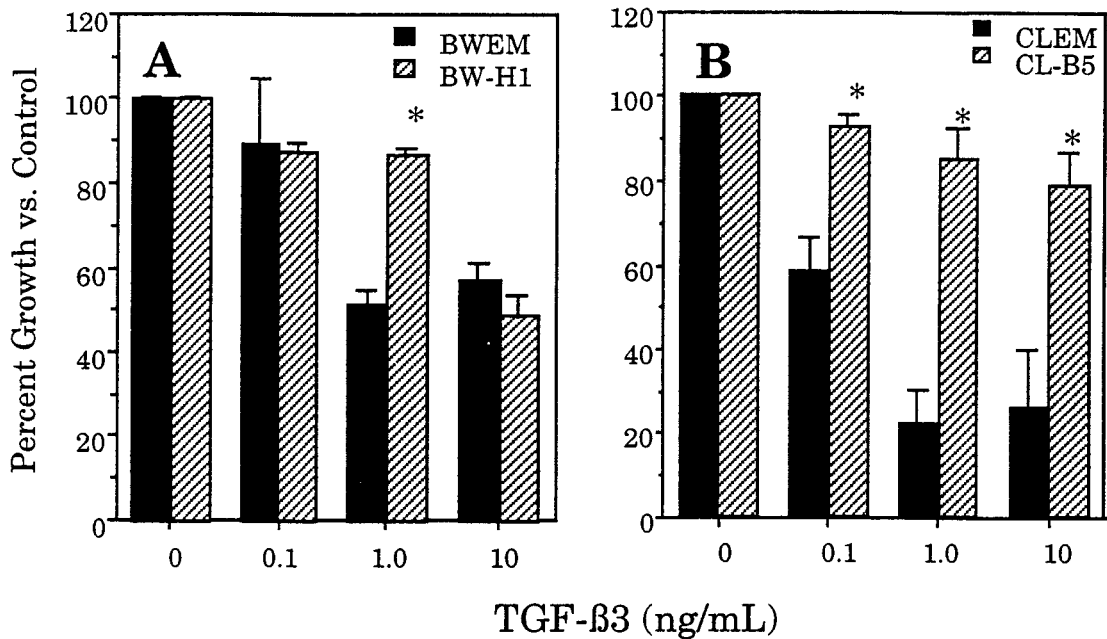


Figure 10: 48 Hour Growth Assays

48 hour growth curves for the BWEM parental and mutant clone BW-H1 (A) and CLEM parental and mutant clone CL-B5 (B) in the presence of 0-10 ng/mL of recombinant TGF-β₃ are shown. Mean data shown are from 4-6 individual assays performed in duplicate and the error bars represent SEM. "Control" refers to those cultures seeing no exogenous TGF-β₃.

* significantly different from parental line (see Table 1)

Table 2: Statistical Analysis of 48 hr. Growth Assay

One way ANOVA was used to determine significance between the percent growth for each parental cell line and its respective mutant at each dose of TGF-β₃. Data are presented as mean ± SEM.

	ng/mL	Parental*	Mutant*	p value
BW's	0.1	89.8 ± 15.3	87.8 ± 1.8	0.897
BW's	1.0	51.0 ± 3.9	86.8 ± 1.9	0.0002
BW's	10	57.0 ± 4.4	48.8 ± 4.9	0.251
CL's	0.1	59.0 ± 7.6	92.8 ± 3.1	0.004
CL's	1.0	22.8 ± 7.6	85.4 ± 6.9	0.0002
CL's	10	26.7 ± 13.4	78.8 ± 7.9	0.011

*Percent Growth Vs. Control (0 ng/mL TGF-β₃, control=100)

As a second TGF- β response index evaluated in the clones, we measured the ability of exogenous TGF- β_3 to induce transcription of a well established TGF- β -responsive gene, PAI-1. Expression from the luciferase reporter construct p3TP-Lux (Figure 11a), which contains three TPA responsive elements (TRE) and a portion of the PAI-1 promoter, was determined. As expected, TGF- β_3 significantly induced luciferase activity in a dose dependent manner in the two parental lines (Figure 11b and 11c, Table 3). Interestingly, even though the BWEM line appeared less sensitive than CLEM to TGF- β_3 's growth inhibition, they showed a greater fold induction of transcription from this promoter: $5.4 \pm 0.4X$ induction for BWEM vs. $3.9 \pm 0.6X$ induction for CLEM at 10 ng/mL TGF- β_3 , although this variation is statistically non-significant (Table 4). While this level of induction is dramatically lower than those observed in epithelial cells (20-40-fold, Wrana et al., 1992; Carcamo et al., 1995; Brand and Schneider, 1995), it is similar to those obtained in fibroblastic cell lines (5-6-fold, Carcamo et al., 1995). The level of induction (fold induction) seen in the parental lines is comparable to the $4.8 \pm 0.002X$ induction seen with primary fetal cultures (Figure 11b), but it appears that the activities measured in the parental lines are higher than the fetal myocytes, but only the difference between the fetal myocytes and BWEM cell line achieved statistical significance (Figure 11c, Table 4).

Similar luciferase assays performed on the BW-H1 and CL-B5 clones showed a markedly decreased sensitivity to TGF- β_3 induction of PAI-1

promoter activity. At 10 ng/mL TGF- β_3 , there was only a $2.3 \pm 0.3X$ induction of PAI activity in the BW-H1 clone and only a $1.6 \pm 0.2 X$ induction of PAI-1 activity in the CL-B5 clone. These values are significantly different (Table 5) from their respective parental lines at equivalent ligand concentrations. Similar to what was seen in the BW-H1 growth assay, PAI-1 reporter activity in the two DN clones at 10 ng/mL TGF- β_3 were not significantly different from the level of induction seen with only 1 ng/mL TGF- β_3 in their respective parental lines. This ~50% reduction in TGF- β_3 -responsiveness at 10 ng/mL TGF- β_3 is less prominent as similar experiments in mink lung epithelial cells where expression of β RII-DN resulted in an approximate 75% reduction in PAI-1 induction (Brand and Schneider, 1995, Carcamo et al., 1995). However, these data substantiate the postulate that the levels of β RII-DN expressed by these mutant clones are rendering them approximately 10X less sensitive than their parental lines to changes mediated by TGF- β_3 induced signal transduction pathways.

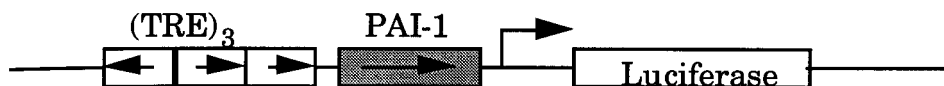
Table 3: Statistical Analysis of TGF- β_3 Dose Response Curves of PAI-1 Induction

One way ANOVA was used to determine significance of the dose response curve of PAI-1 promoter activity.

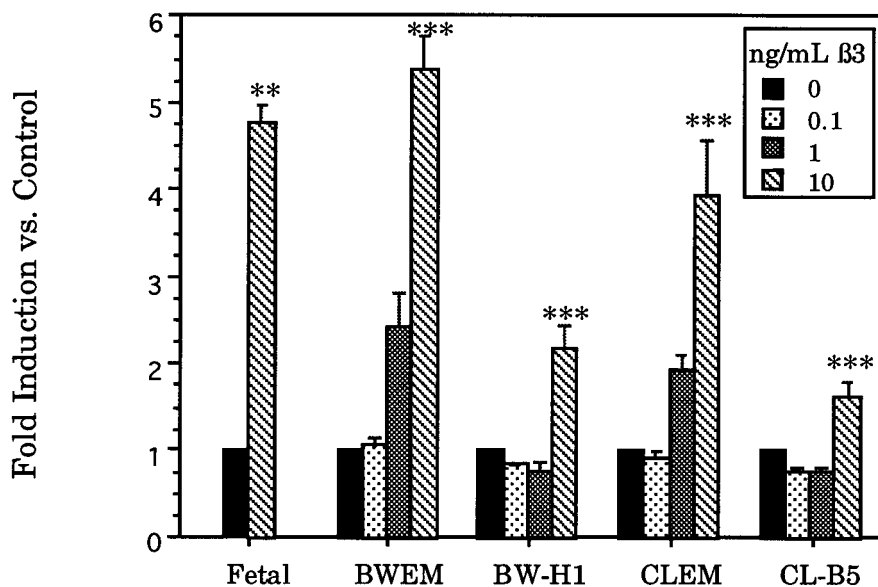
Cell Type	p-value (fold induction)	p-value (raw)
Fetal	0.0031	0.0004
BWEM	<0.0001	<0.0001
BW-H1	<0.0001	0.0991
CLEM	<0.0001	0.1424
CL-B5	<0.0001	0.1428

Figure 11: PAI-1 Promoter Assays

A: PAI-1 Promoter Construct



B: PAI-1 Promoter Activity -- Relative Changes



C: PAI-1 Promoter Activity -- Raw Data

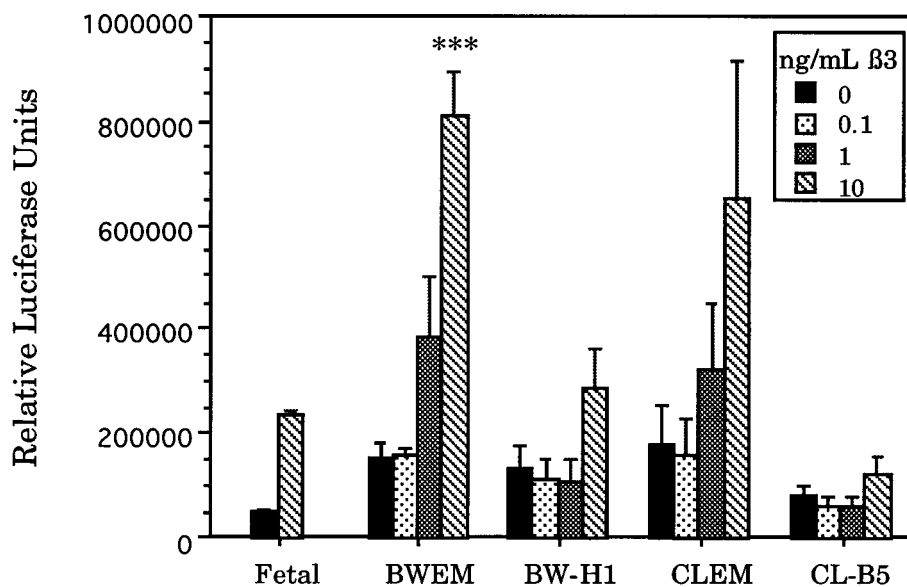


Figure 11: TGF- β_3 Induced Changes in Plasminogen Activator Inhibitor-1 (PAI-1) Promoter Activity

(A) p3TP-Lux reporter construct. This construct contains 3 TPA responsive elements (TRE) and the PAI-1 promoter region upstream of the luciferase gene.

(B & C) 36-hr. luciferase assays. 18 dpc primary fetal myocyte cultures (fetal), each parental line (BWEM and CLEM) and their respective mutant clones (BW-H1 and CL-B5) were transfected with the p3TP-Lux reporter plasmid and treated with 0-10 ng/mL of recombinant TGF- β_3 for 36 hours, after which luciferase activity was assessed as described Chapter 3. Luciferase data were normalized with β -galactosidase activity. Data for the cell lines represent the mean value from 3-4 individual assays and the error bars represent SEM. Data for the fetal cardiomyocytes represent the mean value from 2 individual assays and the error bars represent SD.

(B) Relative changes in activity are presented as fold increases when compared to untreated (0 ng/mL β_3) samples.

(C) Relative luciferase units are presented for each cell line to demonstrate individual expression levels in the different cell lines.

Significance: * $p \leq 0.05$, ** ≤ 0.01 , *** $p \leq 0.001$

Table 4: Statistical Analysis of Fetal and Parental PAI-1 Induction

Unpaired t-test was used to determine significance between results obtained from the different cell types in the PAI-1 promoter assay.

	p-value (RLU-0)	p-value (RLU-10)	p-value (fold induction)
Fetal vs. BWEM	0.052	0.012	0.320
Fetal vs. CLEM	0.345	0.354	0.424
BWEM vs. CLEM	0.805	0.592	0.090

Table 5: Statistical Analysis of PAI-1 Induction - Parental vs. Mutant
 One way ANOVA was used to determine significance between the PAI-1 induction for each parental cell line and its respective mutant at each dose of TGF- β_3 . Data are presented as mean \pm SEM.

	ng/mL	Parental*	Mutant*	p value
BW's	0.1	1.06 \pm 0.11	0.85 \pm 0.01	0.160
BW's	1.0	2.42 \pm 0.40	0.81 \pm 0.12	0.021
BW's	10	5.39 \pm 0.37	2.25 \pm 0.34	0.002
CL's	0.1	0.92 \pm 0.06	0.76 \pm 0.04	0.054
CL's	1.0	1.92 \pm 0.17	0.77 \pm 0.03	0.0002
CL's	10	3.93 \pm 0.63	1.64 \pm 0.17	0.006

*Fold Induction Above Control (0 ng/mL TGF- β_3 , control = 1.00)

Effects of TGF- β_3 on Gene and/or Protein Expression

Having established that the two mutant cell lines BW-H1 and CL-B5 are approximately 10-fold less responsive to TGF- β_3 -mediated changes in cellular proliferation and PAI-1 transcriptional induction, these mutant lines were used, alongside their respective parental lines and primary cultures of fetal cardiomyocytes, to identify the direct effects TGF- β may have on ventricular myocyte development and on their paracrine-mediated promotion of angiogenesis. To test the hypothesis that TGF- β promotes the maturation and differentiation of the cardiomyocyte during the perinatal transition period, the ability of TGF- β_3 to influence the expression, at pre-translational levels, of three contractile proteins, troponin C (TNC), troponin I (TNI), and troponin T (TNT), were determined. As maintaining receptor expression levels are important for TGF- β to mediate its effects during this differentiation process, TGF- β_3 -mediated changes in TGF- β receptor expression were also examined. Finally, the ability of TGF- β_3 to influence the production of the angiogenesis-related protein SPARC was examined to address the third and final hypothesis that TGF- β promotes angiogenesis in the ventricle .

Effect of TGF- β_3 on TNC Isoform Expression

As stated previously, two isoforms of TNC have been identified to date, skeletal (sTNC) and cardiac (cTNC). While sTNC has not been found in the heart at any stage, cTNC can be detected in the heart throughout

development. Unlike the other members of the troponin complex, no fetal isoform of cTNC has been identified. However, while expression of cTNC is low in the fetal heart, its transcript levels increase around birth, in concert with the increase in expression of the TGF- β ligands and β RII. Therefore, we hypothesized that TGF- β enhances the expression of cTNC in the late fetal cardiomyocyte by upregulating transcription from this gene.

Additionally, to verify that the myocyte-like cell lines have maintained their cardiac background and thus support their use as a model system, sTNC expression was examined in the lines as well as primary fetal cardiomyocytes. Therefore, expression of sTNC is expected to be low-to-undetectable in these cells. In contrast, cTNC transcripts are expected to be readily detected and increase significantly in response to TGF- β_3 in a dose dependent manner in each of the parental cell lines. Companion promoter assays are expected to reflect these changes. As the mutant cell lines were found to be 10-fold less responsive to TGF- β_3 in the transcriptional, PAI-1 promoter assay, it is expected that the induction of cTNC in these mutant lines would be significantly less than their respective parental lines.

The two isoforms of TNC, sTNC and cTNC, are derived from separate genes and are moderately homologous (~70%) to each other at the amino acid level (Parmacek et al., 1990). The divergence can be found in three separate domains and allows for discrimination between isoforms by Northern blot analyses using isoform-specific cDNA probes. Total RNA was collected from cultures of each cell type which were treated for 48-72 hours with TGF- β_3 and was used for Northern blot analyses. As expected,

TGF- β_3 treatment resulted in an increase in the steady state level of the 712 bp cTNC transcript in each of the cell lines, both parental and mutant, in a time and dose dependent manner (Figures 12 and 13). However, there appeared to be no difference in the level of induction at the transcript level between the parental lines and their respective mutant clones (Figure 12, Table 6). This was not expected as the mutants appeared to be 10-fold less sensitive to TGF- β_3 -mediated changes in transcription observed in the PAI-1 reporter assay (see Chap. 5 for further discussion). In all four cell lines, increases in steady state cTNC transcript levels were detected at 48 hours, and reached an approximate 2-2.5-fold induction after 72 hours of TGF- β_3 (10 ng/mL). It can also be noted that inherent differences in the expression levels of cTNC can be seen among the cell lines (Figure 13).

Table 6: Statistical Analysis of TGF- β_3 Dose Response Curves of cTNC Northern Blot Analyses

One way ANOVA was used to determine significance of the dose response curves of cTNC steady state transcript levels for each cell type. Two way ANOVA was used to determine differences between dose curves of each parental cell line and its respective mutant.

<u>Cell Type</u>	<u>p-value (48hr.)</u>	<u>p-value (72hr.)</u>
Fetal	0.229	0.023
BWEM	<0.0001	0.144
BW-H1	0.003	0.167
BW vs. BW-H1	0.863	0.749
CLEM	0.059	0.429
CL-B5	0.001	0.005
CL vs. CL-B5	0.034	0.966

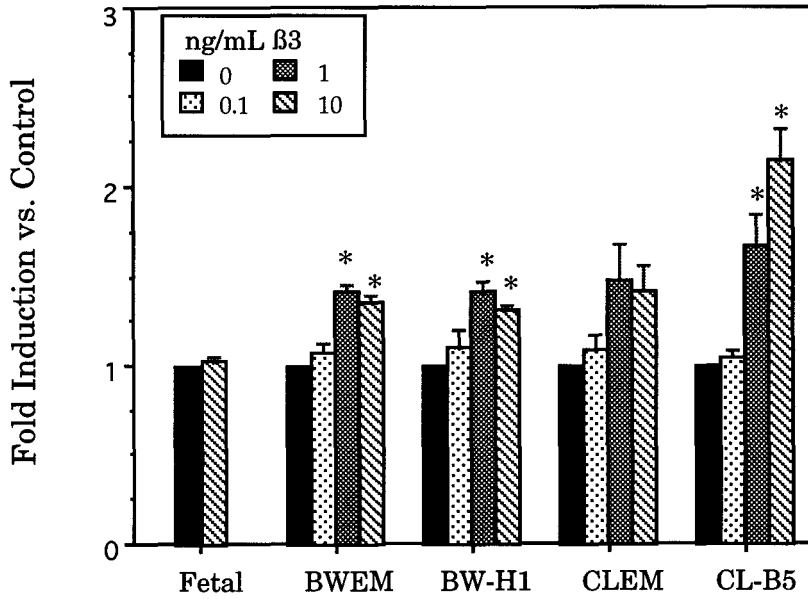
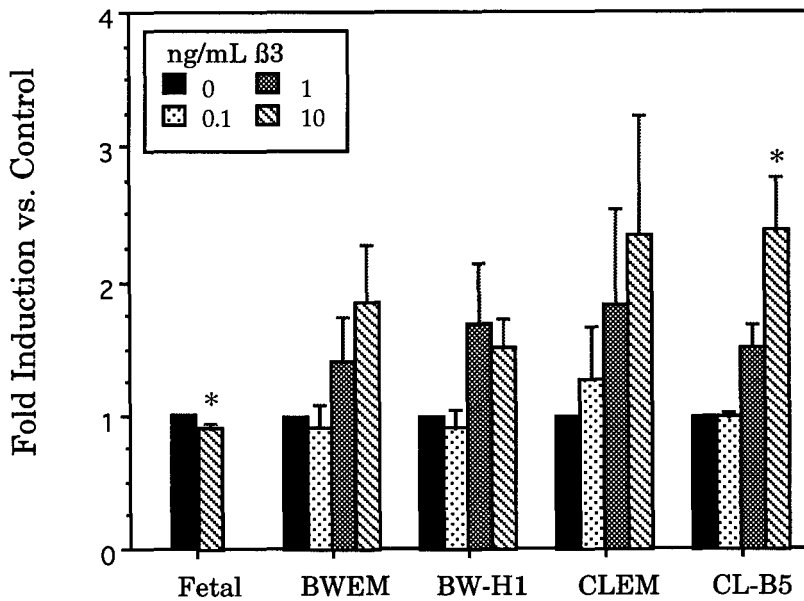
Figure 12: cTNC Northern Blot Analyses**A: 48 hour Steady State cTNC mRNA Levels****B: 72 hour Steady State cTNC mRNA Levels**

Figure 12: TGF- β_3 Induced Changes in Steady State cTNC Transcript Levels

Cultures of all four cell lines and primary cultures of 18 dpc ventricular myocytes were treated with 0-10 ng/mL TGF- β_3 for 48 (**A**) and 72 (**B**) hours. Northern blots were hybridized with a cDNA probe for cTNC. Fold induction of steady state transcript levels as compared to untreated controls is shown. Results shown are from 3-4 individual assays. Expression data were normalized to 18S rRNA oligo hybridizations. Error bars represent SEM.

Figure 13: Northern Blot Analyses of cTNC

Primary cultures of 18 dpc ventricular myocytes and cultures of each of the cell lines were treated with 0- 10 ng/mL TGF- β_3 for 48-72 hours. Northern blots were hybridized the cDNA probe for cTNC. Bars indicate 18S and 28S rRNA bands. Blots shown are from 24 hr. exposure to film at -80°C. 18S rRNA hybridizations shown below each blot.

(A) 18 dpc Fetal RNA. 3 μ g total RNA loaded for each sample.

Lane 1: 48 hr. Control	Lane 2: 48 hr. +TGF- β_3
Lane 3: 72 hr. Control	Lane 4: 72 hr. +TGF- β_3

(B) Total RNA from each of the cell lines treated with or without 10 ng/mL TGF- β_3 . 30 μ g loaded for each sample as indicated.

(C) CLEM and CL-B5 RNA. Representative blot of dose response to TGF- β_3 . 30 μ g total RNA loaded for each sample.

Lane 1: CLEM Control	Lane 2: CLEM 0.1 ng/mL β_3
Lane 3: CLEM 1 ng/mL β_3	Lane 4: CLEM 10 ng/mL β_3
Lane 5: CL-B5 Control	Lane 6: CL-B5 0.1 ng/mL β_3
Lane 7: CL-B5 1 ng/mL β_3	Lane 8: CL-B5 10 ng/mL β_3

To determine if these changes in cTNC transcript levels involved transcriptional upregulation, cTNC-CAT promoter assays were performed after treatment with TGF- β_3 for 48 (Figure 14) and 72 hours (data not shown). Based on the results obtained from the Northern blot analyses, we expected cTNC promoter activity to increase in response to TGF- β_3 . However, cTNC promoter activity in the parental lines significantly decreased by ~40-50% at both 48 and 72 hours in response to exogenous TGF- β_3 treatment (Figure 14, Table 7). Similar to the PAI-1 promoter assays, the negative transcriptional regulation of the cTNC promoter by TGF- β_3 appeared to be inhibited in the DN mutant lines as no significant difference in promoter activity was observed in BW-H1 and CL-B5 in response to TGF- β_3 (Figure 14, Table 7). However, similar to the Northern blot analyses, the cTNC promoter results obtained for the mutants and their respective parental lines were not significantly different (Table 7). These data suggest a complex, multi-level mechanism(s) wherein TGF- β regulates cTNC production in the cell lines by both positive and negative influences. However, these phenomena appear to be a characteristic of the cell lines as the changes in promoter activity and transcript levels in primary fetal cultures do not exhibit these same effects after TGF- β_3 treatment, having very little, or no response to exogenous TGF- β_3 (Figures 12 and 14).

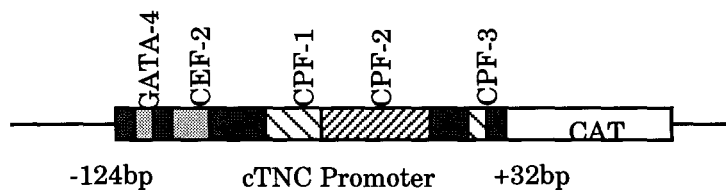
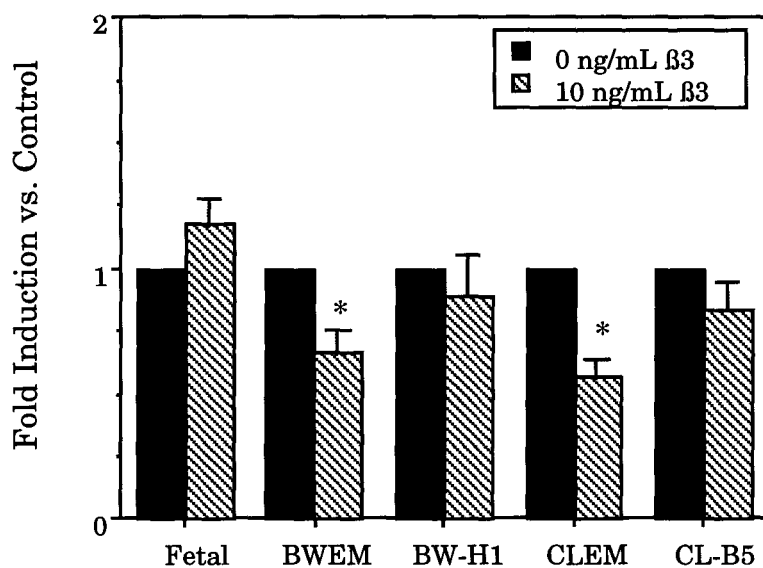
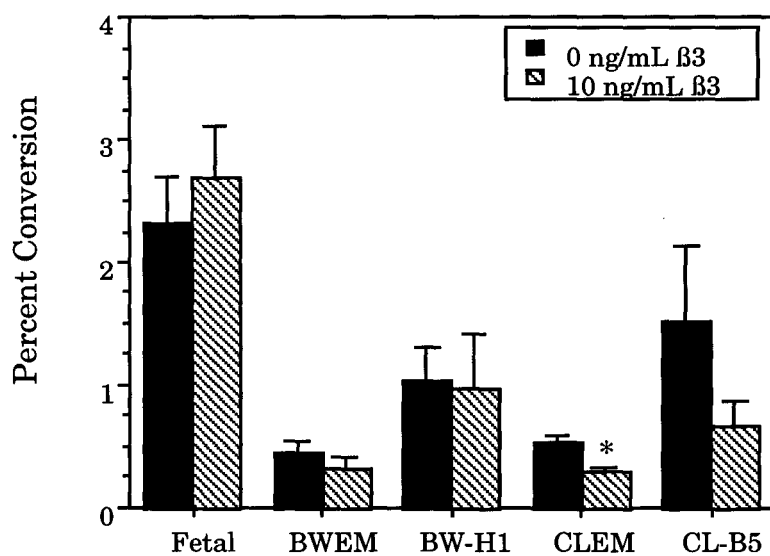
Figure 14: cTNC Promoter Assays**A: cTNC Promoter Construct****B: cTNC Promoter Activity -- Relative Changes****C: cTNC Promoter Activity -- Raw Data**

Figure 14: TGF- β_3 Induced Changes in Cardiac Troponin C (cTNC) Promoter Activity

(A) p-124SV0CAT reporter construct. This construct contains 124 bp of the cTNC 5' flanking region plus 32 bp of the first exon upstream of the CAT gene. Within this segment are 5 cardiac-specific binding elements: GATA-4 binding site, cardiac enhancer factor-2 (CEF-2), and three cardiac promoter factors (CPF-1-3).

(B & C) 48 hour CAT Assays. 18 dpc primary myocyte cultures (fetal), each parental line (BWEM and CLEM) and their respective mutant clones (BW-H1 and CL-B5) were transfected with the p-124SV0CAT reporter plasmid and treated with 0 or 10 ng/mL of recombinant TGF- β_3 for 48 hours. Samples of each were assessed for CAT activity as described in Chapter 3. Data were normalized with β -galactosidase activity. Data represent the mean value from 3 individual assays and the error bars represent SEM.

(B) Relative changes in activity are presented as fold increases when compared to untreated (0 ng/mL β_3).

(C) Raw data are presented as the per cent conversion of ^{14}C -chloramphenicol per 10 μg protein (fetal) or 150 μg protein (cell lines).

Significance: * $p \leq 0.05$, ** $p \leq 0.01$

Table 7: Statistical Analysis of cTNC Promoter Assays

Unpaired t-tests were used to determine significant differences between treated and untreated cultures and between each parental line and its respective mutant.

Cell Type	p-value (fold induction)	p-value (raw)
Fetal	0.534	0.823
BWEM	0.025	0.383
BW-H1	0.540	0.926
BWEM vs. BW-H1	0.282	0.205
CLEM	0.004	0.005
CL-B5	0.165	0.231
CLEM vs. CL-B5	0.101	0.186

To support the use of the BWEM and CLEM myocyte-like cell lines as a model system for cardiomyocyte development, experiments were performed to examine the effects of TGF- β on sTNC expression. As sTNC is not normally expressed in the cardiomyocyte, sTNC transcript levels and promoter activity are expected to be low to undetectable, and decrease in the presence of TGF- β_3 . As expected sTNC transcript levels were low, requiring extended exposure times for detection, but unexpectedly, the BWEM cell line exhibited significant upregulation in the 700 bp sTNC transcript levels after 72 hours exposure to TGF- β_3 (Figure 15, Table 8). The results obtained for the mutant BW-H1 line are significantly different from its parental BWEM line (Figure 15, Table 8). Similar to the Northern blot analyses, no significant changes were observed in sTNC promoter activity at 10 ng/mL TGF- β_3 in the primary fetal cardiomyocyte cultures and each cell line (Figure 16, Table 9). However, caution should be taken when interpreting the promoter data as basal activity in each cell type was exceedingly low. These nearly undetectable levels of sTNC transcripts and promoter activities are reflective of the fact that sTNC is not normally expressed in cardiac myocytes, and therefore supports the use of these cell lines as a cardiomyocyte model.

Table 8: Statistical Analysis of TGF- β_3 Dose Response Curves of sTNC Northern Blot Analyses

One way ANOVA was used to determine significance of the dose response curves of sTNC steady state transcript levels for each cell type. Two way ANOVA was used to determine differences between dose curves of each parental cell line and its respective mutant.

<u>Cell Type</u>	<u>p-value (48hr.)</u>	<u>p-value (72hr.)</u>
BWEM	0.271	0.012
BW-H1	0.671	0.161
BW vs. BW-H1	0.506	0.006
CLEM	0.001	0.266
CL-B5	0.127	0.651
CL vs. CL-B5	0.071	0.442

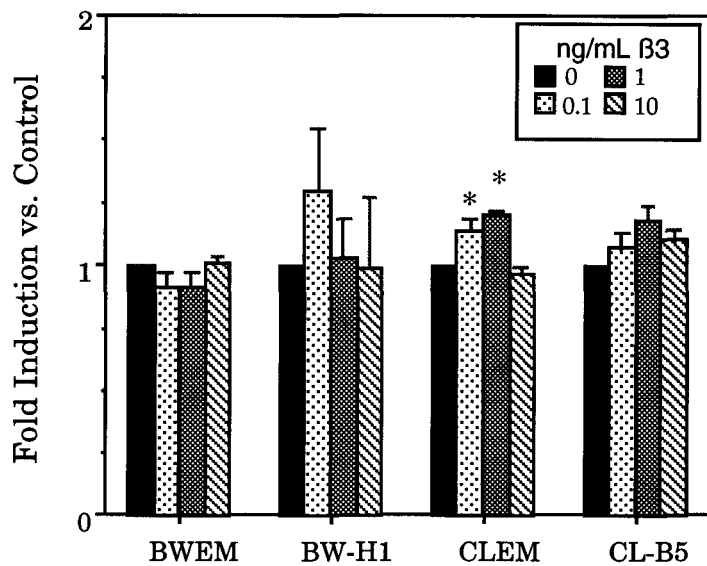
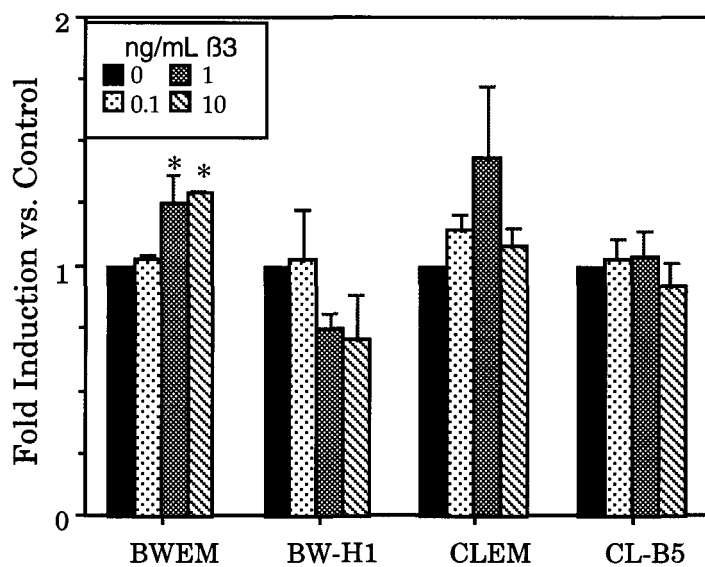
Figure 15: sTNC Northern Blot Analyses**A: 48 hour Steady State sTNC mRNA Levels****B: 72 hour Steady State sTNC mRNA Levels**

Figure 15: TGF- β_3 Induced Changes in Steady State sTNC Transcript Levels

Cultures of all four cell lines were treated with 0-10 ng/mL TGF- β_3 for 48(A) or 72 (B) hours. Northern blots were hybridized with a cDNA probe specific for sTNC. Data are presented as fold induction of transcript levels as compared to untreated controls. Results shown are from 3 individual assays. Data were normalized to 18S rRNA oligo hybridizations. Error bars represent SEM.

* indicate responses significantly different from control (0 ng/mL β_3)

Table 9: Statistical Analysis of sTNC Promoter Assays

Unpaired t-tests were used to determine significant differences between untreated and treated samples for each cell line.

<u>Cell Type</u>	<u>p-value (fold induction)</u>	<u>p-value (raw)</u>
Fetal	0.164	0.811
BWEM	0.928	0.976
BW-H1	0.015	0.154
CLEM	0.403	0.366
CL-B5	0.793	0.547

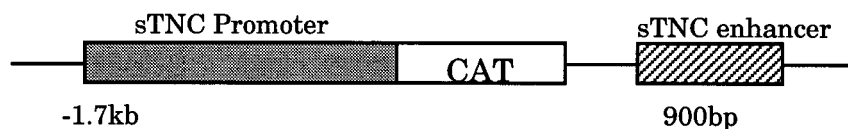
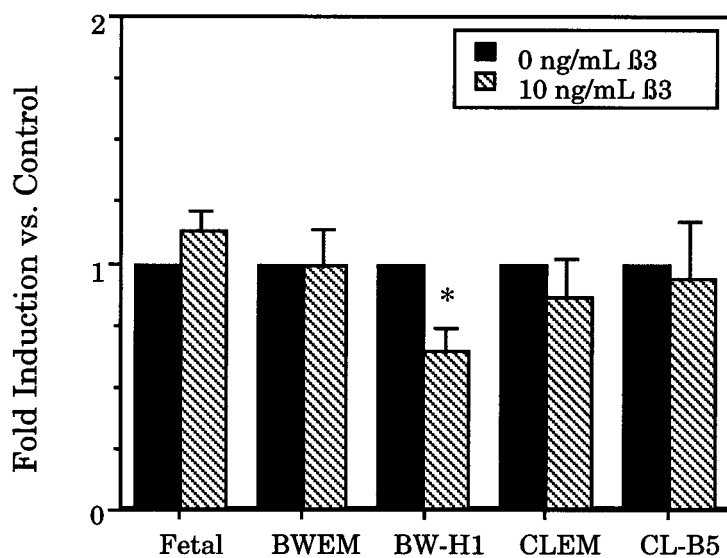
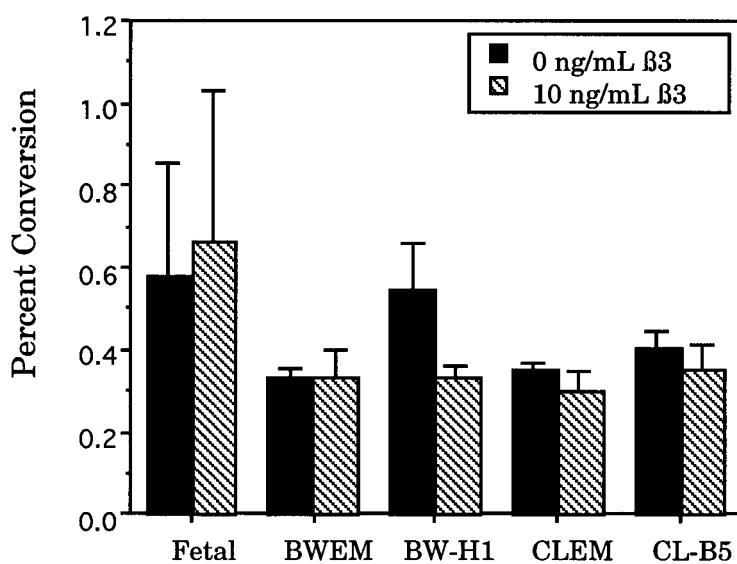
Figure 16: sTNC Promoter Assays**A: sTNC Promoter Construct****B: sTNC Promoter Activity -- Relative Changes****C: sTNC Promoter Activity -- Raw Data**

Figure 16: TGF- β_3 -Induced Changes in Skeletal Troponin C (sTNC) Promoter Activity

(A) 1.7/900-CAT reporter construct. This construct contains 1.7 kb of the sTNC promoter inserted in front of the CAT gene and a 900 bp enhancer element found within the first intron inserted downstream of the CAT gene.

(B & C) 48 hour CAT Assays. 18 dpc primary myocyte cultures (fetal), each parental line (BWEM and CLEM) and their respective mutant clones (BW-H1 and CL-B5) were transfected with the 1.7/900-CAT reporter construct and treated with 0 or 10 ng/mL of recombinant TGF- β_3 for 48 hours. Samples of each were assayed for CAT activity as described in Chapter 3. Data were normalized with β -galactosidase activity. Data represent the mean value from 3 individual assays and the error bars represent SEM.

(B) Relative changes in activity are presented as fold increases when compared to untreated (0 ng/mL β_3) samples.

(C) Raw data are presented as the per cent conversion of ^{14}C -chloramphenicol per 10 μg protein (fetal) or 150 μg protein (cell lines).

* indicates response significantly different from control (0 ng/mL β_3)

Effect of TGF- β_3 on TNI Isoform Expression

Unlike TNC, the two TNI isoforms, skeletal (sTNI) and cardiac (cTNI), are present in the heart at distinct times during development. The transition from sTNI expression to cTNI expression occurs during the perinatal transition period. Therefore, we hypothesized TGF- β promotes this sTNI-cTNI transition, and thus the maturation and differentiation of the cardiomyocyte, through the upregulation of transcription of the adult isoform, cTNI, and downregulation of the fetal isoform, sTNI. To test this hypothesis, sTNI promoter assays and Northern blot analyses of sTNI and cTNI transcript levels were performed.

Similar to the TNCs, the two TNI isoforms, sTNI and cTNI, are derived from separate genes with distinct regulatory sequences within their respective promoters. While a promoter construct was not available for cTNI, a luciferase-based construct containing the 4.2 kb 5' flanking region and exon 1 of the sTNI gene (see Figure 17a) was used to assay the effects of TGF- β_3 on sTNI expression. In support of the hypothesis, TGF- β_3 (up to 10 ng/mL) caused a small, but significant decrease in sTNI promoter activity in primary fetal cardiomyocytes, but not in any of the cell lines (Figure 17b, Table 10). As previously seen with the growth assays, inherent differences can be seen in basal promoter activity between the BWEM and CLEM parental cell lines and their respective DN clones (Figure 17c). To assess the relationship of these data to a more *in vivo*-like system, cDNA probes were available and utilized to determine steady state sTNI and cTNI

transcript levels in 18 dpc primary fetal cardiomyocyte cultures treated with 10 ng/mL TGF- β_3 for 48-72 hours. In contrast to what was observed in the promoter assays, TGF- β_3 significantly ($p = 0.027$) increased the 1.05 kb sTNI transcript level after 72 hours (Figure 18, Table 11). Additionally, TGF- β_3 significantly ($p \leq 0.001$) decreased the cTNI (1.2 kb) transcript levels in the fetal cultures to ~25% of the control levels after 72 hours of treatment (Figure 18). This would suggest that sTNI would become more abundant than cTNI after exposure to TGF- β_3 , possibly resulting in a change in Ca⁺⁺ affinity in the troponin complex. This conclusion would be tentative as Northern blots demonstrating sTNI expression used more RNA and required a longer exposure time (see Figure 18 legend). Nevertheless, these results suggest that TGF- β_3 does not promote cardiomyocyte maturation and differentiation with regards to TNI isoform expression.

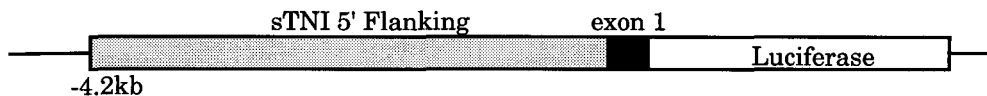
Table 10: Statistical Analysis of TGF- β_3 Dose Response Curves of sTNI Promoter Assays

One way ANOVA was used to determine significance of the dose response curves of sTNI promoter activity in response to TGF- β_3 for each cell type.

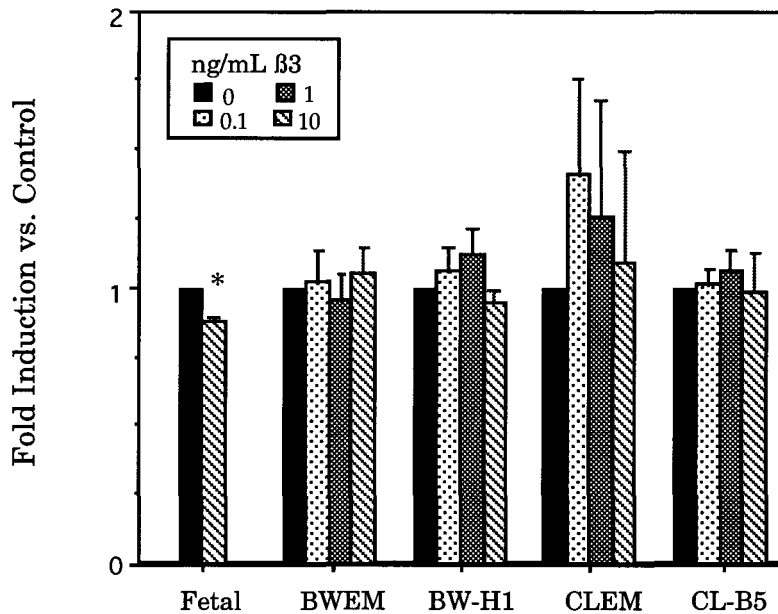
Cell Type	p-value (fold induction)	p-value (raw)
Fetal	0.003	0.394
BWEM	0.849	0.986
BW-H1	0.262	0.961
CLEM	0.823	0.604
CL-B5	0.933	0.974

Figure 17: sTNI Promoter Assays

A: sTNI Reporter Construct



B: Promoter Activity -- Relative Changes



C: Promoter Activity -- Raw Data

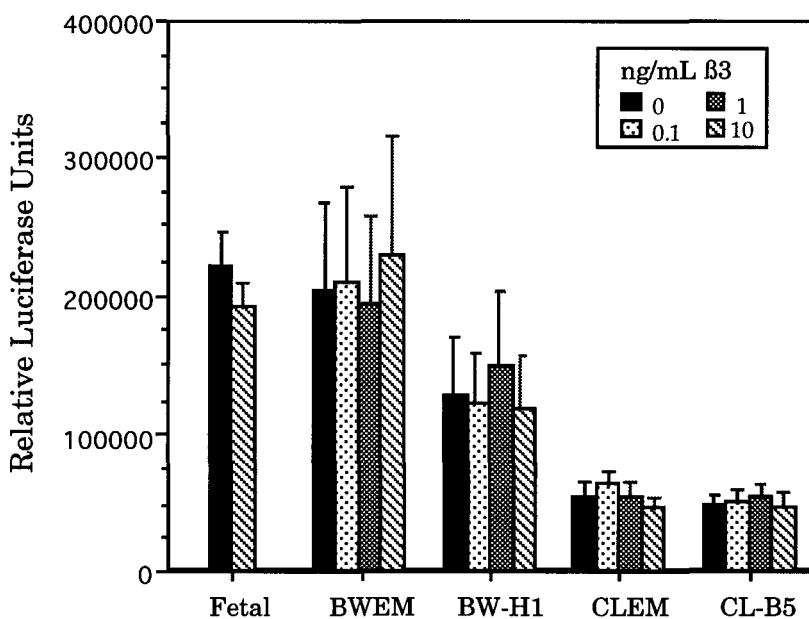


Figure 17: TGF- β_3 Induced Changes in Skeletal Troponin I (sTNI) Promoter Activity

(A) BW147 reporter construct. This construct contains 4.2 kb of the 5' flanking region of the sTNI gene as well as the first exon inserted in front of the luciferase gene.

(B & C) 36 hour luciferase assays. 18 dpc primary fetal myocyte cultures, each parental line (BWEM and CLEM) and their respective mutant clones (BW-H1 and CL-B5) were transfected with the BW147 reporter plasmid and treated with 0-10 ng/mL of recombinant TGF- β_3 for 36 hours. Each sample was collected and luciferase activity was assessed as described in Chapter 3. Luciferase data were normalized with β -galactosidase activity. Data represent the mean value from 3 (fetal) or 5-6 (cell lines) individual assays and the error bars represent SEM.

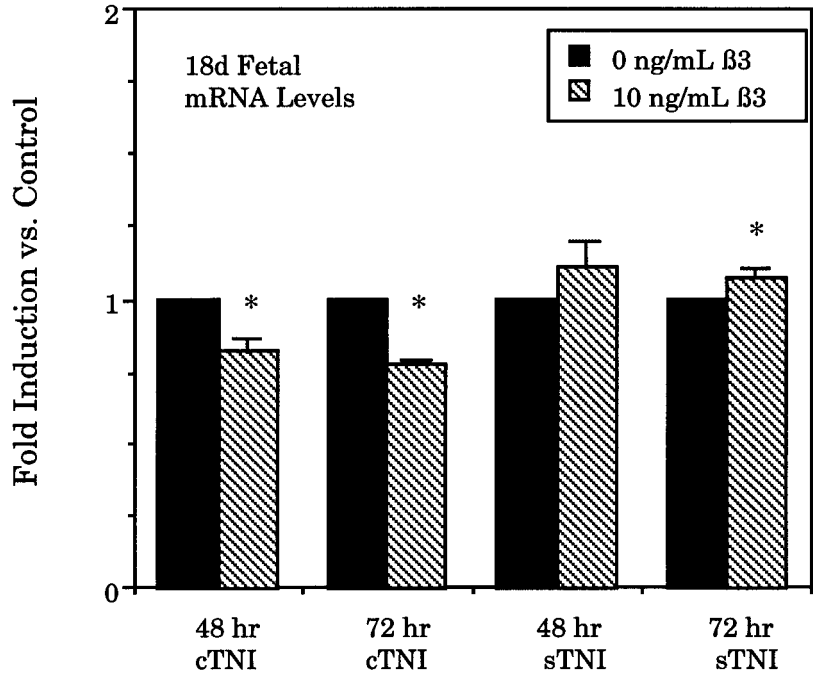
(B) Relative changes in activity are presented as fold increases when compared to untreated (0 ng/mL β_3) samples.

(C) Relative luciferase units are presented for each cell line to demonstrate individual expression levels in the different cell types.

* indicates response significantly different from control (0 ng/mL β_3)

Figure 18: TNI Northern Blot Analyses

A: Steady State mRNA Levels -- Relative Changes



B: Steady State mRNA Levels -- Raw Data

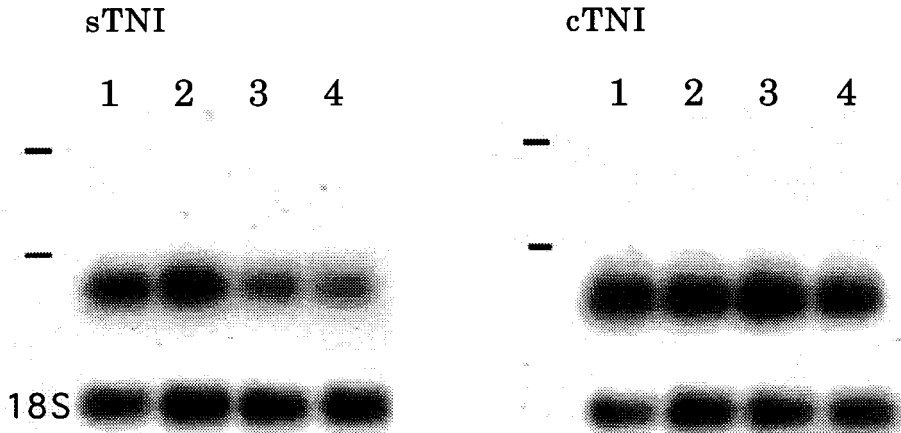


Figure 18: TGF- β_3 Induced Changes in Steady State Skeletal (sTNI) and Cardiac (cTNI) TNI Transcript Levels

Primary cultures of 18 dpc fetal ventricular myocytes were treated with 0 or 10 ng/mL TGF- β_3 for 48-72 hours. Northern blots were hybridized with cDNA probes for cardiac TNI (cTNI) and skeletal TNI (sTNI).

(A) Fold induction of transcript levels compared to untreated controls. Results shown are from 3 individual assays. Data were normalized to 18S rRNA oligo hybridizations. Error bars represent SEM.

* indicates response significantly different from control (0 ng/mL β_3)

(B) Representative Northern blots of each TNI isoform. 5 μ g (sTNI) and 3 μ g (cTNI) total RNA loaded for each sample. Bars indicate 18S and 28S rRNA bands. Blots shown are from 3 day (sTNI) and 24 hr (cTNI) exposures to film at -80°C. 18S rRNA hybridization shown below each blot.

Lane 1: 48 hr. Control

Lane 2: 48 hr. +TGF- β_3

Lane 3: 72 hr. Control

Lane 4: 72 hr. +TGF- β_3

Table 11: Statistical Analysis of TNI Northern Blot Analyses

Unpaired t-tests were used to determine statistical difference between control and treated (10 ng/mL TGF- β_3) 18 day fetal primary myocyte cultures.

<u>Time/Probe</u>	<u>p-value</u>
48hr. cTNI	0.018
72hr. cTNI	<0.001
48hr. sTNI	0.281
72hr. sTNI	0.027

Effect of TGF- β_3 on TNT Isoform Expression

In contrast to TNC and TNI, the two cardiac TNT (cTNT) isoforms, fetal and adult, are products of the same gene and are produced by the alternative splicing of a single 30 bp exon (exon 4 in the rat). This exon is present in the 1.14 kb transcript for the fetal isoform of cTNT, and subsequently absent in the adult isoform. While TGF- β has been shown to affect transcriptional and translational activity as well as mRNA stability in several different systems, no reports have been made on possible effects the TGF- β s could have on alternative splicing, and thus the cTNT isoform switch. Therefore to address TGF- β 's role in cardiomyocyte differentiation in regards to the troponin complex, the effects of TGF- β_3 on cTNT isoform expression were evaluated in primary fetal cardiomyocytes and the four cell lines. As with the other two troponin proteins, we hypothesized that TGF- β would increase the transcript levels of the adult isoform of cTNT, and therefore decrease the level of the fetal isoform.

While promoter analysis is not indicative of cTNT isoform switching, assays were performed not only to determine if TGF- β_3 has an effect on overall transcriptional activity from the cTNT gene, but also to verify the use of the myocyte-like cell lines as a model for cardiomyocyte development. As transcriptional activity is not expected to influence the alternative splicing of the cTNT hnRNA, TGF- β_3 is not expected to cause a significant change in cTNT gene transcription. However, TGF- β_3 (10 ng/mL) appeared to downregulate transcription from this promoter in the parental cell lines,

but not in the primary fetal cardiomyocytes or mutant cell lines (Figure 19b, Table 12). It should be noted that the promoter activity in the fetal cardiomyocytes was significantly ($p = 0.001$) higher than in any of the cell lines (Figure 19c).

To examine the effect TGF- β_3 has on cTNT isoform expression, Northern blot analyses were performed to detect relative changes in cTNT isoform production as the result of alternative splicing. We have hypothesized that the role for TGF- β in cardiomyocyte differentiation is to upregulate the adult isoform of each type of troponin. While the effect on TNC and TNI expression would theoretically be a direct regulation of transcriptional activity, the regulation of cTNT splicing could be regarded as an indirect effect. Nevertheless, we expect that treatment of the cells with TGF- β_3 will result in the accumulation of the adult isoform of cTNT.

Northern blot analyses were performed using oligonucleotide probes which detected either total cTNT transcript levels or just the fetal isoform. As expected from results obtained from Northern blots of ventricular tissue, cTNT transcripts were readily detected in total RNA from primary fetal myocyte cultures (Figure 20b). Consistent with the promoter assay data, 10 ng/mL TGF- β_3 had no significant effect on the level of total cTNT transcripts in primary fetal myocytes after 48 and 72 hours (Figure 20a, Table 13). Using an oligonucleotide probe corresponding to the alternatively spliced exon present only in the fetal isoform, the effect of TGF- β_3 on its steady state transcript levels were determined. The results indicate TGF- β_3

(10 ng/mL) did not significantly alter the expression of the fetal isoform after 48 and 72 hours of treatment (Figure 20a, Table 13). Reflective of the significantly lower promoter activity in the cell lines as compared to the fetal cardiomyocytes, Northern blot analyses on the cell lines proved limiting as neither cTNT isoform could be detected in the cell lines when using 30 µg total RNA, but could be detected if PolyA⁺ RNA was used (data not shown). These results indicate that TGF-β₃ does not affect the alternative splicing or accumulation of the adult isoform of cTNT in the fetal cardiomyocyte as originally hypothesized. Additionally, these data and the data obtained from the TNI studies indicate that there appears to be significant limitations on the use of the BWEM and CLEM myocyte-like cell lines for the study of cardiomyocyte development (see discussion, Chapter 5).

Table 12: Statistical Analysis of TNT Promoter Assays

Unpaired t-tests were used to determine significant differences between control and treated (10 ng/mL TGF-β₃) cultures as well as between BWEM and fetal cells.

<u>Cell Type</u>	<u>p-value (fold induction)</u>	<u>p-value (raw)</u>
Fetal	0.163	0.474
Fetal vs. BWEM	N.D.*	0.001
BWEM	0.004	0.368
BW-H1	0.721	0.727
CLEM	0.026	0.629
CL-B5	0.052	0.537

* N.D. = not determined

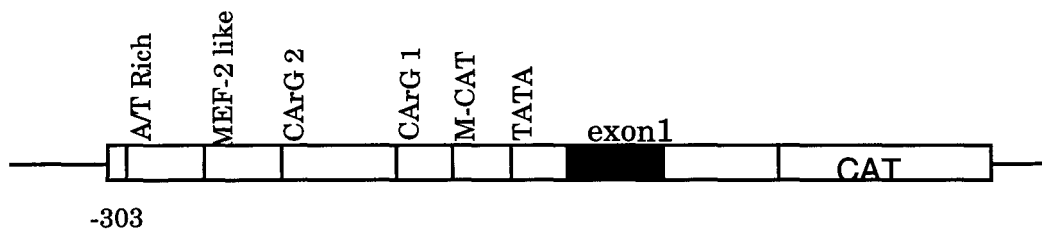
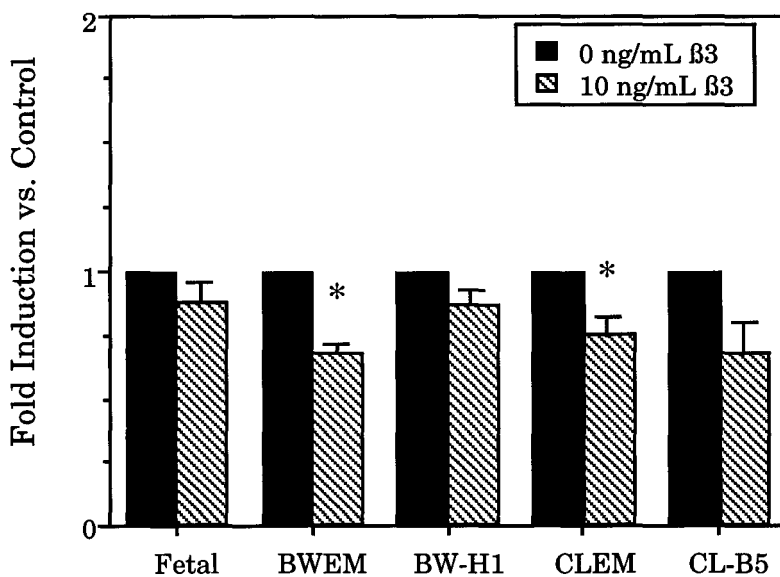
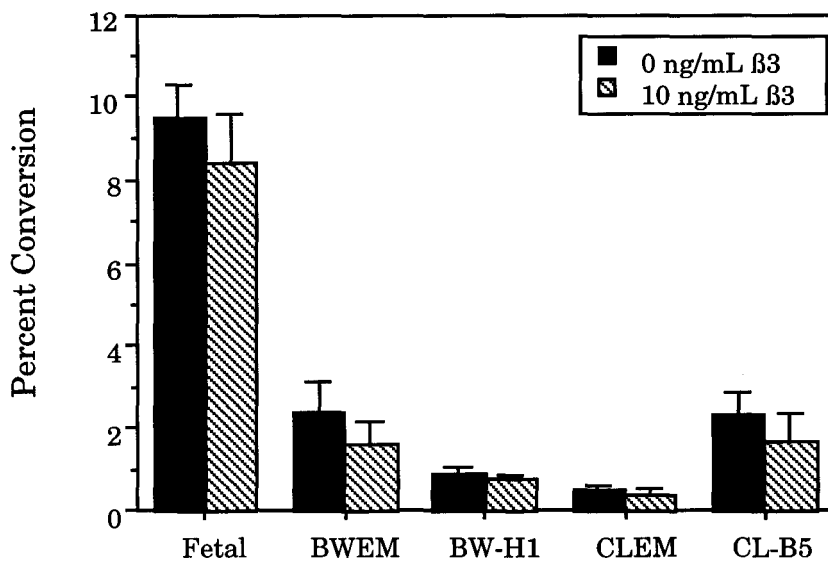
Figure 19: cTNT Promoter Assays**A: TNT Reporter Construct****B: Promoter Activity -- Relative Changes****C: Promoter Activity -- Raw Data**

Figure 19: TGF- β_3 Induced Changes in Cardiac Troponin T (cTNT) Promoter Activity

(A) 303-CAT reporter construct. This construct contains 303 bp of the cTNT promoter inserted upstream of the CAT gene. As noted, this promoter contains several known muscle-specific transcription factor binding sites (CArG, MEF-2, M-CAT).

(B & C) 48 hour CAT assays. 18 dpc fetal primary myocyte cultures, each parental line (BWEM and CLEM) and their respective mutant clones (BW-H1 and CL-B5) were transfected with the 303-CAT reporter plasmid and treated with 0 or 10 ng/mL of recombinant TGF- β_3 for 48 hours. Samples were collected and assayed for CAT activity as described in Chapter 3. Data were normalized with β -galactosidase activity. Data represent the mean value from 3 individual assays and the error bars represent SEM.

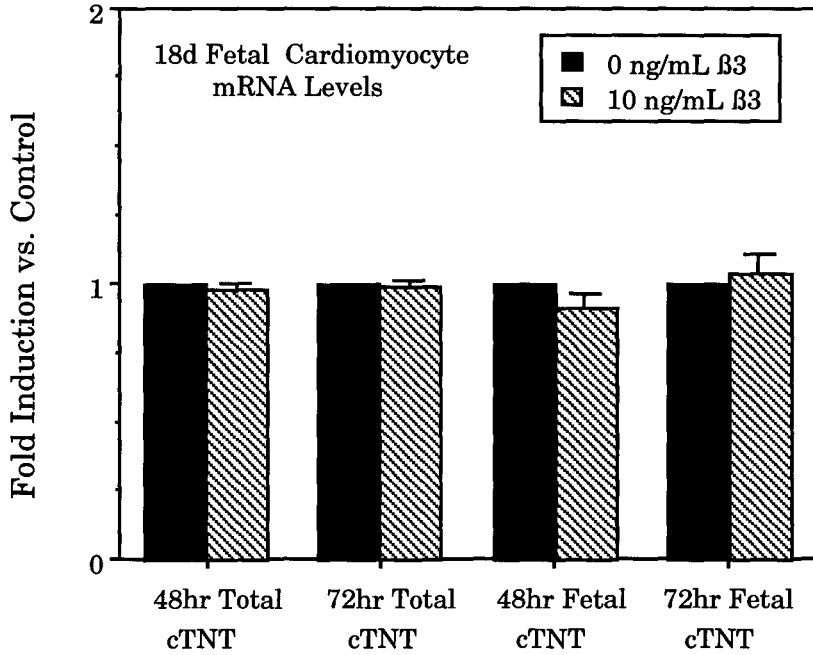
(B) Relative changes in activity are presented as fold increases when compared to untreated (0 ng/mL β_3).

(C) Raw data are presented as the per cent conversion of ^{14}C -chloramphenicol per 10 μg protein (fetal) or 150 μg protein (cell lines).

* indicates response significantly different from control (0 ng/mL β_3)

Figure 20: cTNT Northern Blot Analyses

A: Steady State mRNA Levels -- Relative Changes



B: Steady State mRNA Levels -- Raw Data

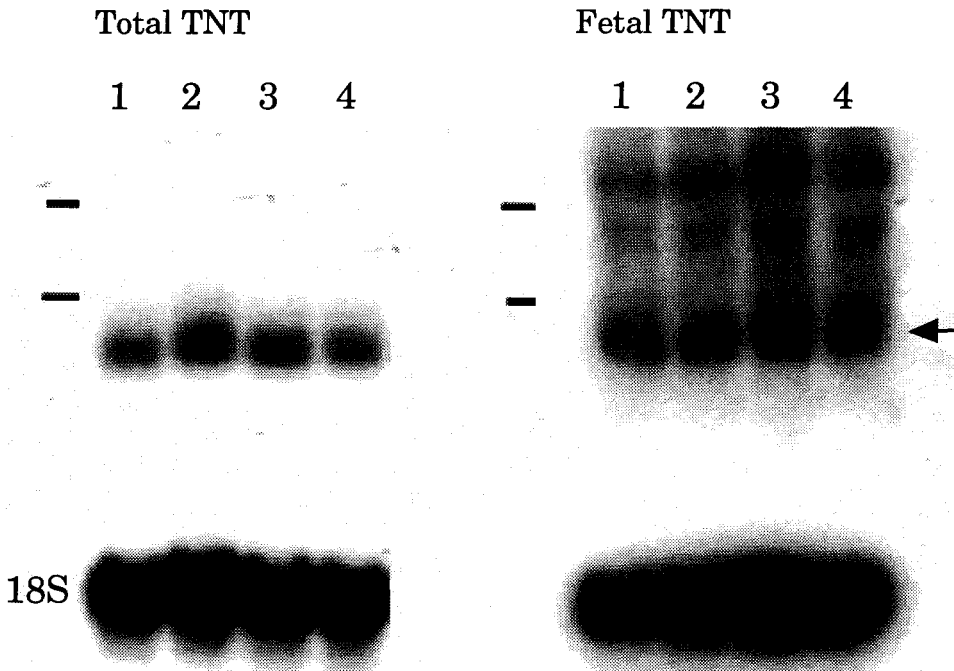


Figure 20: TGF- β_3 Induced Changes in cTNT Steady State Transcript Levels

Primary cultures of 18 dpc fetal ventricular myocytes were treated with 0 or 10 ng/mL TGF- β_3 for 48-72 hours. Northern blots were hybridized with the oligonucleotide probes, CCAR (detects adult and fetal isoforms) and PE4 (detects only fetal isoform).

(A) Fold induction of transcript levels compared to untreated controls. Results shown are from 3 individual assays. Data were normalized to 18S rRNA oligo hybridizations. Error bars represent SEM.

(B) Representative Northern blots of each cTNT isoform. 8 μ g total RNA loaded for each sample. Arrow indicates fetal cTNT band. Bars indicate 18S and 28S rRNA bands. Blots shown are from 4 day exposure to film at -80°C. 18S rRNA hybridizations shown below each blot.

Lane 1: 48 hr. Control
Lane 3: 72 hr. Control

Lane 2: 48 hr. +TGF- β_3
Lane 4: 72 hr. +TGF- β_3

Table 13: Statistical Analysis of TNT Northern Blot Analyses

Unpaired t-tests were used to determine the statistical difference between control and treated (10 ng/mL TGF- β_3) primary 18-day fetal myocyte cultures.

<u>Time/Probe</u>	<u>p-value</u>
48hr. Total TNT	0.248
72hr. Total TNT	0.561
48hr. Fetal TNT	0.129
72hr. Fetal TNT	0.563

Changes in TGF- β receptor expression in response to TGF- β_3

The presence of biologically active ligand and β RII appear to be rate limiting factors for the TGF- β signaling processes to function within the ventricle during the fetal development period (Engelmann et al., 1992; Engelmann 1993, Engelmann and Grutkoski, 1994). As the TGF- β ligands, TGF- β_1 and - β_3 , and β RII begin to be expressed at approximately the same time and increase dramatically at or around birth, the regulation of one or several of these “limiting” components of the TGF- β signaling pathway by TGF- β itself would augment its role in the developmental process. Therefore, changes in TGF- β receptor expression in response to TGF- β_3 treatment were determined in the five different cell types. As β RI appears to be constitutively expressed, its expression level is not expected to be affected by the presence of TGF- β_3 . In contrast, the dramatic increase in β RII expression suggests that its expression may be induced by TGF- β in a pseudo-autoregulatory loop similar to that seen for the ligand TGF- β_1 (Kim et al., 1990).

In support of the developmental data, β RI transcript levels were not significantly affected by TGF- β_3 treatment after 72 hours (Figure 21a, Table 14). However in contrast to that suggested by the developmental data, 72 hours of continuous TGF- β_3 treatment resulted in a significant ($p < 0.05$), dose-dependent decrease in β RII transcript levels in the BW-H1 and CLEM cell lines (Figures 21b and 22, Table 14), with a 25-40% decrease relative to untreated cultures at a dose of 10 ng/mL TGF- β_3 . While there were no

significant differences between BWEM and BW-H1 cell lines, the results obtained for CLEM and CL-B5 were significantly ($p = 0.013$) different. These differences between the two DN lines is reflective of the changes observed in the growth assays, with BW-H1 having overcome the DN effect at 10 ng/mL TGF- β_3 while CL-B5 remained minimally unresponsive to TGF- β_3 treatment. As the antiproliferative effect of TGF- β has been uncoupled from its transcriptional effects, these results suggest that the regulation of β R_{II} by TGF- β_3 is indirect and may share or have similar regulatory pathways as those used to block proliferation. Prolonged exposure to TGF- β_3 was necessary as a significant decrease in transcript levels was not observed after only 48 hours of exposure (data not shown). In contrast to the cell lines, primary fetal cardiomyocytes exhibited a more modest, non-significant decrease in β R_{II} levels after 72 hours of TGF- β_3 (10 ng/mL) treatment (Figure 21b, Table 14), which may be reflective of the trend seen in these studies in which they appear less responsive to TGF- β_3 than the cell lines (see discussion, Chapter 5).

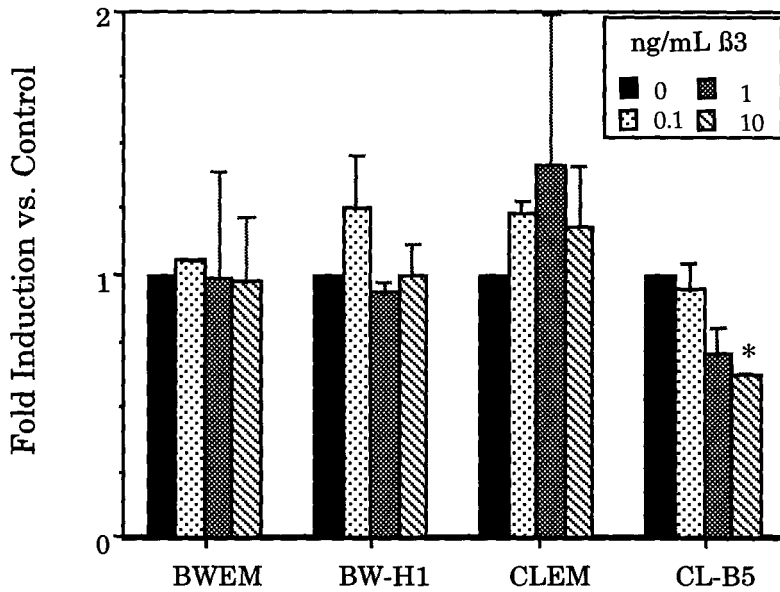
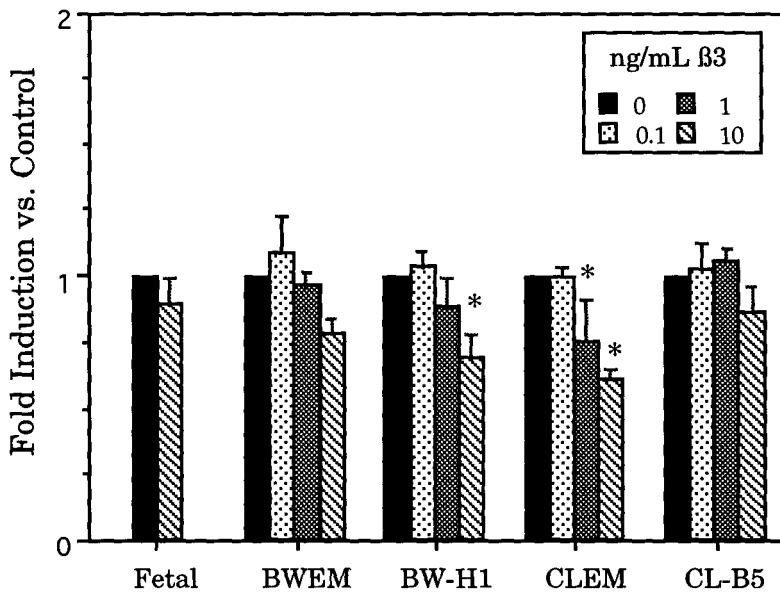
Figure 21: TGF- β Receptor Northern Blot Analyses**A: 72 hour Steady State β RI mRNA Levels****B: 72 hour Steady State β RII mRNA Levels**

Figure 21: TGF- β_3 Induced Changes in Steady State Type I and Type II TGF- β Receptor Transcript Levels

Cultures of all four cell lines and primary cultures of 18 dpc fetal ventricular myocytes were treated with 0-10 ng/mL TGF- β_3 for 72 hours. Northern blots analyses were performed and data were normalized to 18S rRNA oligo hybridizations. Data are presented as fold induction of transcript levels as compared to untreated controls.

(A) β RI. Blots were hybridized with the cDNA probe for rat β RI (R4). Results shown are from 2 individual assays. Data error bars represent SD.

(B) β RII. Blots were hybridized with the cDNA probe for rat β RII. Results shown are from 3-4 individual assays. Data error bars represent SEM.

* indicates response significantly different from control (0 ng/mL β_3)

Table 14: Statistical Analysis of TGF- β_3 Dose Response Curves of TGF- β Receptor Northern Blot Analyses

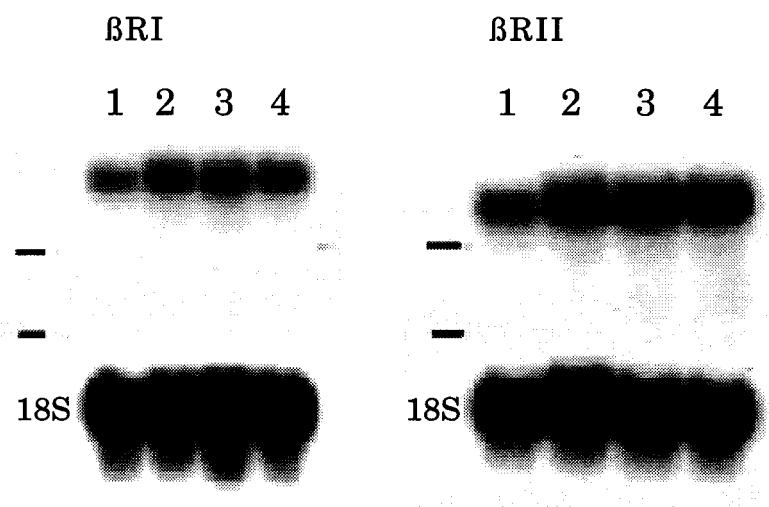
One way ANOVA was used to determine significance of the dose response curves of TGF- β receptor steady state transcript levels for each cell type. Two way ANOVA was used to determine significance differences between the dose curves of each parental line and its respective mutant.

<u>Cell Type</u>	<u>p-value (βRI)</u>	<u>p-value (βRII)</u>
Fetal	N.D.*	0.319
BWEM	0.995	0.066
BW-H1	0.350	0.030
BW vs. BW-H1	0.907	0.816
CLEM	0.824	0.001
CL-B5	0.041	0.132
CL vs. CL-B5	0.446	0.013

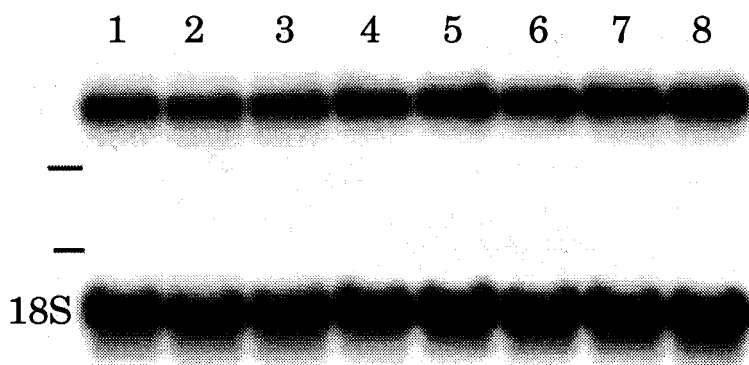
* N.D. = Not Determined

Figure 22: Northern Blots of TGF- β Receptors

A: 18 day Fetal Cardiomyocyte Northern Blots



B: BWEM and BW-H1 β RI Northern Blot



C: β RII changes in all four cell lines \pm 10 ng/mL β_3 :

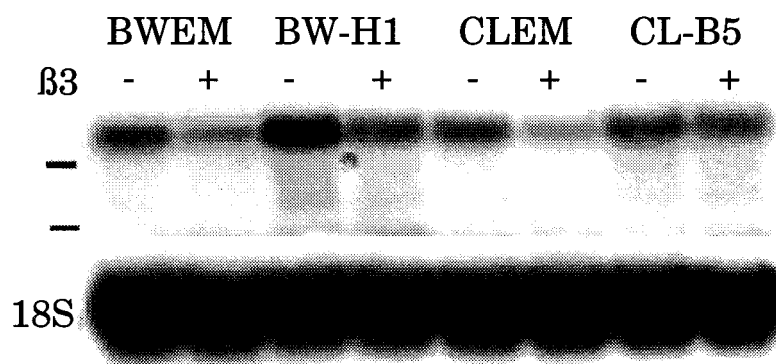


Figure 22: Northern Blots of TGF- β Receptors

Primary cultures of 18 dpc fetal ventricular myocytes and cultures of each of the cell lines were treated with 0-10 ng/mL TGF- β_3 for 48-72 hours. Northern blots were hybridized with the cDNA probe for β RI (R4) or β RII. Bars indicate 18S and 28S rRNA bands. 18S rRNA hybridizations shown below each blot.

(A) 18 dpc Fetal RNA. 3 μ g total RNA loaded for each sample.

Results shown are from 48 hr. exposure to film at -80°C .

Lane 1: 48 hr. Control

Lane 2: 48 hr. + 10 ng/mL TGF- β_3

Lane 3: 72 hr. Control

Lane 4: 72 hr. + 10 ng/mL TGF- β_3

(B) TGF- β RI expression. BWEM and BW-H1 RNA. Representative dose response curve for β RI transcript expression. 30 μ g total RNA loaded for each sample. Results shown are from 48 hr. exposure to film at -80°C .

Lane 1: BWEM Control

Lane 2: BWEM 0.1 ng/mL β_3

Lane 3: BWEM 1 ng/mL β_3

Lane 4: BWEM 10 ng/mL β_3

Lane 5: BW-H1 Control

Lane 6: BW-H1 0.1 ng/mL β_3

Lane 7: BW-H1 1 ng/mL β_3

Lane 8: BW-H1 10 ng/mL β_3

(C) TGF- β RII expression. Total RNA from each of the cell lines treated with or without 10 ng/mL TGF- β_3 . 30 μ g loaded for each sample as indicated. Results shown are from 24 hr. exposure to film at -80°C .

Changes in SPARC expression in response to TGF- β_3

The formation of new blood vessels, or angiogenesis, is a crucial part of the ventricular remodeling which occurs during the perinatal transition period. We have hypothesized that TGF- β promotes this angiogenesis through the induction of the angiogenic factor, SPARC. As TGF- β has been shown to induce SPARC both transcriptionally and translationally, we have chosen to examine the effect of TGF- β_3 on SPARC transcript and protein production. While we expect the end result to be a significant increase in SPARC protein production, it is difficult to predict *a priori* whether that increase is the result of increased transcript production, increased translation from existing transcripts, or both.

To examine the effect TGF- β may have on SPARC transcript levels, we examined changes in SPARC promoter activity and steady state transcript levels after treatment with TGF- β_3 for 48 hours. Using a promoter construct containing 639 bp of the 5' flanking region of the SPARC promoter plus 11 bp of exon 1, transcriptional activity was determined in primary fetal myocyte cultures and each of the cell lines in response to 0 and 10 ng/mL TGF- β_3 (Figure 23). Interestingly, the results appeared to be cell specific. A modest, but significant, increase of ~20% in promoter activity was detected in the fetal myocyte cultures, while a significant decrease was detected in CLEM (~30%) cultures (Figure 23a, Table 15). As a direct result of this, the only significant differences between a parental line and its respective mutant was between CLEM and CL-B5. Additionally, basal

promoter activity in each of the mutant lines appeared higher than their respective parental lines, however, this difference was only significant for the CLEM and CL-B5 cell lines (Figure 23b, Table 15). In contrast to the promoter data, a dose-dependent increase of ~25-40% was observed in the steady state ~2.2 kb transcript levels in the BWEM and fetal myocyte cultures (Figures 24 and 25, Table 16).

Since the changes in SPARC transcript levels are minimal, possible changes in translation rates from existing SPARC transcripts in response to TGF- β_3 were examined. As SPARC is readily secreted by cells in culture after translation, proteins obtained from media conditioned by the cells for 48 hours \pm 10 ng/mL TGF- β_3 were used for Western blot analysis. Similar to the Northern blot analyses, no significant changes in the amount SPARC protein secreted were observed in these conditioned media samples (Figure 26). Similar Western blots analyses were performed on the cellular proteins of the cultures from which the media were obtained, and no increases in intracellular SPARC protein levels were detected (data not shown). Therefore, it does not appear that TGF- β_3 promotes angiogenesis through the induction of cardiomyocyte produced SPARC.

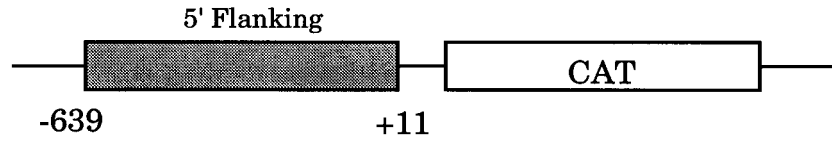
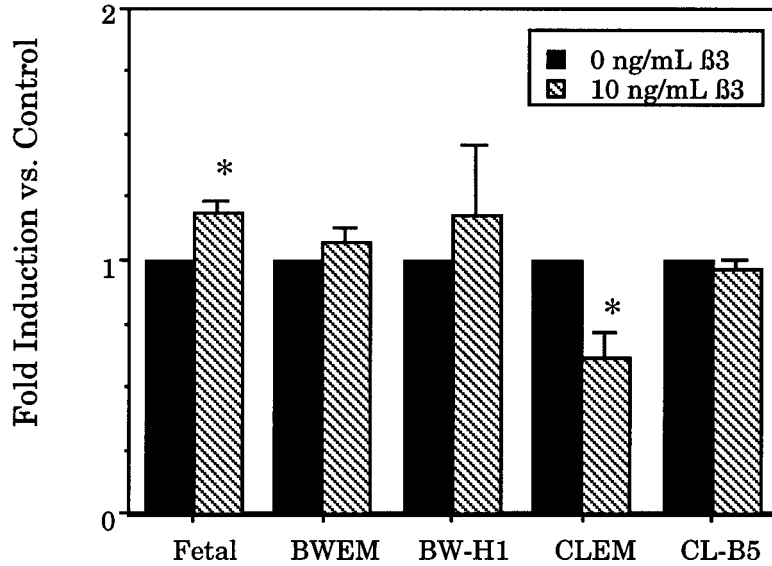
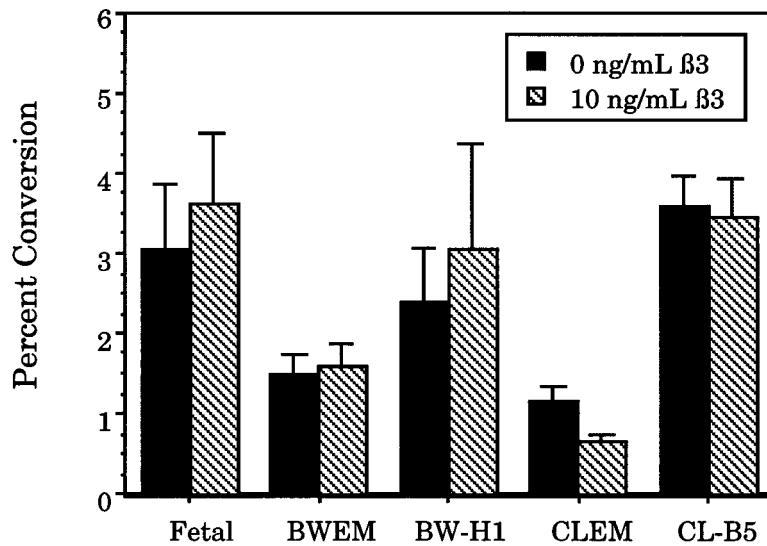
Figure 23: SPARC Promoter Assays**A: SPARC Reporter Construct****B: SPARC Promoter Activity -- Relative Changes****C: SPARC Promoter Activity -- Raw Data**

Figure 23: TGF- β_3 Induced Changes in SPARC Promoter Activity

(A) 639SPARC-CAT promoter construct. This construct contains 639 bp of the SPARC promoter inserted in front of the CAT reporter gene.

(B & C) 48 hour CAT assays. 18 dpc fetal primary myocyte cultures, each parental line (BWEM and CLEM) and their respective mutant clones (BW-H1 and CL-B5) were transfected with the 639SPARC-CAT reporter plasmid and treated with 0 or 10 ng/mL of recombinant TGF- β_3 for 48 hours. Samples were collected and assayed for CAT activity as described in Chapter 3. Data were normalized with β -galactosidase activity. Data represent the mean value from 3 individual assays and the error bars represent SEM.

(B) Relative changes in activity are presented as fold increases when compared to untreated (0 ng/mL β_3).

(C) Raw data are presented as the per cent conversion of ^{14}C -chloramphenicol per 10 μg protein (fetal) or 150 μg protein (cell lines).

* indicates response significantly different from control (0 ng/mL β_3)

Table 15: Statistical Analysis of SPARC Promoter Assays

Unpaired t-tests were used to determine significant differences between control and treated (10 ng/mL TGF- β_3) samples and between each parental cell lines and its respective mutant.

<u>Cell Type</u>	<u>p-value (fold induction)</u>	<u>p-value (raw)</u>
Fetal	0.013	0.662
BWEM	0.313	0.808
BW-H1	0.535	0.654
BWEM vs. BW-H1	N.D.*	0.332
CLEM	0.009	0.041
CL-B5	0.387	0.834
CLEM vs. CL-B5	0.019	0.001

* N.D. = Not Determined

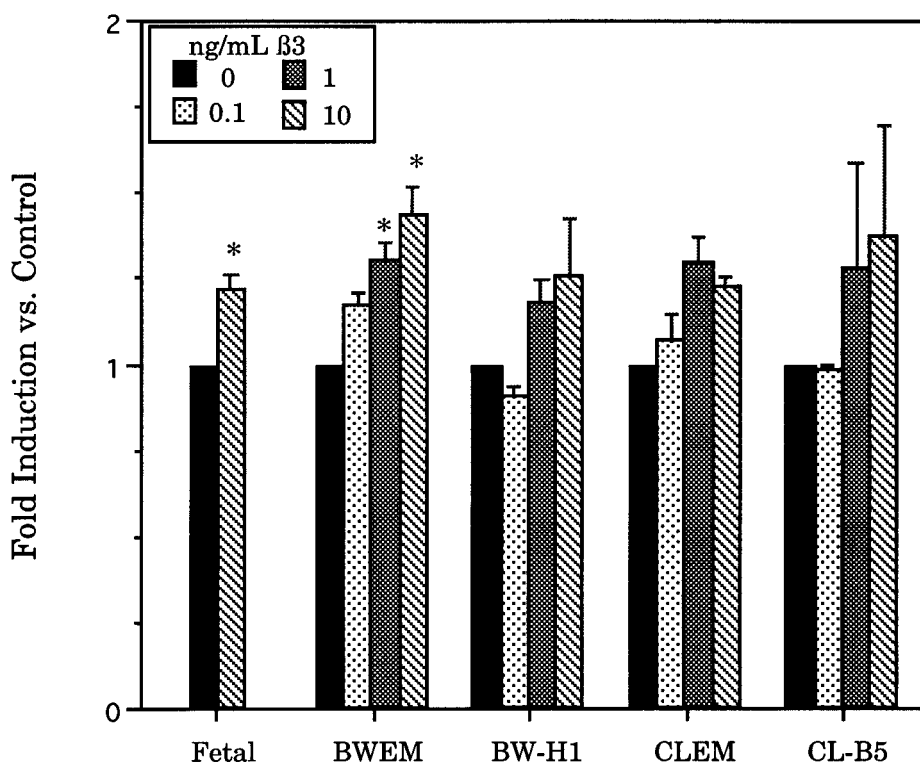


Figure 24: TGF- β_3 Induced Changes in Steady State SPARC Transcript Levels

Cultures of all four cell lines and primary cultures of 18 dpc fetal ventricular myocytes were treated with 0-10 ng/mL TGF- β_3 for 48 hours. Northern blots were hybridized with a cDNA probe for SPARC. Data are presented as fold induction of transcript levels as compared to untreated controls. Results shown are from 3 individual assays. Data were normalized to 18S rRNA oligo hybridizations. Error bars represent SEM.

* indicates response significantly different from control (0 ng/mL β_3)

Table 16: Statistical Analysis of TGF- β_3 Dose Response Curves of SPARC Northern Blot Analyses

One way ANOVA was used to determine significance of the dose response curves of SPARC steady state transcript levels for each cell type. Two way ANOVA was used to determine significant differences between the dose response curves for each parental line and its respective mutant.

<u>Cell Type</u>	<u>p-value</u>
Fetal	0.002
BWEM	<0.001
BW-H1	0.082
BW vs. BW-H1	0.295
CLEM	0.069
CL-B5	0.581
CL vs. CL-B5	0.919

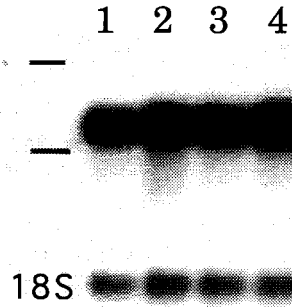
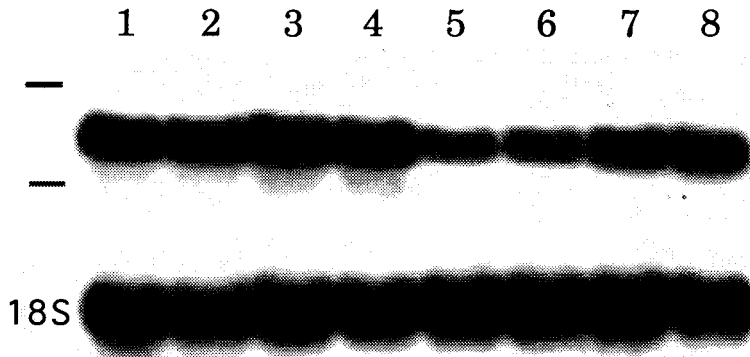
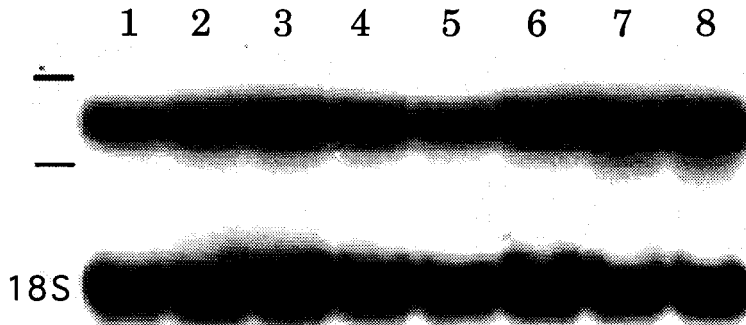
Figure 25: Northern Blot Analyses of SPARC Expression**A: 18 dpc Fetal Cardiomyocyte Northern****B: BWEM and BW-H1 Northern****C: CLEM and CL-B5 Northern**

Figure 25: Northern Blot Analyses of SPARC Expression

Primary cultures of fetal cardiomyocytes and cultures of each of the cell lines were treated with 0 or 10 ng/mL TGF- β_3 for 48 hours. Northern blots were hybridized the cDNA probe for SPARC. Bars indicate 18S and 28S rRNA bands. Blots shown are from 24 hr. exposure to film at -80°C. 18S rRNA hybridizations shown below each blot.

(A) 18 dpc Fetal RNA. 3 μ g total RNA loaded for each sample.

Lane 1: 48 hr. Control

Lane 2: 48 hr. +TGF- β_3

Lane 3: 72 hr. Control

Lane 4: 72 hr. +TGF- β_3

(B) BWEM and BW-H1 RNA. 30 μ g total RNA loaded for each sample.

Lane 1: BWEM Control

Lane 2: BWEM 0.1 ng/mL β_3

Lane 3: BWEM 1 ng/mL β_3

Lane 4: BWEM 10 ng/mL β_3

Lane 5: BW-H1 Control

Lane 6: BW-H1 0.1 ng/mL β_3

Lane 7: BW-H1 1 ng/mL β_3

Lane 8: BW-H1 10 ng/mL β_3

(C) CLEM and CL-B5 RNA. 30 μ g total RNA loaded for each sample.

Lane 1: CLEM Control

Lane 2: CLEM 0.1 ng/mL β_3

Lane 3: CLEM 1 ng/mL β_3

Lane 4: CLEM 10 ng/mL β_3

Lane 5: CL-B5 Control

Lane 6: CL-B5 0.1 ng/mL β_3

Lane 7: CL-B5 1 ng/mL β_3

Lane 8: CL-B5 10 ng/mL β_3

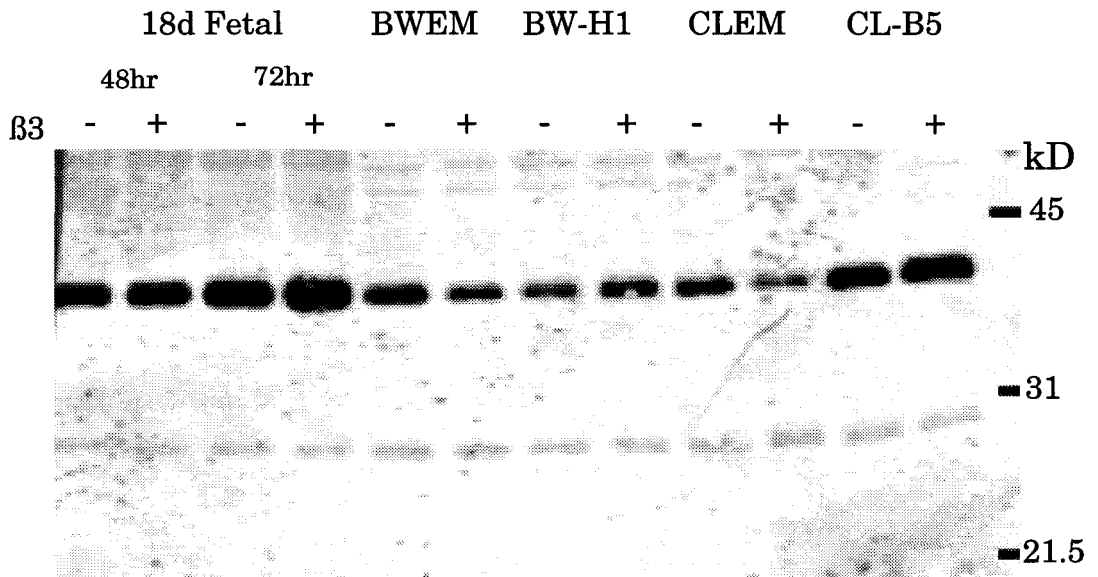


Figure 26: SPARC Western Blot Analyses

Conditioned media proteins from cultures treated for 48 hours \pm 10 ng/mL TGF- β_3 (72 hr. sample also collected for 18 dpc fetal myocyte cultures) were run on 12% SDS-PAGE gels and used for western blot analyses to detect the 43 kD SPARC protein secreted by the cells. 5 μ g of protein was loaded per lane. Markers on right indicate size in kilodaltons as determined by co-running biotinylated standards on the gel. Staining of gel indicated that protein loads was consistent, except for CLEM + β_3 (data not shown).

CHAPTER 5

DISCUSSION

We and others have postulated that TGF- β s are active participants in late fetal and neonatal ventricular development. This is based on the temporal and spatial expression patterns of the TGF- β ligands and receptors which coincide with established changes in myocyte proliferation and differentiation as well as ongoing ventricular remodeling in the postnatal heart. To determine the role TGF- β s may play on cardiomyocyte growth and development at the cellular and molecular level, we have chosen to use two ventricular myocyte cell lines, BWEM and CLEM, as our experimental system. As a means to more clearly define and evaluate the direct effects of TGF- β on the cardiomyocyte, mutant myocyte cell lines have been created which exhibit reduced β R_{II} function due to a dominant negative method of inactivation. These mutant lines, along with their parental counterparts and primary fetal myocyte cultures, were used to study the direct effects TGF- β may have on several developmental changes which occur in the ventricular myocyte population prior to and/or shortly after birth. These results are summarized in Table 17.

Table 17: Summary of Results (10 ng/mL TGF- β_3)

Assay	18d Fetal	BWEM	BW-H1	CLEM	CL-B5	Notes
Growth	ND	↓50%	↓50%*	↓80%	↓20%	*10-fold less sensitive
PAI-1 PA	↑4.8x	↑5.4x	↑2.3x*	↑3.9x	↑1.6x*	*10-fold less sensitive
cTNC NB	↓	+	+	+	+	Increase ~2.5 fold, no DN diff.
cTNC PA	↔	↓	↔	↓	↔	no DN diff.
sTNC NB	ND	+	↔	↔	↔	BWEM/BW-H1 sig. diff.
sTNC PA	↔	↔	↓	↔	↔	
sTNI PA	↓	↔	↔	↔	↔	
sTNI NB	↑	ND*	ND*	ND*	ND*	*Undetectable in total RNA
cTNI NB	↓	ND*	ND*	ND*	ND*	*Undetectable in total RNA
cTNT PA	↔	↓	↔	↓	↔	
t [†] .cTNT NB	↔	ND*	ND*	ND*	ND*	*Undetectable in total RNA
f [†] .cTNT NB	↔	ND*	ND*	ND*	ND*	*Undetectable in total RNA
βRI NB	ND	↔	↔	↔	↔	
βRII NB	↔	↔	↓	↓	↔	CLEM/CL-B5 sig. diff.
SPARC PA	↑	↔	↔	↓	↔	CLEM/CL-B5 sig. diff.
SPARC NB	↑	↑	↔	↔	↔	no sig. DN diff.
SPARC WB	↔	↔	↔	↔	↔	

Table 17: Summary of Results:

Results obtained from each experiment at 10 ng/mL TGF- β_3 (vs. untreated) are given for each cell type.

Table Legend:

NB: Northern blot analyses	↑:	Statistically significant increase
PA: Promoter assays	↓:	Statistically significant decrease
WB: Western blot analyses	↔:	No statistically significant change
ND: Not done	+	Changes at select time points
†: t. cTNT = total cTNT, f. cTNT = fetal cTNT		

Creation and Initial Characterization of
Clonal Dominant Negative Cell Lines:

A eukaryotic expression vector containing a cDNA encoding a kinase deficient, human β R_{II} (Figure 3) was introduced into the parental *v-myc* derived myocyte cell lines. Using G418 selection and screening of single cell clones derived from our initial polyclonal transformants, four mutant myocyte cell lines (BW-F8, BW-H1, CL-B5, and CL-F5) were chosen for further study. Integration of and expression from the truncated, human, Type II TGF- β receptor (ph β R_{II}DN) in the mutant lines and its absence in the parental lines was verified by Southern (Figure 8a) and Northern (Figure 8b) blot hybridization analyses, respectively. The variations in signal intensity on the Southern blot between the BW and CL mutant lines represent differences in the number of integrations of the plasmid into the host genome. Additional studies utilizing alternative restriction enzymes indicate that at least 3-4 individual integrations occurred in the BW-H1 line (data not shown) while CL-B5 appears to have only a single integration of

ph β RIIDN. Similarly, multiple integrations and/or aberrant transcriptional read-through of the cDNA within the plasmid may be responsible for the larger Northern blot transcript(s) detected when using the human β RII-DN cDNA insert as a probe during the hybridizations. Nonetheless, these data clearly show that we have successfully created and isolated clonal β RII mutant cell lines which have stably incorporated the ph β RIIDN plasmid and express the β RII-DN transcript at high, readily detectable levels.

Additionally, Northern (Figures 5 and 7) and Western (Figure 6) blot analyses showed that expression of the endogenous rat β RI and β RII is similar in the parental and transformed cell lines. These data indicate that the cellular transformation mediated by G418 selection and subsequent expression of the human β RII-DN cDNA does not affect the transcript or protein levels for any of the endogenous rat TGF- β receptors. Of greatest importance, β RI expression data inferred that its availability for heterodimerization would be equivalent in the parental lines and mutant clones. As partial confirmation of this, ligand-binding by β RI and β RII (both wild type and mutant) was verified by receptor crosslinking analyses (Figure 9). Similar to the Northern and Western blot analyses, the crosslinking results showed that cell surface expression of the endogenous rat receptors was similar between the parental lines and mutant clones. Since β RI has been shown to bind TGF- β ligands only in the presence of β RII, the strong crosslinking signal by the endogenous β RI suggests that ligand-binding to β RII and/or β RII-DN is under-represented by the

intensity of the image. What is represented with the iodinated ligand predominantly associating with β RI rather than β RII or β RII-DN may reflect binding of non-isotopic ligand or non-uniform separation of labeled vs. unlabeled TGF- β dimers during the stringent reduction step just prior to loading samples on the gels. Nonetheless, we interpret these data to indicate that the detected β RII-DN transcripts are translated into a ligand-binding, truncated, epitope tagged human β RII in the four mutant rat myocyte cell clones. Additionally, we have demonstrated that the levels of expression of the endogenous β RI and β RII genes in the cell lines are comparable to that seen in primary neonatal cardiomyocytes, strengthening the usefulness of these lines for our studies. It does appear that the neonatal cardiomyocytes expressed a significantly greater amount of β RIII than any of the cell lines. Since this is not a "signaling" molecule and our present interests do not include TGF- β_2 , of which ligand binding to β RII is enhanced by ligand presentation by β RIII (Lopez-Casillas et al., 1993), the lower β RIII expression in the cell lines does not represent a limitation to the studies at hand.

Characterization of the direct effects TGF- β elicits in the parental and mutant cell lines was carried out by assaying two fundamental TGF- β -mediated responses which represent the "gold standard" in the literature: inhibition of proliferation and induction of PAI-1 promoter activity. Data from our growth assays show that the mutant clones are significantly less sensitive (~10X, BW-H1) or virtually insensitive (CL-B5) to TGF- β_3 -mediated growth inhibition relative to their respective parental BWEM and CLEM

lines (Table 17). As expected, TGF- β_3 induced transcription from the PAI-1 promoter construct, p3TP-Lux, in a dose-dependent manner in the parental BWEM and CLEM cell lines. Even though the activity from the p3TP-Lux plasmid was significantly higher in the cell lines than the activity seen in the primary fetal cardiomyocyte cultures, the fold induction above the basal (untreated) activity was comparable (Table 17), supporting the use of these cell lines as a model to study effects of TGF- β on cardiomyocyte growth and differentiation during heart development. PAI-1 was also induced in a dose-dependent manner in the mutant clones, BW-H1 and CL-B5. However, the concentration needed to elicit a response in the mutant clones is significantly greater than in the parental lines, requiring an approximately 10-fold higher concentration of bioactive TGF- β ligand to elicit similar levels of PAI-1 induction. Therefore, the expression of β RII-DN in the two mutant cell lines was able to reduce the cells' ability to respond to TGF- β_3 , and suggests that this dominant negative inhibition can provide a means in which to examine the different aspects of TGF- β signal transduction in a cell line of cardiomyocyte origin.

While total ablation of any TGF- β response in the clones would have been ideal, incomplete inhibition through the expression of β RII-DN was not unexpected. Since the dominant negative β RII did not interrupt the expression of the endogenous TGF- β R genes, any endogenous β RII present is still capable of initiating a response if it binds ligand as part of a heteromeric receptor complex. In *Xenopus* embryos, ratios of 1:10 or 1:100 wild type to mutant FGF receptors were needed to see any level of inhibition

(Amaya et al., 1991). While the exact ratio of β RII-DN to β RII was not determined, it appears that the ratio of wild type to mutant β RII falls within the level which exhibits partial inhibition. While not a receptor, dominant negative mutations in the Ras protein demonstrate that different levels of activation/phosphorylation are required for the different responses a protein can elicit (Han and Sternberg, 1991). Therefore, if sufficient TGF- β ligand is present to saturate the mutant receptor or bind the native, functional β RII at a desired "threshold" level, one specific response (such as transcription) will be seen while another (growth inhibition) will not (Brand and Schneider, 1995). While the model presented for TGF- β signal transduction is widely used (see Chapter 2, Figure 2), it is a simplified model which does not address the formation of hetero-oligomers of β RI and β RII upon ligand binding (Yamashita et al., 1994; ten Dijke et al., 1996). As these oligomeric complexes contain at least two molecules of each type of receptor (β RI and β RII), the "thresholds" may involve the ability of a complex containing just one functional β RII (and one β RII-DN) to contain enough activity to trigger one response, while two functional receptors are required for another. Similarly, the association of downstream effectors with this complex may also rely on the interactions of the intracellular domains, with the presence of a truncated mutant possibly interrupting an effector's stable interaction with and activation by β RI or β RII. Nevertheless, the presence of β RII-DN in our mutant cell lines has rendered them ~10-fold less sensitive to exogenous TGF- β_3 than their parental lines.

This reduced sensitivity, as opposed to total ablation of TGF- β responsiveness, in these lines may actually prove to be advantageous. As all TGF- β responses are mediated through β RII and β RI heterodimerizations, a different threshold (a.k.a. different levels of stimulation by ligand) may be required for each individual response. Therefore, these lines may be useful for the characterization of these thresholds in a myocyte-like background. Additionally, the data from the CLEM and CL-B5 lines suggest that the signal transduction pathways utilized by the two responses examined, proliferation and PAI-1 induction, are distinct and would appear to involve significantly different thresholds for activation. This could be one explanation for the virtual insensitivity to inhibition of proliferation in CL-B5, even at 10 ng/mL TGF- β_3 , while there is only an approximate 10-fold reduction of sensitivity to TGF- β_3 as measured by PAI-1 promoter induction.

Through this initial characterization of the cell lines, data have been collected that support two fundamental parts of our hypothesis: 1) TGF- β_3 can inhibit cardiomyocyte proliferation, and 2) by inducing the expression of PAI-1, TGF- β_3 indirectly promotes the accumulation of ECM by inhibiting its degradation. As previously discussed, similar effects of TGF- β on proliferation and PAI-1 induction have been observed in primary neonatal cardiomyocyte cultures. Therefore, our results were not unexpected, and extend these findings to fetal cardiomyocyte cultures and the myocyte cell lines which closely reflect the late fetal stage of myocyte development. However, as standard assays for TGF- β responsiveness in

any cell, these results clearly demonstrate that the parental myocyte-like cell lines are responsive to TGF- β_3 and that the mutant clones have altered (reduced) sensitivity to normal, inhibitory levels of TGF- β_3 . Therefore, these lines are a suitable model in which to examine the effects of TGF- β as it appears that components of the TGF- β signal transduction pathway are not restricted in the parental or mutant cell lines. Additionally, the differences in the signaling pathways or thresholds necessary to elicit the different responses suggests there are several mechanisms by which TGF- β can influence cellular functions in a variety of cell types, including myocytes. These mechanisms may involve processes such as post-translational modification, regulation of translational activity, or direct transcriptional activation. Given the wide variety of second messenger pathways (e.g. G-proteins, MAP kinases, PLC) which have been linked to TGF- β activity, it would not be wise to dismiss any of these mechanisms *a priori*. The mutant cell lines may prove useful in the identification and/or separation of these pathways in the myocyte background. As the induced effects of TGF- β_3 were clearly discernible at 10 ng/mL TGF- β_3 in the parental cells, this dose was used for those experiments in which a dose response curve was not practical or feasible. This is most important for the CAT-based reporter assays in which small changes are not as readily detected as the luciferase-based systems. As most of the effects examined are transcriptional, the greater than 50% reduction in transcriptional activity in the mutant lines as compared to their parental counterparts in the PAI-1 reporter assay should provide a difference in which the DN-effect could be identified.

Effects of TGF- β_3 on Fetal Cardiomyocyte Differentiation/Maturation

The ability of secreted proteins, such as growth factors, to influence the differentiation and maturation of cardiomyocytes is not a new phenomenon. It has been previously shown that innervation of heart tissue results in a switch in expression of the troponin isoforms to the adult forms, and that conditioned media from nerve cultures were able to induce the same changes (Toyota and Shimada, 1983). Similarly, thyroid hormone and the stimulation of the α_1 -adrenergic receptor have also been found to activate the fetal gene program in cardiomyocytes (Long et al., 1990; Bugaisky et al., 1992). Therefore, as TGF- β ligand and β RII mRNA appears and increases during the same period in which many contractile proteins are undergoing the transition from a “fetal” isoform to its “adult” counterpart, we hypothesized that TGF- β would promote the differentiation and maturation of the fetal cardiomyocyte by upregulating the adult isoforms and/or downregulating their fetal counterparts. Therefore, we examined the ability of TGF- β_3 to influence the expression of the different isoforms of three contractile proteins: TNC, TNI, and TNT.

As hypothesized, TGF- β_3 treatment resulted in a dose-dependent increase in steady state mRNA levels for cTNC (2-2.5-fold increase at 10 ng/mL TGF- β_3) after 72 hours in all four of the cell lines, both parental and mutant (Table 17, Figure 12). The increases observed in the mutant lines were not expected to be the same as the increases which occurred in their parental counterparts given the mutant cell lines' 10-fold decrease in

sensitivity to TGF- β_3 stimulation determined in the growth and PAI-1 promoter assays. Conflicting results were obtained from the cTNC promoter assays in each of the parental lines, indicating a 40-50% downregulation of transcription from this gene after exposure to 10 ng/mL TGF- β_3 ; yet only a 10% decrease in the mutant lines (Table 17). These conflicting results between the Northern blot analyses and the promoter assays could be explained by the fact that the reporter construct contains a relatively small fragment of the cTNC promoter (Figure 14a), and the increases observed in transcript levels could be the result of TGF- β_3 induction of cTNC transcription through the use of an enhancer region or positive promoter element located outside this region and which remains intact for the native cTNC gene. However, this construct does appear to have a negative promoter element which is responsible for the decrease in transcription from this promoter in response to TGF- β_3 .

It is interesting to note that the downregulation of transcription from this cTNC promoter construct was not as pronounced in the mutant lines, demonstrating a decrease of only ~10% as compared to the 40-50% decreases observed in the parental lines. This decreased sensitivity to TGF- β_3 -mediated transcriptional changes agrees with the data obtained from the PAI-1 promoter assays. The higher transcription rates for cTNC in the mutant lines when compared to their parental counterparts would lead to the prediction that the cTNC steady state mRNA levels would be greater in the mutant lines than in the parental lines after 72 hours. However, steady state mRNA levels increased at levels similar to their respective parental

lines. This suggests that TGF- β_3 is able to regulate cTNC transcript levels on at least two overlapping levels, transcriptional and post-transcriptional, both of which appear to be disrupted in the mutant lines.

One post-transcriptional mechanism that can partially explain these results is one which involves an increase in cTNC mRNA stability in response to TGF- β_3 , thereby allowing a steady increase in cTNC mRNA accumulation even though transcription rates are reduced. The 3' UTR of cTNC contains a 10 bp sequence (M. Parmacek, personal communication) found to confer TGF- β_1 -mediated mRNA stability to the mRNA for RHAMM (receptor for hyaluronan mediated motility gene, Amara et al., 1996). Therefore, studies utilizing the transcriptional inhibitor actinomycin D were initiated to address the effects TGF- β_3 may have on cTNC mRNA stability. Unfortunately, a relatively long half life of the cTNC mRNA in the myocyte cell lines (~15 hours, data not shown; Zhu et al., 1991) and reduced cell viability in the presence of actinomycin D for extended periods of time restricted our ability to clarify this issue. Alternative approaches such as metabolic labeling and gel shift analyses could be considered to more clearly define any role TGF- β_3 may have on cTNC mRNA stability.

Additionally, a more thorough examination (e.g. nuclear run-on analyses or the use of larger promoter constructs) of the transcriptional regulation of cTNC should be attempted to clarify or verify the effects of TGF- β_3 . Identification of TGF- β -responsive elements (β RE), both positive and negative, may be able to explain the results obtained in the myocyte cell lines and possibly negate a role for TGF- β in cTNC mRNA stability. As

previously discussed, TGF- β is able to influence the expression of several contractile genes in primary myocyte cultures. As contractile gene expression involves both cardiac/muscle-specific factors as well as ubiquitous factors, the regulation of their activity by TGF- β is of great importance. While TGF- β has been shown to increase general transcription factors such as *c-jun* and *c-fos*, less is known about its effects on cardiac transcription factors. One transcription factor which may be of particular interest is GATA-4, a zinc-finger protein which appears to be important for cTNC transcription and can drive its expression in non-muscle cells (Ip et al., 1994). Since GATA-4 is inducible by factors such as retinoic acid (Arceci et al., 1993), one could postulate that this factor, as well as other muscle-specific factors, could be regulated by growth factors such as TGF- β .

Although the complex regulation of cTNC expression that occurs in the cell lines is intriguing and deserves further attention, these studies do not lie within the focus of this dissertation. As a focus of this dissertation, it must be stressed that the data obtained from the primary fetal cardiomyocyte cultures indicated that cTNC promoter activity and mRNA levels are not significantly affected by 10 ng/mL TGF- β_3 , with the cTNC mRNA levels decreasing slightly after 72 hours of TGF- β_3 treatment. Thus, the effects observed in the lines may be cell line specific and may not fully represent what occurs *in vivo*.

To further test the hypothesis that TGF- β promotes differentiation and maturation through the induction of the “adult”/cardiac isoforms and

downregulation of “fetal”/skeletal isoforms of contractile proteins, the effect of TGF- β_3 on the expression of cTNI, sTNI, and sTNC was examined. As sTNC is not normally expressed in the heart, the low promoter activities observed in the primary cultures were not unexpected. The similar results observed in the cell lines suggest that they have maintained their cardiac-specific background, and therefore, support their use as a cardiomyocyte model system. However, mRNA transcripts for sTNC were detectable in the cell lines, albeit at very low levels. As cTNC is avidly expressed in all of the lines, both parental and mutant, and low stringency washes were required for successful sTNC hybridizations, cross-hybridization of the sTNC cDNA probe to cTNC transcripts similar to that reported in heart tissue remains a possibility. However, the insignificant change in transcript levels detected for sTNC after 72 hours of TGF- β_3 treatment does not support that possibility. Therefore, the sTNC probe identified a transcript distinct from cTNC, and suggests that sTNC is transcribed at a low, negligible level in the cell lines.

In contrast to our hypothesis, it appears that TGF- β_3 may have a negative effect on the maturation of the cardiomyocyte. While sTNI promoter activity in the primary fetal cultures is slightly reduced (~15%) in response to 10 ng/mL TGF- β_3 , a slight increase (10-15%) was detected in sTNI transcript levels at 48 and 72 hours. In concert with these results, a 25% decrease in cTNI transcript levels was observed in primary fetal myocyte cultures after 72 hours of treatment with 10 ng/mL TGF- β_3 (Table 17, Figure 20). In contrast, sTNI promoter activity, did not significantly

change in any of the cell lines, even at 10 ng/mL TGF- β_3 , however, the individuality of the cell lines is once again demonstrated as the basal activity in the BWEM lines was higher than that observed in the CLEM lines, with little difference between the mutants and their parental lines. The implications of the changes observed in the primary cultures and the results obtained in the cell lines are discussed below.

While the transition between the two cTNT isoforms primarily occurs just after birth, we hypothesized that TGF- β_3 promotes the differentiation of the cardiomyocyte through the upregulation of the adult isoform of cTNT. Additionally, we used promoter assays to determine if TGF- β_3 has an effect on overall transcriptional activity from the cTNT gene and to verify the use of the myocyte-like cell lines as a model for cardiomyocyte development. Promoter analyses demonstrated that 10 ng/mL TGF- β_3 did not increase transcription from the cTNT promoter construct in either primary fetal myocyte cultures or the cell lines (Table 17). However, while cTNT promoter activity was high in the primary cardiomyocyte cultures, cTNT promoter activity was significantly lower in all of the cell lines. Similarly, cTNT transcripts were readily detectable in total RNA collected from primary cardiomyocyte cultures, yet undetectable in the cell lines by Northern blot analyses using 30 μ g total RNA. These results along with the results obtained for TNI suggest that the cell lines have limited utility as a cardiomyocyte developmental model. These limitations are discussed further below.

To directly examine effects of TGF- β_3 on the expression of the different isoforms of cTNT, Northern blot analyses were performed on RNA collected from primary fetal cardiomyocytes treated with 0 or 10 ng/mL TGF- β_3 . Consistent with the promoter data, the transcript levels of total cTNT expression did not change after 48 or 72 hours of treatment with TGF- β_3 (Table 17). To test the hypothesis that TGF- β promotes fetal myocyte maturation, we examined the ability of TGF- β_3 to influence the alternative splicing of the cTNT transcript to favor the production of the alternatively spliced, adult isoform in late fetal cardiomyocytes (18 dpc). A probe specific for the fetal isoform of cTNT was used to determine any changes in the ratio of fetal to adult isoforms. The results revealed that TGF- β_3 did not significantly affect the transcript levels of the fetal isoform and, thus, the adult isoform in the fetal cardiomyocyte cultures. This is in contrast to what was observed in neonatal cardiomyocytes. Northern blot analyses of RNA obtained from primary cultures of 2-day neonatal cardiomyocytes treated with 1 ng/mL TGF- β_1 for 48-72 hours demonstrated an increase in total cTNT expression and an increase in the alternatively spliced adult isoform of cTNT (Dr. J. Lin, personal communication). As previously discussed, TGF- β_1 and TGF- β_3 elicit nearly identical effects *in vitro*, and as such, it would be unlikely that these differences could be attributed to the TGF- β isoform used. Since cTNT isoform switching predominantly occurs during the immediate post-natal period, these data suggest that TGF- β can augment the switch in its natural developmental time frame, but cannot induce the switch prematurely in the fetal cardiomyocyte. Alternatively,

the differences observed between the fetal and neonatal primary myocyte cultures could be attributed to a developmental change in the TGF- β signal transduction pathway (see below for further discussion). While selective degradation of the fetal cTNT transcript may result from TGF- β_3 treatment, these data provide evidence suggesting TGF- β may have the ability to influence gene expression at a post-transcriptional level by directly or indirectly affecting the alternative splicing of a specific gene product.

Since the expression of TNC, and possibly other contractile genes, is predominantly controlled by the production and accumulation of mRNA transcripts (Gahlmann et al., 1988), it appears that TGF- β_3 has little effect on the expression of TNC, TNI, or TNT isoforms in fetal cardiomyocytes. The downregulation observed for the adult isoform of TNI (cTNI) by TGF- β_3 is an extension of what has been observed in neonatal cardiomyocytes treated with TGF- β . In these cells, several "fetal" proteins, α Sk-actin, β -MHC, β -tropomyosin, and ANF, are induced while the adult isoform of myosin, α -MHC, is downregulated (Schneider and Parker, 1990; Schneider et al., 1992; Lembo et al., 1995). These changes in expression resemble those that occur in the hypertrophic response to pressure overload commonly induced *in vivo* by aortic banding or *in vitro* by stretching of myocytes in culture (Schneider and Parker, 1991; Komuro et al., 1991; Chien et al., 1991; Schneider et al., 1992) but not in exercise or thyroid induced hypertrophy (Long et al., 1990). Additionally, the absence of change in sTNI expression in the cell lines or small changes in expression in the primary fetal myocyte cultures treated with TGF- β_3 corresponds to

the lack of re-expression of sTNI in the ventricle in the adult heart challenged by pulmonary hypertension or ischemic heart disease (Sasse et al., 1993).

These similarities in changes in myocyte gene expression induced by TGF- β and pressure overload have resulted in a postulated role for TGF- β in the hypertrophic response of the adult myocyte. It has been demonstrated that TGF- β expression is upregulated *in vivo* in response to pressure overload (such as that induced by aortic banding) (Komuro et al., 1991; Schneider et al., 1992; Lembo et al., 1995), but not through the induction of hypertrophy utilizing other methods (Klein et al., 1995). In this regard, TGF- β is considered to be part of a complex mechanism in which changes in the cells' environment can be transmitted to the cells, either directly influencing changes in gene expression and/or protein production, or by augmenting the "signal" imposed by the pressure overload. In addition to its apparent role in mediating a stress response, the TGF- β s have been implicated to play a role in recovery from myocardial infarction. While TGF- β_1 is undetectable in the damaged myocytes, the viable myocytes surrounding the injured cells exhibit increased TGF- β_1 expression (Schneider and Parker, 1990; Lembo et al., 1995). Due to its established roles in ECM biosynthesis and tissue repair, TGF- β_1 has been postulated to play a role in the induction of compensatory hypertrophy of the myocytes surviving or surrounding an infarct as well as building up the ECM to increase tensile strength of the damaged area (Schneider and Parker, 1990; Lembo et al., 1995). This is similar to what is observed during wound

repair in skin tissue, where TGF- β ligands and receptor expression is high in the tissues surrounding a wound, and absent within the wound and the migrating epithelium (Gold et al., 1997).

In light of the data regarding TGF- β treatment of neonatal cardiomyocytes as a model for pressure overload hypertrophy, the results obtained in the cell lines and fetal cardiomyocytes suggest that our original hypothesis for the direct role of TGF- β in ventricular development in regards to modulating myocyte maturation is false and needs to be modified. The data obtained for the TNT isoform switch suggests that TGF- β may be able to promote the “adult” phenotype after birth, but not in the developing fetal heart. The inability of TGF- β_3 to induce similar changes in cTNT expression in 18 day fetal myocytes as was observed in the neonatal myocytes suggest two possible scenarios. The first option is that the developmental program for the isoform switching that occurs in the heart cannot be prematurely induced by TGF- β_3 , but once the isoform switch is initiated, TGF- β_3 has the ability to augment the change. However, the lack of response observed for cTNC and the downregulation of cTNI in the fetal myocytes cannot be attributed to TGF- β acting “out of frame” as the isoform switch is already underway for these proteins during the late fetal period, which encompasses the age at which the primary myocytes were collected. This suggests an alternative scenario: the fetal myocytes lack or have a low abundance of a rate-limiting factor in the TGF- β_3 signal transduction pathway. While β R2 expression is limiting *in vivo* at 18 dpc, Northern blot analyses of RNA obtained from primary fetal myocyte cultures suggests

neither receptor is rate limiting *in vitro* (Figure 22). However, other members of the signal transduction pathway may be limiting. Since members of the TGF- β signal transduction pathway have only recently begun to be identified, this possibility cannot be addressed at this time. However, the ability of the primary fetal myocytes to increase PAI-1 transcription in response to TGF- β_3 suggests these cells contain some of the necessary components to mediate transcriptional regulation by TGF- β . As PAI-1 is not a cardiac specific gene, a cardiac- or muscle-specific factor, such as a transcription factor or a positive or negative trans-acting regulator, which can be modulated by TGF- β may not be present or exist at a low level in the fetal myocyte, and which is developmentally upregulated in the neonatal myocyte conferring TGF- β responsiveness. This could explain the overall lack of or low response to exogenous TGF- β_3 treatment observed in the studies described.

Similar arguments can be used to explain the differences observed between the cell lines and the fetal myocytes. As transformed cell lines, BWEM and CLEM have lost some aspect(s) of their cardiac-specific transcriptional machinery demonstrated by the fact that they do not express contractile proteins such as myosin heavy chain. As demonstrated here, similar losses in expression have occurred for two other contractile proteins, TNT and TNI, being readily detected in primary fetal myocyte cultures but low or undetectable in the cell lines. Additionally, as transformed cell lines, BWEM and CLEM express *v-myc* and potentially several other unique transcription factors. As an extension of this, the

β RII-DN cell lines, having been transformed again with two exogenous plasmids which integrated randomly, may have yet another molecular milieu which may or may not be attributed to the expression of β RII-DN. The altered transcriptional machinery available in the nucleus of all of these cell lines may confer differential responses to exogenous TGF- β_3 than those responses elicited in the primary cells. Using cTNC as an example, the decrease in promoter activity detected in the cell lines may be the result of the activation of a TGF- β -responsive regulatory protein that binds to a negative element in the cTNC promoter construct. However, the primary fetal myocytes did not demonstrate a similar decrease in cTNC promoter activity in response to TGF- β_3 . This suggests that the cell lines have acquired or upregulated a negative regulator not normally present in the cardiomyocyte. Finally, differences in responses, specifically the level of response, between the cell lines and the primary myocytes may be the result of differential expression of the receptors. While β RII receptor expression is not rate limiting in any of the lines, the low expression levels of β RIII in the cell lines may be one possible factor responsible for the differences observed between the cell lines and fetal myocytes. Even though membrane bound β RIII is thought only to present ligand to β RII, β RIII can be shed from the cell surface and act to sequester TGF- β dimers (Attisano et al., 1994). As β RIII is highly expressed in primary fetal myocyte cultures as well as *in vivo*, heightened sequestration of exogenous TGF- β_3 may be responsible for the lower responses seen in the primary myocyte cultures.

Based on a lack of effect on the filament isoform expression, it appears that the developmental effects of TGF- β_3 on the fetal cardiomyocyte are minimal. In as much as the studies described here only address the direct effects TGF- β_3 has on the expression of these three contractile proteins, indirect effects cannot be ruled out. As a modulator of ECM formation, cell-cell interactions, and cell-ECM interactions, TGF- β may promote cardiomyocyte differentiation and/or maturation through the induction of morphological changes complemented by its ability to induce cellular quiescence by its potent anti-proliferative properties retained in all cell types examined.

Effects of TGF- β_3 on Angiogenesis

A number of growth factors have been found to regulate angiogenesis *in vivo* and, as discussed previously, one of these is TGF- β . While TGF- β has been hypothesized to work as a chemoattractant and regulator of capillary morphogenesis and apoptosis (Choi and Ballermann, 1995), TGF- β may also control angiogenesis by regulating the production of an angiogenic factor, SPARC. In order to test the hypothesis that TGF- β promotes angiogenesis in the ventricle by increasing cardiomyocyte-produced SPARC, cultures of primary fetal cardiomyocytes and each of the cell lines, wild type and mutant, were used to measure the changes in the expression of SPARC and the release of its gene product into the media upon exposure to TGF- β_3 . Although lower than expected, 10 ng/mL TGF- β_3

induced a 20-40% increase in steady state SPARC transcript levels in all of the cell lines as well as the primary fetal myocytes. Therefore, it appears that TGF- β_3 minimally upregulated SPARC production through transcription. Post-transcriptional upregulation in the absence of any transcriptional induction has been demonstrated for SPARC in other cell types (Wrana et al., 1991). Therefore, if our hypothesis that TGF- β significantly increases SPARC production by cardiomyocytes and therefore promotes angiogenesis in the ventricle is correct, TGF- β_3 would have to mediate its effects translationally as the increases in transcripts were minimal. To determine whether TGF- β_3 increased translation from pre-existing SPARC transcripts, Western blot analyses were performed using conditioned media proteins to detect changes in the levels of SPARC secreted by the cells. Measuring the amount of SPARC protein secreted into the media has been a standard determinant of the translation from SPARC transcripts by a cell in culture (Iruela-Arispe et al., 1991b; Wrana et al., 1991). As such, Western blot analyses indicated that no further upregulation of SPARC secretion occurred through increased translation from the existing SPARC transcripts in response to TGF- β_3 in any of the cell types. While promoter data for the fetal and BWEM lines support these data, the CLEM and CL-B5 lines have conflicting results. Similar to the cTNC study, CLEM exhibited a decrease of ~35% in promoter activity, while there was no significant change in activity in the CL-B5 line in response to TGF- β_3 . However, unlike cTNC, it would be difficult to explain these results in terms of mRNA stability as SPARC has a half life of ~50 hours (Wrana et

al., 1991). It is also of interest to note that the basal promoter activities for CL-B5 are significantly higher than those for CLEM (Figure 23c). This is reflected in the Western blot analyses (Figure 25) in which it appears that CL-B5 secreted higher levels of SPARC protein as compared to CLEM. However, this is not reflective of the relative transcript levels. In contrast to CL-B5, there is no significant difference between BW-H1 and BWEM for SPARC promoter activity, transcript or protein levels. The higher basal promoter activity in CL-B5 may be attributed to an altered transcription factor or regulatory protein content in this mutant line. While the results obtained from these experiments vary slightly from cell line to cell line, a general conclusion can be drawn that TGF- β_3 does not significantly upregulate SPARC expression in and secretion from the fetal cardiomyocytes or the myocyte-like cell lines.

It should be noted that the basal expression of SPARC, both mRNA and protein, is very high in both the primary myocytes and the cell lines. It has also been shown that secreted SPARC protein *in vivo* is rapidly degraded, and thus, the majority of SPARC found in tissues is stored within the cell until it is needed (Sage et al., 1989; Reed and Sage, 1996). Therefore, it would be unwise to conclude that cardiomyocyte-produced SPARC plays no role in promoting angiogenesis or that TGF- β doesn't have a role in this process. However, it can be concluded that TGF- β_3 does not significantly increase SPARC production by the cardiomyocyte under the conditions examined. As SPARC is stored in cytoplasmic granules *in vivo*, TGF- β may provide a regulatory "trigger" which releases SPARC into the

extracellular space when appropriate, selectively increasing the level of SPARC into the ECM. As discussed previously, SPARC mediates most of its actions, particularly angiogenesis, only after cleavage by proteases such as plasmin. This provides another mechanism for TGF- β to promote angiogenesis in relation to SPARC. As a modulator of ECM remodeling, TGF- β has been shown to increase the production of several secreted proteases. Since some of these same proteases may be involved in SPARC activation, TGF- β may promote angiogenesis by inducing the production and/or secretion of these SPARC activators from the cardiomyocytes. It should also be stressed that TGF- β may also promote SPARC production/secretion and angiogenesis in the ventricle by inducing similar changes by paracrine mechanisms of action in the surrounding non-myocyte cells whose numbers increase dramatically around and after birth.

Use of Myocyte-like Cell Lines and Their Respective Mutants

The BWEM and CLEM myocyte-like cells lines are, theoretically, a model system in which aspects of cardiomyocyte development and muscle-specific activities can be evaluated. As stated previously, these cell lines present certain limitations since they have lost some myocyte-specific markers, such as the expression of myosin heavy chain, and their spontaneous contractile activity after their transformation with *v-myc*. While other contractile proteins such as cTNC are readily detected in these

lines, in the experiments described here, transcripts for two other thin filament proteins, TNI and TNT, were found to be very low or undetectable using total RNA. Similarly, the basal promoter activities for the reporter constructs of these two contractile proteins examined were significantly lower than those observed in the primary cultures. As such, these low transcript levels and basal promoter activities are difficult to interpret, especially when a downregulation in expression level or activity is anticipated or observed. Conversion of these promoter constructs to the more sensitive luciferase based reporter may result in the accumulation of data from the cell lines, as well as the primary myocytes, which are more clearly defined and easier to interpret. Additionally, as demonstrated for cTNC, the results obtained in these cell lines may not reflect what occurs in the primary fetal cardiomyocytes. Therefore, the use of these cell lines as a cardiomyocyte developmental model has limitations dependent on the particular aspect being examined.

It has been demonstrated that these cell lines are useful in examining aspects of cardiomyocyte developmental function such as the roles cardiac and muscle-specific transcription factors play in contractile gene expression (G.L. Engelmann, unpublished data). Having low TNI and TNT transcript levels, these cell lines may be useful to identify transcription factors involved in the activation of these or other genes directly related to the contractile machinery of the beating cardiomyocyte. Theoretically, through the introduction of exogenous transcription factors by transfection of selective expression vectors, those factors involved in TNT

or TNI transcription may be identified by their ability to increase levels of these transcripts to those seen in primary cardiomyocytes. Since these cells are much easier to manipulate than primary fetal and neonatal cardiomyocytes, both knock-out and knock-in experimental systems are available as tools to study molecular events involved in cardiomyocyte gene regulation in a myocyte-like background.

As described here, these cell lines can be useful to study and possibly identify components of the TGF- β signal transduction pathways in a myocyte-like background. While not the focus of this dissertation, the results obtained for cTNC in regards to the dual mechanisms of control revealed by promoter and Northern blot analyses suggest that these cell lines, both parental and mutant, provide a system in which the mechanism(s) used to elicit the pleiotropic effects of TGF- β can be examined in greater detail. As cell numbers are not a rate-limiting factor, co-treatment of cell cultures with TGF- β along with drugs, toxins, or other chemicals can be used to interrupt various known signaling mechanisms (G-proteins, kinases, etc.) and identify those pathways that may elicit the specific TGF- β response. Using the transcriptional and post-transcriptional regulation of cTNC expression as a model, these types of experiments could identify the type of pathways which regulate the two different responses. Additionally, with the recent, and future, identification of proteins which are involved in the signaling pathways of the TGF- β superfamily, the dominant negative mutant cell lines could be used to identify those proteins which may confer a specific response.

Overexpression of an active form of these proteins in the DN lines may result in the rescue of only one, transcriptional or post-transcriptional, mechanism of cTNC regulation; an effect which may not be easily discernible in the parental lines; and very difficult to examine in an *in vivo* situation.

Conclusion

It appears that the role of TGF- β in the late fetal/ early neonatal period of heart development is not as direct as we originally hypothesized. We and others have provided *in vitro* data which supports the first part of our hypothesis: biologically active TGF- β can directly inhibit proliferation of ventricular cardiomyocytes. While the expression patterns of TGF- β_1 , TGF- β_3 , and β RII temporally coincide with the changes in cTNC, TNI, TNT, and SPARC expression in the developing ventricle, they do not appear to be functionally related to each other. It is important to keep in mind that the increased production of TGF- β ligand as detected at the transcript and protein level during this period may not be an indicator of TGF- β bioactivity as TGF- β is usually secreted as a latent complex. *In situ* data have been obtained from avian embryonic hearts which demonstrate that a majority of TGF- β_3 located in the ECM is in its latent form (Ghosh and Brauer, 1996). Therefore, functional activity of TGF- β in heart development may be regulated by carefully restricting its level of activation, and not by its general expression levels or that of its required receptor, β RII.

As a mediator of the hypertrophic response to pressure overload, TGF- β may also be a mediator of the mechanical stress that will be exerted after birth as the newborn has to adjust to an air breathing environment. As such, the onset of expression of TGF- $\beta_{1/3}$ and β RII during the late fetal period may serve to prime the myocardium for a proper response to this stress or other physiological stimuli after birth. The onset/increase of expression of β RII deems the cells competent to respond to TGF- β activation. Similarly, as the majority of TGF- β released into the ECM is biologically inactive, or latent, the onset/increase in expression of TGF- $\beta_{1/3}$ in the late fetal period provides a large “stock” of TGF- β which can be readily activated as conditions require. While the exact mechanism of activation *in vivo* is unclear, TGF- β can be activated *in vitro* by extreme pH, heat, or proteolytic enzymes.

In contrast to our hypothesis which states that TGF- β affects ventricular remodeling through the promotion of ECM accumulation and angiogenesis, it would appear that cardiomyocyte-derived TGF- β may act more as a negative regulator for these processes, keeping each in check. Through the induction of PAI-1, TGF- β both promotes and inhibits ventricular remodeling, allowing ECM accumulation, yet inhibiting the transient breakdown of ECM components needed for remodeling and cellular migration. Similarly, the induction of PAI-1 may actually inhibit angiogenesis with regards to the mechanism of activation of SPARC. As mentioned previously, SPARC must be cleaved by plasmin to induce angiogenesis, and PAI-1 inhibits the formation of active plasmin.

Therefore, TGF- β , while having some pro-angiogenic activities, also provides a regulatory mechanism to keep the process in check.

Interestingly, TGF- β through two separate mechanisms, appears to keep its own activity in check. While TGF- β_1 has been found to autoinduce its expression (MacLellan et al., 1993; Flanders et al., 1995), co-induction of PAI-1 inhibits the activation of plasmin, one of several proteolytic enzymes found to release bioactive TGF- β from its latent form, thus downregulating TGF- β 's activity. Secondly, as demonstrated here, TGF- β_3 appears to downregulate a critical component of its own signal transduction machinery by reducing the steady state transcript levels of β RII after prolonged exposure. TGF- β Rs have been found to undergo rapid internalization upon ligand binding, being replaced almost immediately by new receptors (Massague and Kelly, 1986; Centrella et al., 1996). As a result, downregulation of the steady state transcript levels for β RII may gradually slow down its replenishment at the cell surface, thereby downregulating the cells' ability to respond to TGF- β . Therefore, it appears that a complex web of positive and negative controls involving TGF- β exist which could play a regulatory role in the proper development of the cardiomyocyte and maintenance of ventricular tissue architectural and functional homeostasis.

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The final copies have been examined by the director of Patricia S. Grutkoski and the signature which appears below verifies the fact that any necessary changes have been incorporated and that the dissertation is now given final approval by the committee with reference to content and form.

The dissertation is, therefore, accepted in partial fulfillment of the requirements for the degree of Doctor of Philosophy.

3-31-97

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