Effects of CT-1, TGF-β_1, and PDGF on the proliferation of primary cardiac myofibroblasts and wound contraction in cell culture

A Thesis submitted to the Department of Graduate Studies
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For the partial fulfillment of requirement for the degree of
Master in Science in Physiology

By

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A Thesis/Practicum submitted to the Faculty of Graduate Studies of The University of Manitoba in partial fulfillment of the requirement of the degree

of

MASTER OF SCIENCE

This thesis is dedicated to the memory of

Roxanne Chartier

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Abstract

The incidence of heart failure among the world population has reached epidemic levels and myocardial infarction (MI) is a major contributor to both morbidity and mortality levels worldwide. After MI, cardiac myofibroblasts play a major role in wound healing and collagen production that occurs in the infarcted and remnant myocardium, thereby contributing to infarct scar formation and cardiac fibrosis. Activation of cytokine and growth factor signaling pathways mediate many processes implicated in post-MI wound healing leading to cardiac fibrosis and therefore represent therapeutic targets for intervention and modification of inappropriate wound healing and/or cardiac fibrosis. The current literature offers some knowledge of the individual effects of cytokines and growth factors in cell culture. However, very little research has been focused on the combined effects of the specific factors on myofibroblast function.

Our rationale to carry out studies on combinations of mitogens and cytokines that influence myofibroblast function is that these factors are often present in significant concentrations at the same point in healing post-MI myocardium. Some factors such as TGF-β_1 and PDGF are overt pro-fibrotic stimuli, whereas the emerging role of CT-1 may be to suppress some aspects of fibrosis.

Specifically, we sought to investigate the effects of transforming growth factor-β_1 (TGF-β_1) on cardiotrophin-1 (CT-1) and platelet derived growth factor (PDGF) regulated proliferation of adult rat primary cardiac myofibroblasts by measuring the rate of DNA synthesis, by analysis of the cell cycling protein levels, and localization of PCNA. We also investigated the effects of TGF-β_1, CT-1, and PDGF combinations on collagen type I gel deformation (measured as gel contractions) by cardiac myofibroblasts.

We hypothesize that TGF-β_1 opposes CT-1 and PDGF induced proliferation of primary rat cardiac myofibroblasts. We suggest that TGF-β_1 induced down-regulation of proliferation is due to the alteration in the cell cycling protein levels, cyclin E and cdk2, and cell cycle inhibitor p27. We hypothesize that CT-1 does not induce deformation of collagen type I gel whereas TGF-β_1 and PDGF will stimulate deformation of collagen type I gel.

Our findings confirm that CT-1 (10 ng/ml) and PDGF (1 ng/ml) induce proliferation of cardiac myofibroblasts whereas TGF-β_1 (10 ng/ml) suppresses their proliferation. In addressing the influence of TGF-β_1 on CT-1 and PDGF mediated effects, we observed that TGF-β_1 (10 ng/ml) ablates CT-1 induced proliferation and significantly suppresses PDGF induced cell proliferation. Further work indicated that TGF-β_1 increased expression of p27, cell cycle regulatory protein, and decreased expression of cyclin E and cdk2..

In studies of myofibroblasts mediated gel deformation, we found that CT-1 (10 ng/ml) had no effect on the gel shape when compared to control. On the other hand, TGF-β_1 (10 ng/ml) and PDGF (10 ng/ml) treatment of seeded myofibroblasts induced significant collagen gel contraction. Based on our current collagen gel deformation data and previous findings indicating that CT-1 reduces collagen synthesis, we postulate that CT-1 might serve as an anti-fibrotic factor *in vivo* that does not induce deformation of collagen type I gel whereas TGF-β_1 and PDGF will stimulate deformation of collagen type I gel.

II. Introduction

Heart disease is the leading cause of death in the developed world, and as such, is the focus of considerable research effort. Myocardial infarction remains the most common etiology that gives rise to heart failure. Wound healing that ensues in post-MI heart is characterized by time-dependent changes in ventricular architecture not limited to the infarcted site of the myocardium, but also occurring in the non-infarcted (remnant) myocardium (1). Changes in ventricular architecture are facilitated by cellular proliferation and abnormally high collagen secretion. In this regard, cardiac fibroblasts and differentiated myofibroblasts play a central role in this process as they contribute to the expansion of the interstitium (cardiac fibrosis). These cells migrate from the infarct/noninfarct border zone myocardium and enter the infarcted region where they proliferate, thereby restoring cellularity of the affected zone (57). Early after MI (4-6 days) cardiac fibroblasts differentiate into cardiac myofibroblasts (2, 3). Characteristic of cardiac myofibroblasts is their hypersecretion and deposition of collagen, which provides tensile strength to the wound and functions to prevent scar rupture (4, 5). At the same time, cardiac myofibroblasts contribute to progression of cardiac fibrosis by adding excessive collagen deposition, thereby stiffening the myocardium, reducing the pumping ability of the heart and leading ultimately to heart failure.

The availability of cytokines and growth factors in the infarcted region greatly contribute to the progression of wound healing. Specifically, transforming growth factor-β_1 (TGF-β_1) is well described and is a know pleiotropic growth factor that down-regulates proliferation of neonatal cardiac myocytes (6) and of cardiac fibroblasts (7). TGF-β_1 also acts as a stimulus for the transition of cardiac fibroblasts to myofibroblasts (8) as

well as a pro-fibrotic factor that is responsible for increased collagen production and deposition (9). Cardiotrophin-1 (CT-1) is a recently discovered cytokine that is implicated in regulating cell proliferation, cell migration, and production of collagen (10). Protein levels of CT-1 are elevated in serum of patients diagnosed with heart failure (11), unstable angina (12), and aortic stenosis (13). Finally, platelet derived growth factor (PDGF) is behaves as a pro-fibrotic factor in cardiac fibroblasts where it induces increased collagen production and deposition (14) and is found to be a major mitogen (15) component in serum. In vascular smooth muscle cells, PDGF is responsible for induction of DNA synthesis (16). PDGF is also a stimulator of cardiac fibroblast to myofibroblast phenotypic switch (17) and is a potent inducer of cardiac myofibroblast proliferation (18). Due to the known effects of these factors, we have chosen to focus on their interactions in the current study.

Whereas the individual attributes of each factor are known, how these cytokines and growth factors function together and whether they maintain their individual effects has not yet been addressed. We have compared the effects of CT-1, TGF-β_1 and PDGF on the induction of cardiac myofibroblast proliferation and collagen gel deformation. In addition, we hypothesize that TGF-β_1 will oppose the effects of CT-1 and PDGF in cell proliferation assays by affecting the expression of the cell cycling proteins crucial to the progression of cell proliferation. Also, we hypothesize that CT-1 will have no effect on the contraction of 3D collagen matrix where TGF-β_1 and PDGF will induce contraction of the collagen matrix.

Our approach was to examine individual and net effects of cytokines on the proliferation of cardiac myofibroblasts, expression of cell cycling proteins,

contraction of the 3D collagen matrix, and localization of proliferating cell nuclear antigen (PCNA). In addition, we have examined the net effects of co-incubating CT-1, TGF- β_1 and PDGF on proliferation and collagen deformation and have compared these co-incubations to the individual effects of each cytokine.

We found that CT-1 and PDGF stimulate proliferation of cardiac myofibroblasts, weakly and strongly respectively, and that TGF-β_1 inhibits proliferation of these cells. Moreover, TGF-β_1 down-regulates expression of cyclin E and cdk2, which are needed for the cell cycle progression, while increasing the expression of cell cycle inhibitor p27, and in this way is expected to contribute inhibition of cell proliferation. As hypothesized, treatment of cells with CT-1 did not induce deformation of collagen gel, which could be attributed to CT-1's anti-fibrotic feature. Furthermore, TGF-β_1 and PDGF both induced significant gel deformation (reduction in surface area), and thus may be potent inducers of extra cellular matrix (ECM) reorganization.

III Statement of Hypothesis

Our lab has previously documented that in cardiac myofibroblasts, TGF-β_1 acts as a strong pro-fibrotic cytokine (increases production and deposition of collagen) while CT-1 acts to reduce net collagen secretion in these cells. In keeping with this theme, we hypothesize that TGF-β_1 functions to oppose CT-1 and PDGF induced proliferation of primary rat cardiac myofibroblasts. In addition, we hypothesize that CT-1 will have no effect or perhaps exert cellular relaxation and thus have minimal effects on gel deformation whereas TGF-β_1 and PDGF will stimulate collagen gel deformation (in this case manifest as gel contraction). Finally, we also hypothesize that TGF-β_1 induced down-regulation of proliferation is due to increased expression of the cell cycle inhibitor protein p27 and decreased expression of cyclin E and cdk2.

IV Literature Review

1.0 Cardiac disease and its impact on society

Cardiac diseases represent a leading cause of death and a major financial burden on the healthcare system in Canada. According to the Public Health Agency of Canada[1] 37% of the total number of deaths in Canada were attributed to cardiac diseases in 1995. Estimations of the financial impact on the health care system (based on the assessment of the direct and indirect costs related to illnesses) of all illnesses was $159 billion in 1998, wherein direct costs (hospitalization, prescribed drugs, medical care and research) associated with heart diseases amounted to $6.8 billion. More tellingly, the indirect cost of cardiac diseases (value of lost productivity due to illness or disability, and loss of future earnings due to premature death) was equivalent to $11.7 billion. Heart disease surpasses the combined total cost ascribed to injuries, cancer and respiratory diseases combined. Given the magnitude of this fiscal burden placed on society, it is important to pursue research into the pathological mechanisms of the major contributing etiologies to enable development of improved treatments.

1.1 Development and Causes of Heart Failure

Heart failure was defined by Braunwald as "a pathophysiological state in which abnormality of the heart is responsible for the failure of the heart to pump blood at a rate commensurate with the requirements of metabolic tissues" (19). Aside from aging, heart failure is initiated by pre-existing cardiac conditions such as myocardial infarction (MI), cardiomyopathies, valvular heart disease and hypertension. Despite the wide range of

[1] Public Health Agency of Canada:Economic Burden of Illness in Canada 1998. www.phac-aspc.gc.ca/public/ebic-femc98/pdf/ebic1998.pdf

contributing etiologies that contribute to heart failure, MI is the major factor behind mortality and morbidity of heart failure in developed nations (20) and thus this review focuses on the processes that occur post-MI, and in particular with the relationship between myofibroblast function, wound healing, and post-MI cardiac fibrosis. These cells are vital for infarct scar formation eg. wound healing as well as progressive interstitial fibrosis that occurs in the non-infarcted region of the late stage in the post-MI heart. As myocardial fibrosis in post-MI heart is a known contributor to cardiac dysfunction, we will discuss the function of both fibroblast and myofibroblasts.

1.2 Myocardial infarction (MI) and heart failure

Reduced blood flow (ischemia) to a given portion of the myocardium induces an imbalance between blood supply and its demand in myocardial tissue. Most often reduced blood flow is caused by left coronary artery occlusion (21). Occlusion of the artery may develop due to an excess in collagen deposition or from the atherosclerotic plaque build up in the walls of arteries (22). Minutes after the unblocking of the artery, functional abnormalities occur within the reperfused myocardium and can be observed for an additional 24-48 hours (23). Reperfusion of the ischemic myocardium, when oxygen is re-introduced to the tissue, causes a sudden burst of free oxygen radical production. Oxygen radicals function to create other damaging reactive species such as hydrogen radicals, hydrogen peroxide and peroxynitriles that also contribute to the myocardial injury. Over time, injuries due to the lack of oxygen cause irreversible damage to the myocardium, namely myocardial infarct (21).

1.3 Wound healing of infarcted myocardium

Healing of myocardium after myocardial infarct is a complex and active process that involves four stages. The first stage of the healing process is the myocyte dropout that occurs through either apoptosis or overt necrosis (24). Hallmarks of the myocyte death are the release of Troponin-T, fatty acids binding protein and creatine kinase.

Induction of immune response by necrosis marks the second stage of wound healing. Reactive oxygen species also have the potential to trigger inflammatory response by the induction of cytokines (25, 26). The first cells activated after myocardial injury are monocytes that infiltrate the damaged area within an hour of the hypoxic insult. When monocytes reach the area of the injury they are thought to undergo a phenotypic switch into macrophages (23). Macrophages are the source of interleukins that function to induce cell proliferation, phenotype switching of the resident cells and scar formation. Release of interleukin-6 (IL-6) and IL-8 distinguishes this stage from others in wound healing (27, 28). Activation of cells of the immune response, cytokines and neutrophylic granulocytes results in removal of dead myocytes.

The third stage of the healing process is the formation of the replacement tissue and initiation of remodeling of the infarct site in the myocardium. This replacement tissue is called granulation tissue due to its pink granular appearance which arises from the numerous new capillaries that invade the wound stroma (29). It is characterized by proliferating myofibroblasts, new capillaries and nerve growth, and increased activity of matrix metalloproteinases (4). Myofibroblasts are hypersecretory phenotypic variants of fibroblasts that are normally found within healthy myocardium (30). Increased synthesis of collagen and other ECM proteins (proteoglycans, fibronectin, laminin)

contributes to the expansion of interstitial mass (cardiac fibrosis). Neovascularization is the process of formation of new blood vessels that restores blood flow to the damaged tissue. Enhanced activity of matrix metalloproteinases leads to degradation of extracellular matrix (31) and promotes infarct expansion and cardiac rupture.

The fourth stage of wound healing is marked by the formation of the infarct scar that replaces granulation tissue. Infarct scar is not inert but is a metabolically active entity populated with myofibroblasts, new blood vessels, neurons. Myofibroblasts do not undergo apoptosis but persist in this region well after the scar has been formed (3). Cardiac myofibroblasts are important for the function of the heart in the post-MI period as they contribute to the repopulation and contractility of the region that has become devoid of cardiac myocytes. In addition, function of cardiac myofibroblasts is illustrated through their constant involvement in matrix remodeling of the post-MI heart.

1.4 Changes in Post-MI Myocardium

The extent of changes imposed on the post-MI myocardium depends on the size of the infarct zone. An increase in muscle mass (cardiac hypertrophy) is initiated to compensate for the loss of the contractile cells after MI (32). Thus, the extent of cardiac hypertrophy depends on the size of infarction (33, 34). In the event of a large MI, ventricular chambers remodel by increasing in volume (35) and severe hypertrophy that is associated with increased myocyte size and decreased intrinsic cardiac performance (36). Changes that occur in the infarct zone are in many ways mirrored in the non-infarcted (remnant) myocardium. In the post-MI setting, the entire heart is affected by the loss of function of the infarcted region of the myocardium. The infarcted region is unable to contribute to the rhythmic contractions of the heart. As a result, the remnant

myocardium starts to undergo changes to compensate for this loss of function. Non-ischemic myocardium adapts to the increased workload via changing in its architecture (37). Muscle fibers of the heart change their alignment resulting in deterioration of the cardiac pump. Moreover, the connective tissue fraction of the heart is significantly increased in post-MI hearts (38). Change in the architecture of the myocardium impacts the cells composing the myocardium. The increased mechanical load placed on the myocardium (4, 39) influences the function of cardiac myocytes, epithelial cells and fibroblasts. Cardiac myocytes undergo hypertrophy in order to compensate for the loss of the contractile cells in the scar region. In addition, epithelial cells that line vascular vessels proliferate but this is not enough to compensate for the loss of cardiomyocytes and the phenotype of the myocytes reverts to a fetal state. Myocyte fetal state phenotype is identified by lower energy consumption accompanied by a decrease in myocardial function (4). Conversely, fibroblasts are responsible for secretion of ECM components namely collagen type I (5, 40). As they lay down ECM, fibroblasts function in transduction of mechanical stimuli from the ECM to the interior of the cell (myocyte, fibroblast). Parallel to cardiomyocyte hypertrophy, the effect of mechanical load on fibroblast function could be seen in the phenotypic switch of cardiac fibroblasts into their hypersynthetic variants, myofibroblasts, responsible for an increase in synthetic output (41, 42).

1.5 Cardiac Myofibroblast

The main function of myofibroblasts in the infarcted heart is to synthesize and secrete collagen which contributes to the prevention of the scar rupture. The origin of

myofibroblasts can be traced to fibroblasts that are found as a a heterogenous population under normal physiological conditions (41, 43, 44). Cardiac myofibroblasts are described to have a well developed rough endoplasmic reticulum, collagen granules (41, 45) and gap junctions (17). Furthermore, myofibroblasts express α-smooth muscle (α-SM) actin, vimentin, angiotensin I (AT1) receptors, transforming growth factor-β (TGF β) receptors, leukemia inhibitory factor receptor/glycoprotein 130 (LIFR/gp130) receptor, angiotensin converting enzyme (ACE) and fibrillar collagens (46-51). However, the distinguishing feature of cardiac myofibroblasts from cardiac fibroblasts is the expression of smooth muscle embryonic isoform of actin (SMemb) that is observed in the infarct scar (52). Expression of α-SM actin is postulated to contribute to the ability of cardiac myofibroblasts to give tonus to the scar (5) and to play a role in the wound contraction and closure (53). When cardiac fibroblasts are isolated and plated on the compliant substratum in the presence of serum, differentiation into cardiac myofibroblasts is soon to follow. Known stimulators of myofibroblast phenotype induction are PDGF, stem cell factor (SCF) (17) and TGF-β_1 (8). Seeding of cardiac fibroblasts at low density also contributes to a transition into myofibroblasts *in vitro* (8, 54, 55). In addition, mechanical tension has been identified as an *in vivo* stimulator of fibroblast to myofibroblast phenotype switch (56).

1.6 Wound Contraction

The exact mechanism mediating wound contraction is not yet clearly understood. Most of the present knowledge about wound contraction has been influenced by our understanding this process in the dermal cells. Presently there are two popular theories dealing with wound contraction, namely cell contraction and cell traction (57). The

theory of cell contraction relies on the presence of myofibroblasts within the wound. The ability of myofibroblasts to pull the wound together depends on their ability to pull the collagen fibrils toward the cell body. However, the cell traction theory of wound contraction credits fibroblasts' ability to re-organize collagen fibrils while migrating (58) and in this way contribute to the wound closure (59).

The process of wound contraction is the last stage in wound healing after an injury. In the case of MI, wound contraction is the last step in closure of the scarred region. In closure of cutaneous wounds, the cells implicated in wound contraction are activated dermal fibroblasts (60, 61, 62) that are normally found quiescent in the skin. When activated, these fibroblasts from bordering zones of the affected region pull the wound closed. Following wound closure skin myofibroblasts undergo apoptosis. On the other hand, fibroblasts in cardiac tissue migrate from the borderzone of the site of injury to the affected region where they persist even after the process of wound closure has ended (1). These fibroblasts are phenotypically differentiated into myofibroblasts, and the latter cells participate in wound contraction. Phenotypic change appears to require more than one step. Recently, the existence of two populations of myofibroblasts has been postulated (53). Early stage myofibroblasts, which are partially differentiated, are termed as protomyofibroblasts, and represent cells that do not yet express α –SM actin but have the ability to form stress fibers as it is the case with myofibroblasts. Instead of α -SM actin, stress fibers of proto-myofibroblast contain β and γ-actins (56). Examples of these early myofibroblasts could be found *in vivo* as well as *in vitro.* For instance, in lung alveolar septa and in early phases of formation of granulation tissue, myofibroblast features such as expression of stress fibers, but no α -SM actin, are present alluding to

proto-myofibroblasts (53, 60, 61). When fibroblasts are plated onto a plastic surface in the presence of serum they acquire the characteristics of proto-myofibroblasts (53). From *in vivo* and *in vitro* observations it seems that mechanical tension is an important stimulus in mediating the fibroblast to proto-myofibroblast transition (56).

Proto-myofibroblasts are stimulated by TGF-β_1 to transform into myofibroblasts (8) featuring expression of α -SM actin in *de novo* fashion (62-64). When cells are stimulated with TGFβ_1, the cellular fibronectin splice variant ED-A is needed for transition from proto-myofibroblast to myofibroblast (8, 65, 66). Aside from cells of the immune system, TGF-β_1 is produced by myocytes and fibroblasts which helps in preservation of fibrogenic activity and and acts as a stimulus for phenotypic switching once the inflammatory stimulus ends (53, 67, 68). Also, TGF-β_1 is produced by epithelial cells thus exerting a paracrine function on fibroblasts (69).

So far, three systems of collagen gel deformation have been utilized in representation of *in vitro* models of wound contraction: floating matrix contraction (reduction in gel diameter), anchored matrix contraction (reduction in height) and stress fiber relaxation. The difference between the varius models of gel contraction lies in the different distribution of tension. In the model of floating matrix, tension is distributed isotropically whereas in stress fiber relaxation mechanical stress develops while matrix is anchored but it dissipates after the matrix is released (70). In the case of anchored gel matrix, plated fibroblasts orient along the lines of tension and develop stress fibers, fibronexus junctions and resemble myofibroblasts (71, 72). Therefore, cell phenotype could be affected by the contraction that a particular type of gel will facilitate (73).

2.0 Myofibroblast Cell Cycle

2.1 General Introduction

The role of the cell cycle is the accurate duplication of genomic DNA and subsequent equal distribution of chromosomes to daughter cells (74). DNA duplication occurs during the synthesis (S) phase (75) and takes about 10-12 hours in a mammalian cell. Segregation of duplicated DNA occurs through the process of mitosis that takes place in M phase (76). This process is the shortest phase of the cell cycle as it takes approximately one hour to complete in mammalian cells. There are seven stages of mitosis namely interphase, prophase, metaphase, anaphase, telophase and cytokinesis, which marks the end of cell division (77). Proliferating cells require time to duplicate organelles and double the protein mass which is needed to accommodate the formation of two cells at the end of cell division. In order to provide the time and conditions necessary for the cell growth there are two gap (G) phases inserted between the S and M phase. G1 phase is involved in progression of the cell cycle from mitosis to S phase. When the cell is found in G1 phase it is subject to extracellular stimuli, which if unfavorable can delay cell cycle progression. From G1 phase cells can either enter G0 phase, which is associated with a latency period in cell proliferation, or progress to the next stage of cell cycle (78, 79). For some cell types that are thought to be terminally differentiated, it is speculated that these cells have entered G0 phase. When conditions are favorable a cell can re-enter G1 phase and progress towards S phase. However, before S phase is reached, the cell has to meet favorable conditions and pass a checkpoint termed 'restriction point' (80). After cells move beyond the restriction point, DNA replication will take place even if external stimuli are removed (78). After DNA is replicated, the cell enters G2 phase

during which it undergoes mitosis. At the beginning of the mitotic process the cell has already completed G1, S phase, and G2 which are collectively known as interphase.

2.1 Control of the cell cycle

Control of the cell cycle is exerted through positive and negative stimulation of cell cycle progression (81). A focus of this review will be directed toward the cell cycle and its changes in non-cancerous cells. Progression of the cell cycle is dependent on the presence of different protein complexes that function in alleviating internal blocks to cell cycle progression, namely cyclins, cyclin dependent kinases (CDKs), and cyclin dependent kinase inhibitors (CDKIs).

CDKs are a stably expressed family of proteins that govern the phase-to-phase transition of the cell cycle. The activity of CDKs is directly proportional to the levels of cyclins and CDKI whose expression fluctuates depending on the stage of the cell cycle (82, 83). Cyclins are 56 kDa proteins that belong to a family of proteins structurally identified by containing a conserved box regions (84). There are 12 cyclins described to date and they are categorized into five groups from A to E (77).

Cyclins are positive effectors of CDK and as such are needed to form a protein complex together with CDKs that is required for cell cycle progression. Formation of cyclinD/cdk4 or cdk6 complex is crucial in the early stage of G1 whereas cyclinE/ cdk2 complex is necessary in passing of a restriction point to S phase (85). The net result of G1 CDKs activity is phosphorylation of retinoblastoma (Rb) protein. Unphosphorylated Rb protein sequesters E2F transcription factors preventing them from inducing transcription of target genes involved in the S phase progression. Rb is increasingly phosphorylated through mid to late G1 phase first by cyclinD-cdk4 and cdk6 and later by

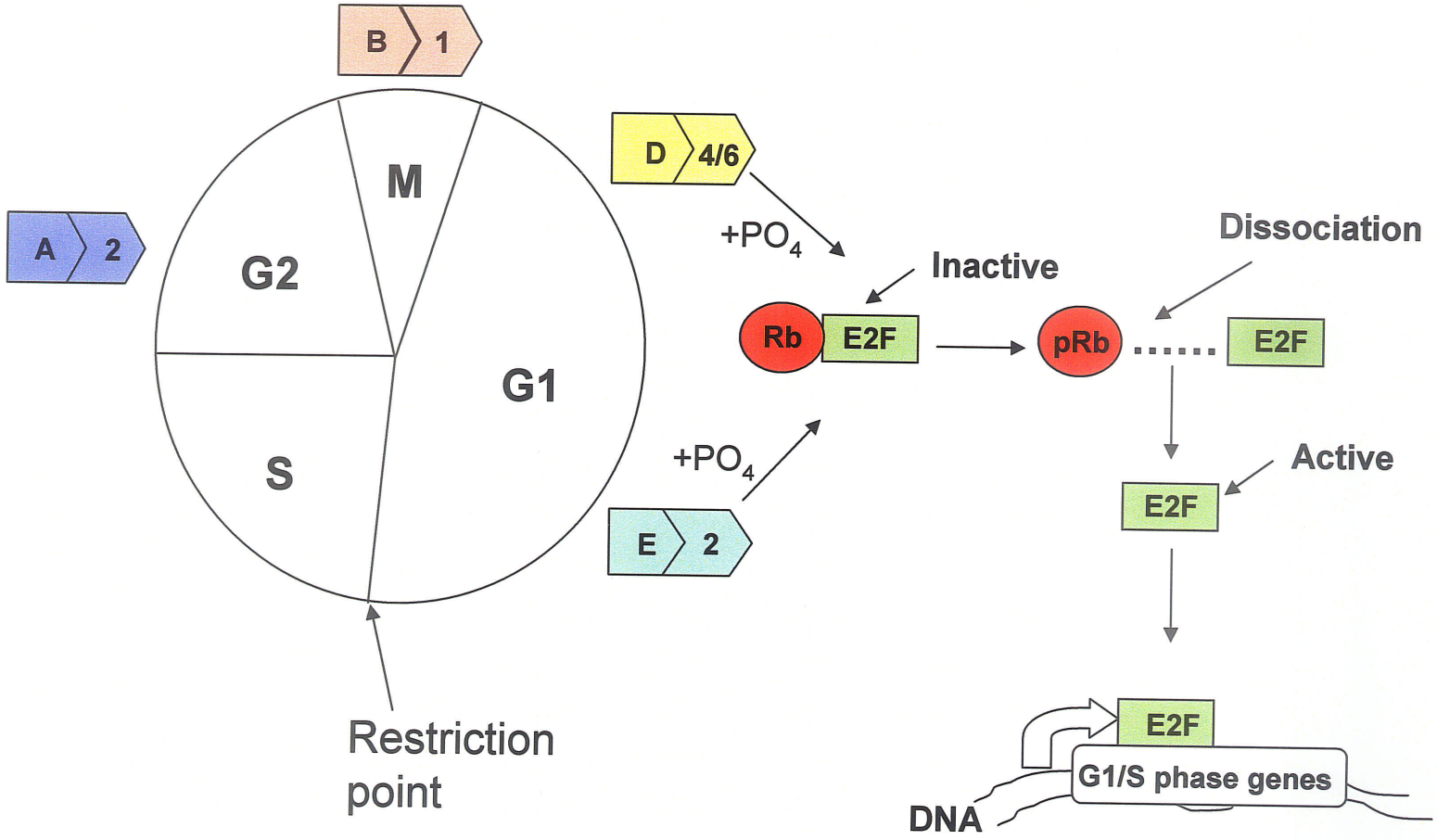


Figure 1. Schematic representation of the cell cycle. G-gap phase, M-mitosis, S-synthesis phase. A,B,D,E refer to specific cyclin proteins; 2, 4, 6 refer to specific cdk associated with a particular cyclin protein; Rb-retinoblastoma protein; PO_4 - phosphate group.

cyclinE/cdk2 complex (75, 85, 86). Thus, the hyperphosphorylated state of Rb is an indicator that the cells are highly proliferative. Expression of cyclin A and cyclin B is low throughout most of the cell cycle but increases during transition from G2 to M phase (83). After entry into S phase cyclinA/cdk2 complex functions to maintain the hyperphosphorylated (inactive) state of Rb and cyclinB/cdk1 functions in the same way during the M phase ensuring an undisturbed traverse from S phase into M phase.

Cyclin-dependent kinase inhibitors are negative effectors of CDKs. In mammalian cells, two classes of inhibitors exist, namely the Ink4 and Cip/Kip family that provide a tissue-specific mechanism by which cell cycle progression is restrained by extracellular and intracellular signals (87). The Ink4 family is composed of p15, p16, p18, and p19 proteins and they specifically inhibit formation of cyclinD/cdk4 or cdk6 complexes. The Cip/Kip family includes p21, p27, and p57 and they preferentially bind to G1/S class of CDKs (inhibiton of cyclin E/cdk2 complex). Following addition of TGF-β_1 it has been observed that p27 inhibits the activity of cyclinE/cdk2 causing a cell cycle arrest (88). Alterations in the expression of CDK inhibitors lead to prevention of G1to S transition thereby inhibiting cell proliferation. In non-transformed cells, progression of the cell cycle is also dependent on adherence of the cell to the substratum (89). Non-adherent cells fail to phosphorylate retinoblastoma (Rb) protein and to activate cyclinE/cdk2 complex (90). However, cells need to be momentarily detached to complete mitosis and cytokinesis. In the case of detachment from substratum, focal adhesions (FAs) must be assembled and disassembled which is mediated by focal adhesion kinase (FAK) (91-93).

3.0 Cardiotrophin-1 (CT-1) introduction

CT-1 was isolated in 1995 based on its ability to induce hypertrophy of neonatal cardiac myocytes *in vitro* (94). CT-1 has been placed into interleukin-6 (IL-6) superfamily based on the similarity in its structure to the rest of the IL-6 members. Other members of the family, besides interleukin-6 (IL-6) and CT-1, include leukemia inhibitory factor (LIF), ciliary neurotrophic factor (CNTF), oncostatin M (OSM), and IL-11. All of these proteins share a common transmembrane signal transducer named glycoprotein 130 (gp130) (95-98) which results in to the functional redundancy of the IL-6 family (99).

3.1 Expression of CT-1 and its *in vivo* function

CT-1 is a pleiotrophic cytokine that modulates the transmitter phenotype of sympathetic neurons (100), promotes survival of dopaminergic neurons and spinal motoneurons, inhibits differentiation of embryonic stem cells, induces acute phase protein expression in hepatocytes and to induce osteoclast differentiation (101, 102). In addition CT-1 inhibits growth of mouse myeloid leukemic M1 cells (99). Protein levels of CT-1 are expressed at high levels in several tissues in adult mice and in large quantities in human heart and skeletal muscle, lungs, kidneys and brain giving rise to the possibility of pleiotrophic effects of CT-1 *in vivo* as well as in cultured cardiomyocytes (99). Aside from stimulating growth of the heart, CT-1 induces liver, kidney and spleen growth (103). The effect of CT-1 administration intravenously is evident in spontaneously hypertensive rats (104) where it causes a drop in mean arterial pressure and reflex increase in heart rate without affecting cardiac output (105). Administration of CT-1 by intraperitoneal injection leads to increased liver, spleen, and kidney weight as

well as to increased heart to body ratio while reducing thymus weight (103).

3.2 CT-1 signaling pathways

CT-1 signaling is transmitted through a receptor complex composed of LIFR and gp 130. As neither of the receptors have any intrinsic kinase activity CT-1 stimulation results in activation and auto/trans-phosphorylation of the gp130 associated Janus kinase 1 (Jak1), Jak2 and Tyk2 (99, 106-108). Once activated Jaks will phosphorylate the cytoplasmic domain of gp130 creating docking sites for SH2 domain proteins such as signal transducer and activator of transcription 1 (STAT1) and STAT3 (99, 109-111). Stats become tyrosine phosphorylated (99, 111) and form homo or heterodimers that translocate into the nucleus where they will bind DNA and cause transcription of target genes (112). Intracellular pathways triggered by CT-1 include extracellular signal regulated kinase (ERK), mitogen activated protein kinase (MAPK), the janus kinase (JAK), signal transducer and activator of transcription (STAT), and phosphoinositide-3K (PI-3K) (113). Downstream mediators of CT-1 signaling effects include nuclear factor-κB (NF-κB), and heat shock proteins 56, 70, 90. CT-1 induction of NF-κB was implied as the mechanism of cardioprotection activated by CT-1 during hypoxia reperfusion injury (114).

3.3 CT-1 expression in pathological settings

Elevated levels of CT-1 protein have been found in the serum of patients diagnosed with heart failure and unstable angina (12), myocardial infarction (11). In addition, patients with aortic restenosis had elevated levels of CT-1 (115). Patients with end stage cardiomyopathy undergoing heart transplantation had elevated levels of CT-1

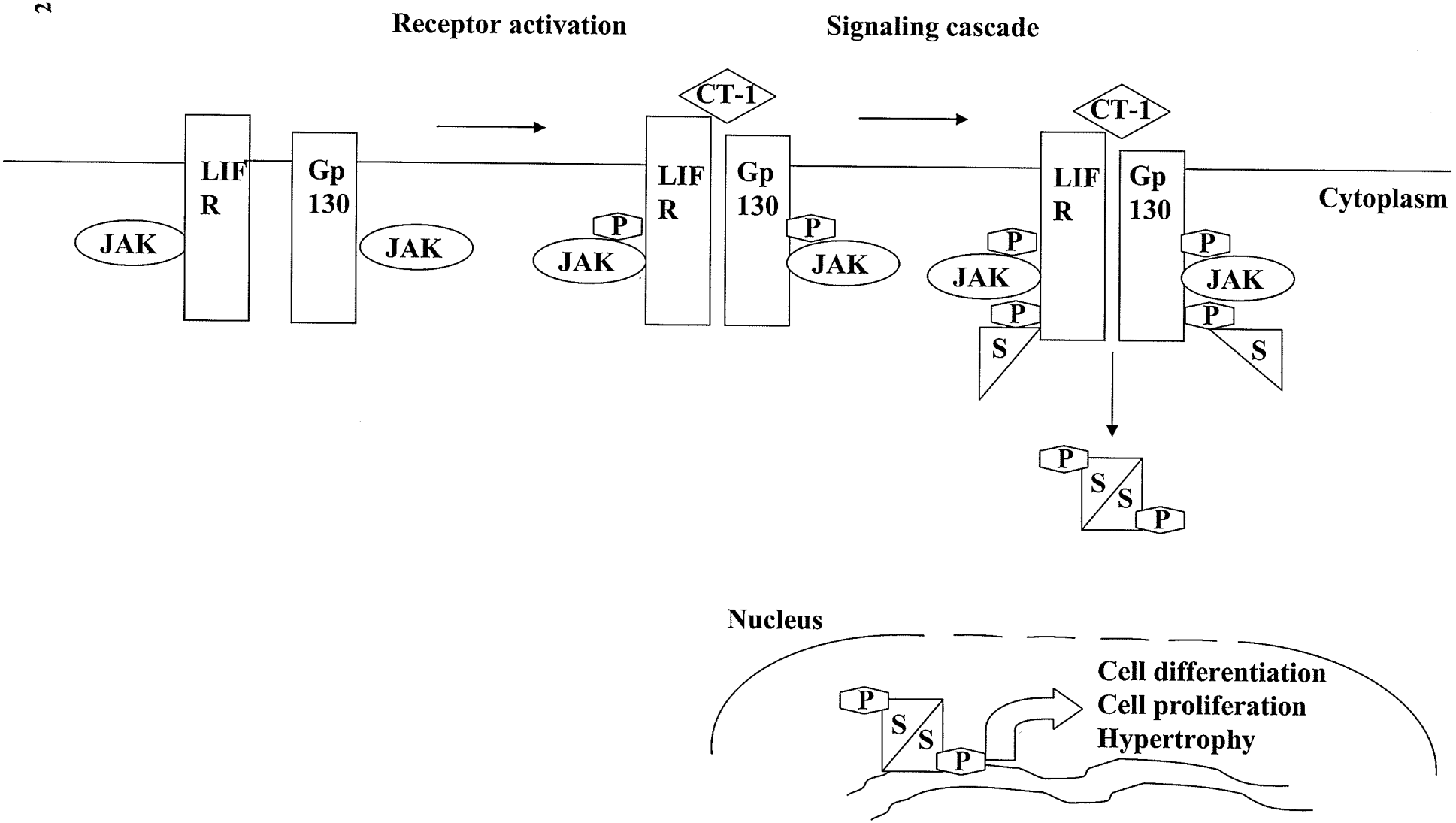


Figure 2. Schematic depicting CT-1 activation of the gp-130 receptor and downstream signaling events. JAK-janus activated kinase, LIFR-leukemia inhibitory factor receptor, P-phosphate group, S-STAT.

mRNA and protein (116). Moreover, receptors of CT-1 eg. gp130 and LIFR have been observed in relatively high levels in the ventricles from rats with myocardial infarction as well as throughout the chronic phase of wound healing (117). In the ventricles of genetically hypertensive rats increased levels of CT-1 mRNA have been detected (118, 119) as well as in the ventricles of rats subjected to acute pressure overload induced by ligation of abdominal aorta distal to renal arteries (120). Increase in the expression of CT-1 mRNA is induced by mechanical stretch in cardiomyocytes (121). Conversely, CT-1 protein is induced by angiotensin II in cardiac fibroblasts(122). In addition, norepinephrine (123), isoproterenol (98) and hypoxic stress increase mRNA of CT-1 (124).

3.4 Cytoprotection by CT-1 and induction of cardiac hypertrophy

CT-1 was identified as a potent stimulator of cardiomyocyte hypertrophy *in vitro*. However, the hypertrophy induced by CT-1 results in assembly of sarcomeres in series leading to an increase in cardiomyocyte cell length (94, 125). Therefore, CT-1 causes a different hypertrophic program than all of the other hypertrophic agents studied that stimulate assembly of sarcomeres in parallel rather than in series. Activation of the CT-1 receptor results in transduction of the signals for transcription of hypertrophic genes in the cardiomyocytes, and at the same time stimulate transduction of cardio-protective pathways. Some of the mediators of the transduction of the signaling pathways induced by CT-1 include kinases such as Src, PI3, MAPK, and STATs whose activation leads to hypertrophy and protection from ischemia/reperfusion injury (126). Cytoprotection by CT-1 is evident in the prevention of apoptosis of serum starved neonatal ventricular myocytes by induction of PI3K (126) and MAPK (127, 128). In the case of the ischemia

reperfusion model, CT-1 was able to act as a cytoprotective agent when added before simulated ischemia or when added at re-oxygenation (128, 129). CT-1 also appears to be cytoprotective in other organs namely brain, where it was found to delay onset of motor impairment and axonal degeneration (130). Furthermore, subcutaneous administration of CT-1 in a motor neuron disease model reversed the decline in neurological and muscle function (131).

4.0 Introduction to TGF-β Signaling Pathway

Members of TGF-β superfamily are categorized into different families based on the similarity in their structure and function into: TGF-βs, activins and inhibins, bone morphogenetic proteins (BMPs), growth and differentiation factors (GDFs), and more distantly related Mullerian inhibitory substance (MIS) and glial cell line-derived neutrophic factor (GDNF) (132). TGF-β signaling regulates cell proliferation, differentiation, migration, apoptosis, the extracellular matrix (ECM) production, and angiogenesis (133). TGF-β is expressed in the normal and hypertrophied myocardium (134, 135). TGF-β impairs cell motility (8, 136) and down-regulates proliferation of cardiac fibroblasts (7). Disturbance of the TGF-β pathway leads to a loss of cell growth regulation which can trigger the occurrence of various diseases such as cancer, tissue fibrosis, vascular disorders and auto-immune diseases (133, 137, 138). In addition, alterations in TGF-β pathway lead to initiation and progression of cardiac hypertrophy, ventricular remodeling and heart failure (5, 7, 46, 137).

4.1 TGF-β isoforms and receptors

The TGF-β superfamily consists of more than thirty proteins (BMP and others) of similar structure. Three TGF-β isoforms are identified in mammalian heart tissues, i.e. TGF-β_1, TGF-β_2, and TGF-β_3 (139). Type II receptors are 70 kDa glycoproteins that are members of a family of trans-membrane serine-threonine receptor kinases (140). Type II receptors have three domains: a cysteine-rich extracellular domain, a single membrane spanning domain, and an intracellular Ser-Thr kinase domain followed by a Ser-Thr-rich C-terminal extension. The kinase domain is capable of autophosphorylation on Ser-Thr residues *in vitro*. Type I receptors belong to a family of related transmembrane Ser/Thr kinases. These 55 kDa glycoproteins have four regions: an extracellular extension, cytoplasmic juxtamembrane region, a serine-glycine repeat region and a C-terminal Ser-Thr kinase domain (141). Type II and type I receptors are related to each other through conserved cysteines that are found in their extracellular part. Diversity in extracellular sequences of these two types of TGF-β superfamily receptors is thought to contribute to the specificity of ligand-receptor interactions.

4.2 Mechanism of receptor activation

TGF-β signaling is initiated through ligand binding to the receptor that subsequently undergoes oligomerization. Ligand binding to the type II receptor initiates recruitment of the type I receptor (142, 143). When the type I receptor finds itself in proximity to the activated type II receptor transphosphorylation occurs. Multiple serine residues (144) need to be phosphorylated to propagate the signal in TGF-β receptors where TβRII transphosphorylates TβRI (140). TβRI is activated when it becomes phosphorylated and is then capable of transmitting the TGFβ signal.

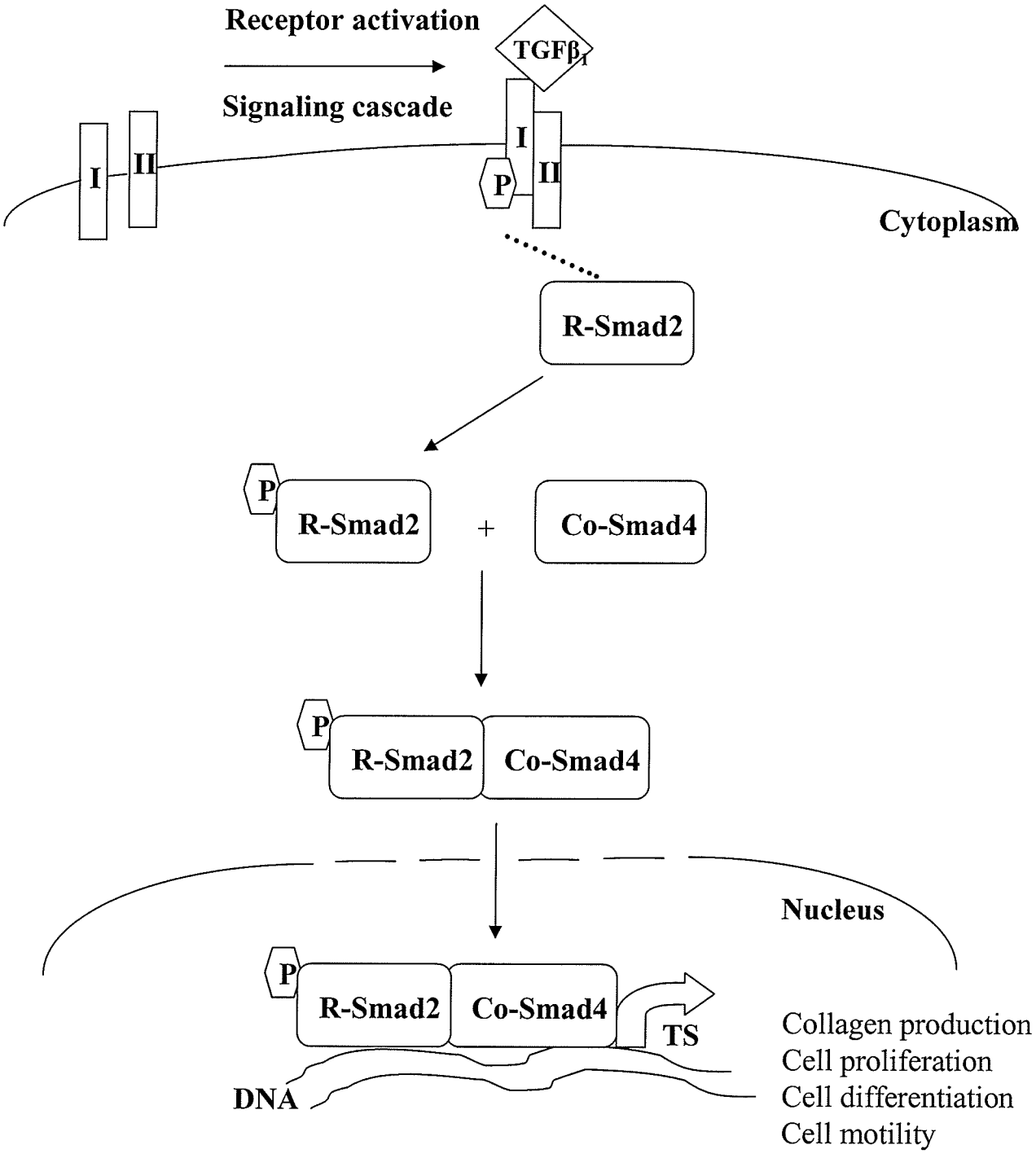


Figure 3. Schematic depicting TGF β_1 signaling in myofibroblasts. I-TGF β receptor I, II-TGF β receptor II, P-phosphate group, TS-transcription.

4.2.1 Fate of activated receptors

A common fate of the receptor upon ligand binding is its internalization from the plasma membrane after which the receptor is recycled back to the cell surface. However, another way of regulation of receptors levels after ligand binding involves receptor down-regulation through endocytosis (145). In the terms of TGFβ ligand binding, transphosphorylation of the type I receptors leads to rapid down-regulation of ligand-bound receptors mediated by inhibitory-Smad (I-Smad) which are bound to Smurf ubiquitin ligases (146).

4.3 Signal transducers in TGF β pathway

The main mediators of the TGF-β superfamily signaling are Smad proteins. The name for these signal transducers was drawn from *sma* and *Mad* genes that came from genetic studies of *C.elegans* and *Drosophila* respectively (147). To date there are three categories of Smads: the receptor-regulated Smads (R-Smads), the common Smads (Co-Smads), and inhibitory Smads (I-Smads) which are the major intracellular mediators of TGF β signaling (141). R-Smads and Co-Smads are found in the cytoplasm. These two intracellular proteins have the Mad homology 1 and 2 (MH1 and MH2) regions that are highly conserved sequences at the N- and C- termini and a linker section. I-Smads are similar to R-Smads with the exception that their MH1 domain is poorly conserved.

4.3.1 Mechanism of Smad signaling and regulator of receptor availability

Transduction of TGF-β_1 signaling is initiated by type I receptor phosphorylation of R-Smads. Phosphorylation of R-Smad 2 by TGF-β_1 and activin receptors on two terminal serines in the SSXS motif is required for its activation (148). Disabled-2 (Dab-2)

is an adaptor molecule that associates with TGF-β receptors, Smad2 and Smad3 and is necessary for the phosphorylation of R-Smads (149).

There are two proteins known to interact with R-Smad2 and R-Smad3: Smad anchor for receptor activation (SARA) (150) and Hgs (151). Each of these proteins contain a double zinc-finger, and a FYVE domain, that binds phosphatidyl inositol-3-phosphate (PI-3P) in the plasma membrane (152). SARA is specific to the TGFβ-activin-responsive Smads but not to the BMP responsive Smads. Specificity of SARA to R-Smad2 and R-Smad3 is conferred by the presence of arginine residues in these R-Smads that are lacking in Smad1 (153).

4.3.2 Nuclear entry of R-Smad2/Co-Smad4 complex

Phosphorylation of R-Smad2 leads to heteromerization of this protein with Smad4. Smad4 belongs to the common receptor subgroup of Smads and interacts with TGF-β and BMP-activated R-Smads (154) as well as with TβRI. Smad4 can not be phosphorylated by TβRI because it lacks a SSXS sequence. Instead, Smad4 is shuttled to the membrane by TβRI-associated protein-1 (TRAP1) which strongly associates with TGF-β and activin receptors and is released upon receptor activation. The heterotrimeric Smad complex enters the nucleus upon its formation (155).

A number of mechanisms regulate subcellular localization of Smads. In the case of Smad3 a nuclear localization signal in the N-terminus is responsible for its subcellular localization (156). On the other hand, MH2 domain of Smad2 is speculated to allow for its nuclear localization. In Smad4 the main mechanism for subcellular localization is a nuclear export sequence (NES) found in the linker region (157). Once in the nucleus, R-Smad/Co-Smad complex can interact with DNA. In the case of Smad3/Smad4

complex, interaction with DNA is achieved through direct binding to specific sequences termed Smad-binding elements (SBE; 5'-CAGAC-3'). SBEs are found in many TGF-β responsive promoter regions such as PAI-1, type VII collagen, and α2(I) procollagen. Binding of Smad4 to SBE is done via the MH1 domain of Smad4, which contains a β hairpin loop necessary for indirect DNA contact (133). In addition, the R-Smad2/Co-Smad4 complex can bind DNA indirectly with the help of DNA-transcriptional adaptors.

4.3.3 Regulators of Smad transcription

Smad complex binds its binding elements (5'-CAGAC-3' and 5'-GTCT-3') under physiological conditions with a low affinity. Therefore, DNA partner binding is needed to confer specificity of Smad complex binding to the target genes. Binding co-factors are a group of proteins with a high affinity for activated Smads when they are associated with specific DNA sequences (141). One group of proteins functions as DNA-binding adaptors and the other group as transcriptional partners. DNA-binding adaptors lack intrinsic transcriptional activity and they include Olf-associated zinc finger (OAZ) for R-Smad1, Forkhead Activin signal transducer (FAST) known as FoxH1 (158) and Mixer for R-Smad2 (141). Transcriptional partners differ from adapters because they have the ability to function independently from R-Smads. Transciptional partners of R-Smads are Jun B, transcription factor binding to immmonoglobulin heavy constant mu enhancer-3 (TFE-3), core-binding factor A/acute myeloneous leukemia proteins (CBFA/AML), lymphoid enhancer-binding factor-1/T-cell-specific factor (LEF/TCF). In addition, depending on activation or repression of specific genes R-Smads will bind other specific factors to facilitate their binding to DNA such as co-activators p300, CREB binding

protein (CBP), FOXH1 and co-repressors such as TG 3-interacting factor (TGIF), Sloan-Kettering Institute proto-oncogene (Ski) and Ski-related novel gene N (Sno N) (141). Intensity in the response to a given TGF-β/R-Smad signal is determined by the activation levels of co-repressor and co-activators of TGF-β_1 signaling (159).

5.0 Platelet Derived Growth Factor (PDGF) - an introduction

PDGF was originally purified from platelets (160). However, it is known that PDGF is expressed by cells that infiltrate the arteries in response to pathological stimuli (161). PDGF has been described as a potent mitogen for cells of mesenchymal origin including myofibroblasts (162). In addition, PDGF induces chemotaxis, actin reorganization and prevents apoptic cell death (163;164). PDGF has been observed to increase synthesis of collagen and to have the ability to stimulate remodeling of extracellular matrix (165).

5.1 Structure and expression of PDGF and receptors

PDGF is composed of two polypeptide chains –A and –B. Members of PDGF family occur in homo- or heterodimer form (166). The three dimensional structure of PDGF is similar to that of VEGF and TGF β is not similar to TGF-β at the amino acid level. Synthesis of PDGF is stimulated by low oxygen tension (167) in target cells and addition of various growth factors, cytokines and thrombin (168).

PDGF isoforms exert their effects on target cells by activating two protein tyrosine kinase receptors, α- and β-receptors (169). PDGF isoforms bind both receptors simultaneously resulting in dimerization upon receptor binding. PDGF-AA homodimer binds both receptors with high affinity while PDGF-BB homodimer binds only β-receptor

with high affinity. Another combination of receptor binding creates PDGF-AB which is caused by dimerization of one α- and one β-receptor. Different isoforms of PDGF have different effects on the target cells. Homodimers contribute most of the information throught the PDGF receptors whereas heterodimers are thought to confer unique properties in the PDGF signaling. In turn, fibroblasts and vascular smooth muscle cells express α- and β-receptors (170). However, response of the cell to PDGF stimulation is going to depend on the type of PDGF receptors present in the cell. Furthermore, the level of the expression of PDGF receptors in cells is not constant. For instance, stimulation of vascular smooth muscle cells with bFGF increases expression of α-receptor but not the β-receptor (171) whereas lipopolysaccharide treatment upregulates β-receptor but not α-receptor in rat lung myofibroblasts (172). In addition, TGF β_1 stimulation of fibroblasts leads to a decreased expression of the α-receptor (162, 173, 174). Conversely, PDGF stimulation of adult rat cardiac fibroblasts results in induction of cell proliferation (18).

5.1.1 Activation of PDGF receptors

Simultaneous binding of two receptors by PDGF ligand constructs a bridge between the two receptors. Bridging of the receptors brings them into the close proximity and enables autophosphoylation to occur. Autophosphorylation serves two functions: phosphorylation of conserved tyrosine residue inside the kinase domains (Tyr-849 in the α-receptor and Tyr-857 in β-receptor) leading to an increase in the catalytic efficiencies of the kinases (175, 176); autophosphorylation of tyrosine residues located outside the kinase domain creates docking sites for signal transduction molecules containing SH2 domain (177).

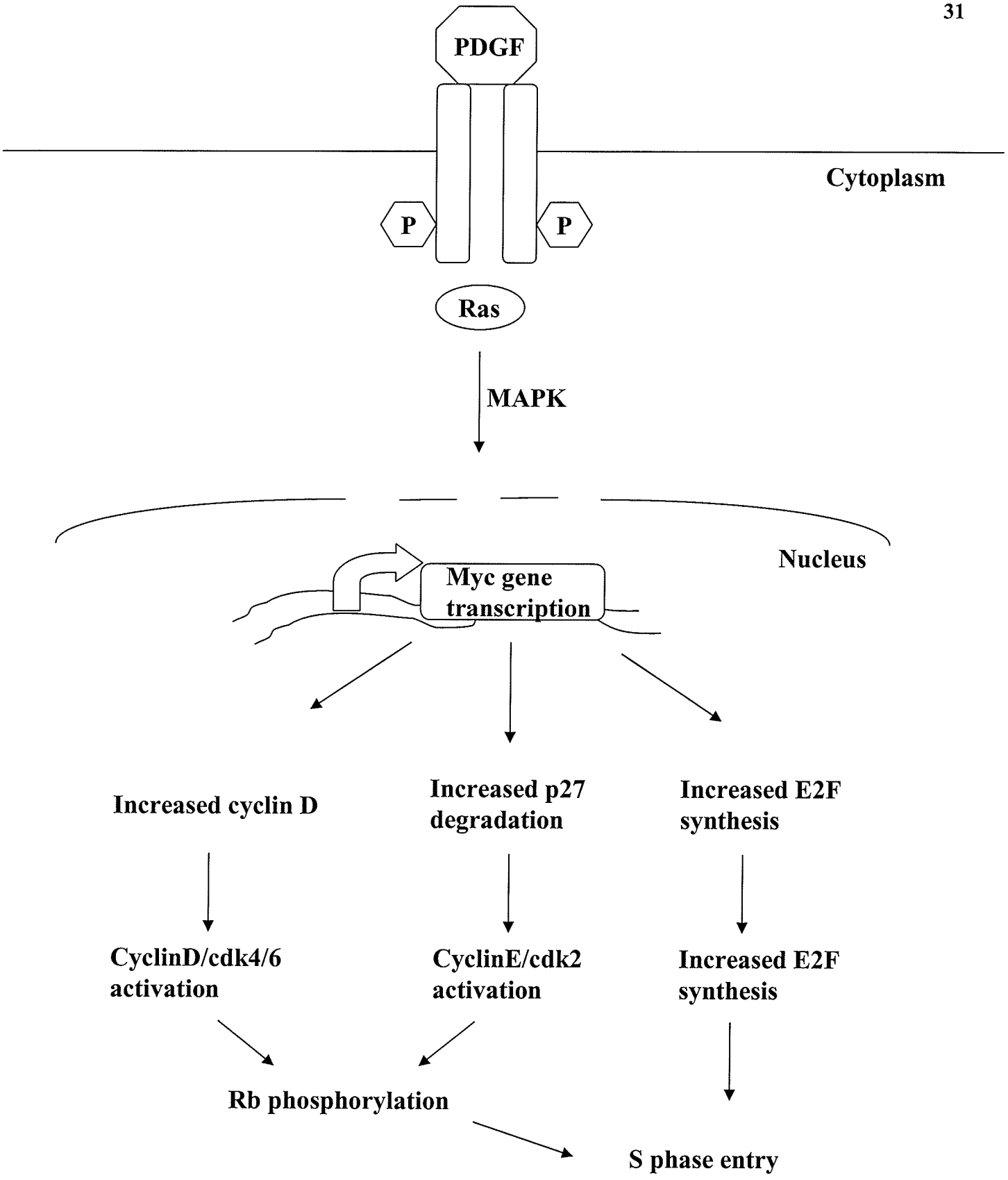


Figure 4. Schematic depicting PDGF signaling in the cardiac myofibroblasts. MAPK-mitogen activated protein kinase; P-phosphate group; Rb- retinoblastoma protein; S-synthesis phase

5.1.2 Binding of SH2 domain proteins to activated PDGF receptors

The SH2 domain is a conserved amino acid sequence of about 100 amino acid residues that can bind phosphorylated tyrosine residues under specific conditions. For example, phosphatidylinositol-3 kinase (PI-3K), phospholipase C (PLC-γ), the Src family of tyrosine kinases, tyrosine phosphatases SHP-2 and GTPase activating protein (GAP) for Ras (178) are some of the proteins that can be activated. Adaptor molecules binding PDGF receptors include Grb2, Grb7, Nck, Shc, and Crk. These proteins function in linking the receptors to downstream catalytic molecules. Members of STAT family also bind PDGF receptors, undergo dimerization and translocate to the nucleus affecting the transcription of specific genes (179).

5.1.3 Receptor fate upon ligand binding

Mitogenic response to PDGF is proportional to the availability of its receptors. Upon ligand binding, the ligand-receptor complex is internalized into endosomes (180). Once the receptor is internalized into the endosome it can have two fates: the ligand-receptor complex dissociates and the receptor is recycled back to the cell membrane, or the endosome fuses with lysosomes where the ligand-receptor complex is degraded. Moreover, the PDGF receptor can undergo cytoplasmic degradation in proteosomes after ubiquination (181). Internalization of PDGF receptor is dependent on the kinase activity of the receptor.

5.2 Control of PDGF signaling

Signaling through PDGF receptors is controlled in a positive and negative manner by activation of stimulatory and inhibitory signals. Tyrosine phosphorylation

induced by PDGF receptor dimerization is counteracted by activation of tyrosine phosphatases (182). In addition, extracellular modulation through interaction with matrix proteins occurs as well as intracellular modulation through cross-talk with different signaling pathways. PDGF stimulates cell cycle progression and cell growth. Namely, the mitogenic and growth factor effect of PDGF are mediated by activation of the Ras protein leading to activation of mitogen activated protein (MAP) kinase and PI3-K respectively (183). In turn, MAPK activation also regulates the activity of Ras protein by phosphorylation and inactivation of Sos protein resulting in a decreased activity of Ras (184). Moreover, angiotensin II influences PDGF function by delaying PDGF induced DNA synthesis in vascular smooth muscle cells (185).

5.3 PDGF function *in vivo*

PDGF is essential for the proper embryonic development of kidneys, blood vessels, lungs and central nervous system (CNS). PDGF receptors were found to be expressed on capillary endothelial cells (186, 187). In addition, PDGF was shown to have a pro- angiogenic effect (188). In the instance of wound healing, PDGF presence at the site of the wound is required. In turn, PDGF is responsible for mitogenic and chemotactic effect on fibroblasts and smooth muscle cells as well as for the chemotaxis of neutrophils and macrophages (189). Specifically, in PDGF-null mice there is a lack of capillary pericytes and alveolar proto-myofibroblasts (53, 187). Furthermore, PDGF stimulates production of collagen (190), fibronectin (191), proteoglycans (192), and hyaluronic acid (193) production. Upon stimulation with PDGF fibroblasts secrete collagenase which could impact on thereorganization of the extracellular matrix (194). A role of PDGF in

wound contraction is strengthened with the evidence of induction of collagen matrix contraction (*in vitro* model of wound contraction) (165, 195).

V. Materials and Methods

1. Materials

Culture media (Dulbecco's Modified Eagle Medium, DMEM/F-12, and Minimum Essential Medium, SMEM), fetal bovine serum (FBS), and antibiotics (penicillin, streptomycin) were purchased from GIBCO BRL (Grand Island, NY). Culture plates and multiwell culture dishes were obtained from Fisher Scientific (Whitby, ON, Canada). Collagen bovine solution was purchased from Stem Cell Technologies (Vancouver, BC, Canada). Primary antibodies specific for PCNA were ordered from Upstate Biotechnology (Lake Placid, NY). Primary antibody for cyclin E and cdk2 were obtained from Biosource. Primary antibody specific to p27 was ordered from Cell Signaling Technology, Inc.(Beverly, MA). Prestained low-molecular-weight marker; secondary Antibodies (anti-mouse, anti-rabbit) were obtained from Bio-Rad Laboratories (Hercules, CA). Polyvinylidene difluoride (PVDF) blotting membranes were obtained from Roche Diagnostics (Laval, QC, Canada). The enhanced chemiluminescence (ECL+ Plus) and the protein assay kit were purchased from Sigma-Aldrich (Oakville, ON, Canada). Recombinant human CT-1, TGF β_1 and PDGF were purchased from R&D Systems (Minneapolis, MN). Primary antibody against actin was acquired from Santa Cruz Biotechnology (Santa Cruz, CA). Surgical handle and the blades were obtained from Becton-Dickinson Acute Care (Franklin Lakes, New Jersey, US). Whatman pH indicator paper was obtained from Whatman International Ltd.

2. Isolation and Culture of Rat Cardiac Myofibroblasts

Male adult Sprague-Dawley rats in the weight range of 150-175 grams were sacrificed for preparation of cardiac fibroblasts according to the methods of Brilla *et al* with minor modifications (196). Rat hearts were subjected to Langendorff perfusion at a flow rate of 5 ml/min at 37°C with recirculatory Joklik's medium containing 0.1% collagenase and 2% bovine serum albumin (BSA) for 25-30 min. Liberated cells were collected by centrifugation at 2,000 rpm for 5 min, and resuspended in DMEM/F-12. Cells were seeded on 100-mm non-coated culture flasks at 37°C with 5% CO_2, and grown in DMEM/F-12 supplemented with 10% fetal bovine serum (FBS), 100 U/ml penicillin, 100 μg/ml streptomycin, and 1μl/ml ascorbate. Cells which attached to the bottom of the flasks during 3 hour incubation were further maintained in serum supplemented media. Non-adherent cells including myocytes were removed by the media change after 3 hours of incubation. Cells used for this study were of passage one (p1). The purity of fibroblasts was found to be ≥95% using routine phenotyping methods as previously described (69, 197). Briefly, endothelial cells were labeled with the use of a monoclonal antibody against factor VIII, and we found that less than 1% of cultured cells stained positively for this protein. Less than 1% of cells were positive for desmin, a protein specific for smooth muscle cells (SMCs), and less than ~ 1% of cultured cells stained positively for (α-SMA), which is produced in SMCs and myofibroblasts. However, more than 95% of cells stained positively for procollagen type I, which is a major protein product of fibroblast cells.

3. Protein Extraction and Assay

Cell stimulation with CT-1, TGF β_1, PDGF was stopped by rinsing the cells twice

with ice cold phosphate buffered saline (PBS). Lysis of the cells was accomplished by addition of 120 µl of RIPA lysing buffer (pH= 7.6), containing 150 mM NaCl, 1.0% nonidet P-40 (NP-40), 0.5% deoxycholate, 0.1% sodium dodecyl sulfate (SDS), 50 mM Tris, phosphatase inhibitors (10 mM NaF, 1 mM Na_3VO_4, and 1 mM EGTA), and protease inhibitors (4 µM leupeptin, 1 µM pepstatin A, and 0.3 µM aprotinin). Collected cells were allowed to lyse in RIPA buffer on ice for 1 hour. Subsequently, cells were sonicated for 5 seconds. Insoluble portion (membrane fraction) was removed by centrifugation at 14,000 rpm for 15 minutes at 4° C. Supernatant was collected, and stored at -20°C. The total protein concentration of all samples was measured using the bicinchoninic acid (BCA) method as previously described (198).

4. Western Blot Analysis of Target Proteins

The samples of cell lysates were mixed with Laemmli loading buffer (final concentration; 125 mM Tris-HCl (pH=6.8), 5 % glycerol, 2.5 % SDS, 5 % 2-mercaptoethanol, and 0.125 % bromophenol blue), and boiled for 5 min. Equal amounts of protein samples (15 µg) were resolved by 10 % SDS-polyacrylamide gel electrophoresis (SDS-PAGE) with the help of prestained low-molecular-weight marker (10 µl). Separated proteins were electrophoretically transferred onto a 0.45 µM polyvinylidene difluoride (PVDF) membranes. PVDFs were blocked overnight at 4°C in Tris-buffered saline with 0.2 % Tween 20 (TBS-T) containing 3% BSA, and probed with primary antibodies for 1h at room temperature. Primary antibodies were diluted 1:500 in 3% BSA and incubated overnight at 4° C. The incubation period of secondary antibodies was 1 h at room temperature with the dilution 1:2000 in 0.2 % TBS-T containing 1%

BSA. Secondary antibodies included horseradish peroxidase (HRP) anti mouse for recognition of cyclin E and cdk2 and HRP-labeled anti-rabbit for detection of p27. Protein bands on Western blots were visualized by ECL+ Plus according to the manufacturer's instructions, and developed on X-Ray film. Equal protein loading was confirmed by immunoblotting analysis against actin and by incubating PVDF membranes in Ponceau S solution (0.1 % Ponceau S in 5 % acetic acid) for 5-10 seconds.

5. Tritiated Thymidine Incorporation (^{3}H thymidine)

Approximately 2.5 x 10^4 cells per ml (counted with a hemacytometer) were resuspended in DMEM/F12 containing 10% fetal bovine serum (FBS), 100 U/ml penicillin, 100 μg/ml streptomycin, and 1μl/ml ascorbate. Cells attached to the bottom of each well of a 24-well culture plate. Cells were allowed to adhere for 24 hours and then rendered quiescent by incubation in DMEM containing 100μM ascorbate and 100 mM penicillin/streptomycin for 24 hours. Subsequently, cells were stimulated for 24 hours by addition of TGFβ_1 (10 ng/ml), CT-1 (10 ng/ml), PDGF (1 ng/ml) in DMEM containing 1% FBS. For the last 4 hours of stimulation cells were pulse labeled with tritated (^{3}H) thymidine. Cold 20% trichloroacetic acid (TCA) was used to precipitate DNA from cell lysates which were filtered through GF/A filters (Fisher). 3 ml of scintillation fluid (ICN Pharmaceuticals, Costa Mesa, CA) was added to each vial where a dried filter was placed. Beta emission was measured with a scintillation counter (LS6500, Beckman Coulter, Fullerton, CA).

6. Immunohistochemistry

Adult primary cardiac fibroblasts were plated on coverslips, and allowed to grow

for 24 h in DMEM/F-12 containing 10 % FBS until ~60% confluent. Cell growth was arrested by the addition of serum-free media for 48 h. Immunofluorescent staining was performed as previously described (46). This technique was applied to detect PCNA nuclear localization in cells being exposed to (1) TGF-β_1 stimulation; (2) CT-1 stimulation; and (3) PDGF stimulation, (4) stimulation of the cells with CT-1 and TGF-β_1, (5) stimulation of the cells with CT-1 and PDGF, (6) stimulation of the cells with TGF-β_1 and PDGF. Briefly, cells were washed twice with cold PBS, fixed in 4 % paraformaldehyde, rendered permeable with 0.1 % Triton X-100 in PBS, and incubated with PCNA primary antibody over night at 4°C. After being washed with cold PBS, cells were incubated with the anti mouse Texas Red conjugated secondary antibody. The incubation period of the secondary antibody was 90 min at room temperature. Prmary antibody was diluted to 1:50 and secondary antibody was diluted 1:20 in PBS containing 1 % bovine serum albumin (BSA). After being washed with cold PBS, cells were immersed in the nuclear dye Hoescht No. 33342 (10 μg/ml) for visualization of cellular nuclei, and subjected to additional wash with PBS. The slides were examined under a microscope equipped with epifluorescence optics with a digital camera.

7. Collagen Gel Deformation Assay

Briefly, collagen type I gel was prepared by gently mixing 7 ml of cold collagen I solution (3 mg/ml) and 2 ml 5-fold concentrated cold DMEM. The pH of the solution was adjusted with 1M NaOH to 7.4. The final volume was adjusted to 10 ml. 600 μl aliquots were added to each well of the 24-well culture plate (Falcon) and set at 37°C over night to solidify. Once the collagen gel solidified it was measured that 600 μl gel aliquots produced 3 mm thick gel. Suspension of cardiac myofibroblasts ($10x10^4$

cells/ml of DMEM containing 10%FBS) was plated onto each well containing solidified collagen type I gel. Cells were allowed to adhere and grow at 37°C for 24 hours. Cells were rendered quiescent by serum starving them for 24 hours at 37°C. Prior to the cell stimulation collagen type I gel was detached from each well by using a surgical blade (Fisher Scientific). Cells were then stimulated by addition of various cytokines to the media. Wells were photographed at 0 hour, 2hr, 4hr, 6hr and 24 hours after initiation of contraction. Gel area was determined for each well and each treatment using Measure Gel custom made software. Area was plotted as Means ± Sem. Student t-test was used for statistical assessment.

8. Determination of the location of the cells seeded on the collagen matrix

Primary rat adult cardiac myofibroblasts (p1) were seeded on top of the collagen type I gel prepared as described in collagen gel contraction assay. Determination of the cell location in the gel was performed by phase contrast microscopy. Photographs were taken with a NIKON camera attached to a phase contrast microscope (NIKON, Diaphot 300) with total magnification of 100x. Two methods were employed to distinguish the location of the cells i.e. are the cells penetrating the gel on which they are seeded or are they confined to the top of the gel. The first method consisted of putting an eyelash on top of the gel and photographing the field while focusing further away from the top of the gel. The second method employed marking of the bottom of the culture dish. Photographs were taken from the top of the gel while focusing away from the top of the gel towards the bottom of the culture dish.

9. Statistical Analysis

All values are expressed as means ± SE. Student t-test and one-way analysis of variance (ANOVA) were used to compare differences among multiple groups. Significant differences among groups were defined as $P \leq 0.05$.

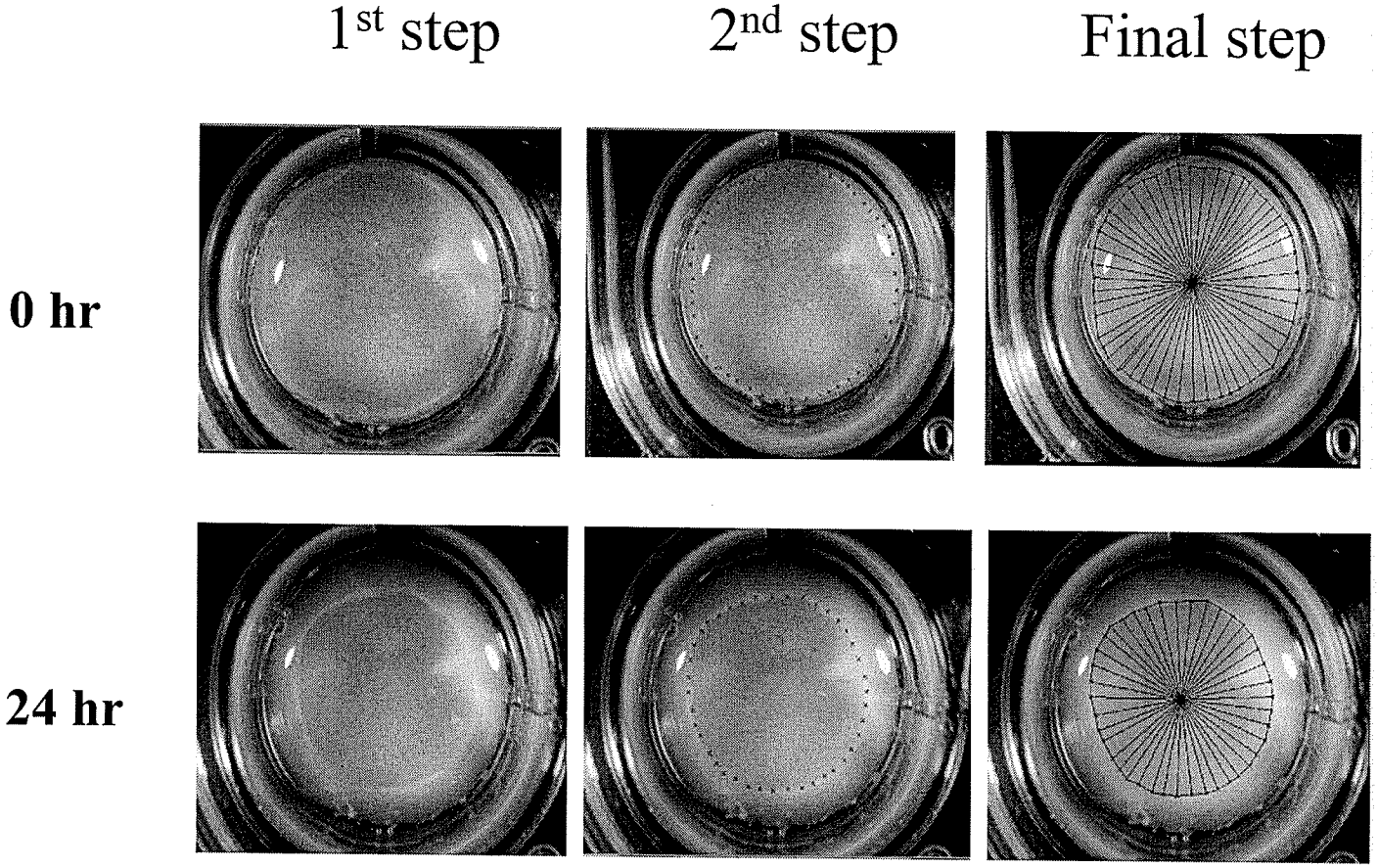


Figure 5. The example of the gel surface area determination using Measure Gel software. Shown are the samples of non-treated cells that have been photographed at 0hr and 24 hr. The same method was used to acquire gel surface area for each treatment and control wells.

VI Results

1. Effect of CT-1, TGF-β_1 and PDGF on P1 cardiac myofibroblast DNA synthesis

In order to assess cell proliferation rates in p1 cardiac myofibroblasts we have measured the incorporation of ^{3}H-thymidine into DNA. We seeded p1 cardiac myofibroblasts into 24 well dishes in 10% FBS DMEM/F12 until they reached 40-50% cell confluency. Cells were rendered quiescent by incubation with 0% FBS DMEM for 24 hours. Stimulation of the cells was performed with CT-1 (10ng/ml), TGF-β_1 (10ng/ml), PDGF (1ng/ml) in the presence of 1% FBS DMEM and incubated for 24 hours. In the last 4 hours of incubation with the cytokines was used to label cells with ^{3}H thymidine. Subsequently, cells were left overnight to be lysed in the lysis buffer.

As shown in our results Figure 6 (and also in Fig. 8) CT-1 and PDGF (Fig. 7 and 8) stimulated DNA synthesis when compared to the control group (1% FBS). In contrast, TGF-β1 inhibited DNA synthesis when compared to the control (Fig. 6, Fig. 7). The stimulatory effects of CT-1 and PDGF were ablated by simultaneous addition of TGF-β1 (Fig. 6 and 7 respectively). Simultaneous addition of CT-1 and PDGF stimulated DNA synthesis to a higher extent than either factor added alone (Fig. 8), suggesting a synergistic effect.

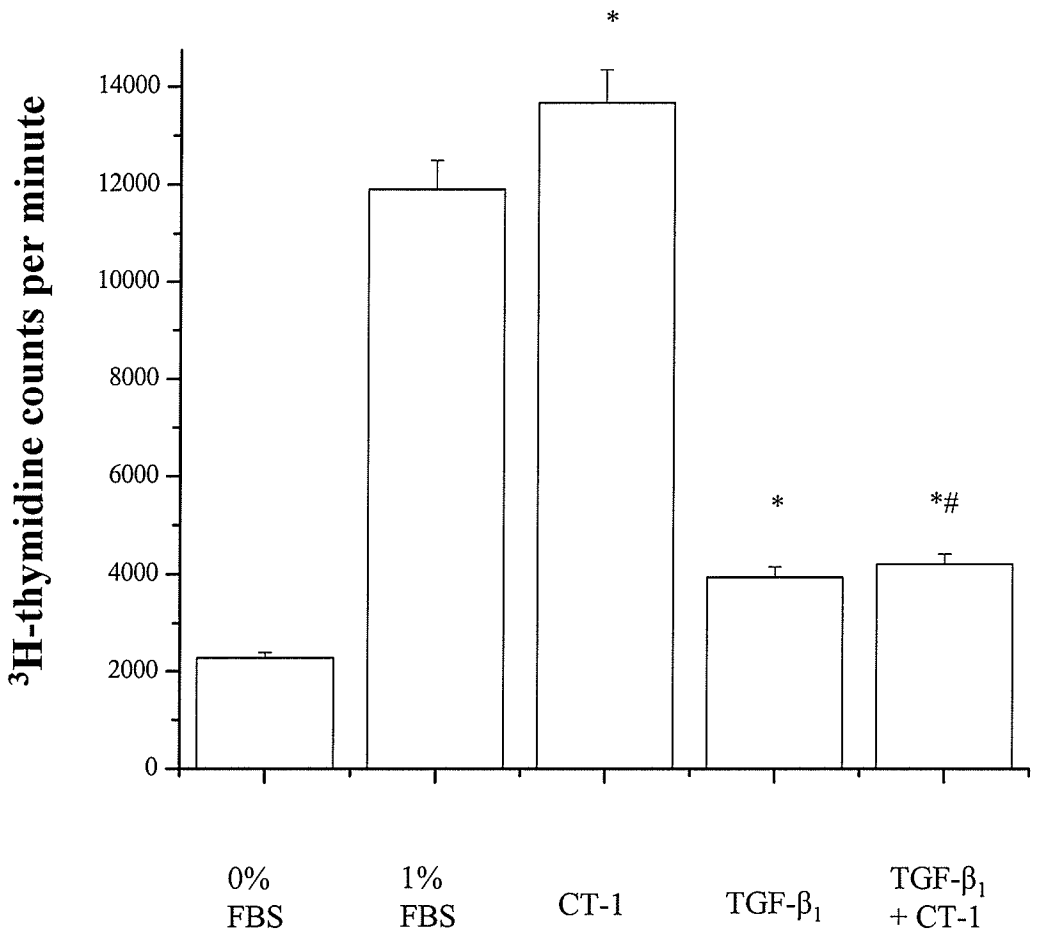


Figure 6. Incorporation of ^{3}H-thymidine into DNA by cardiac myofibroblasts. Cells were grown for 24 hours in 10%FBS DMEM and rendered quiescent by 24 hr incubation in 0%FBS DMEM. Cytokines (CT-1 10 ng/ml; TGF β_1 10 ng/ml) were added in the presence of 1%FBS DMEM and incubated for 24 hours. *$p \leq 0.05$ vs control (1% FBS); # $p \leq 0.05$ vs CT-1. Data are expressed as mean + SEM, n=5. Student t-test was used to check for statistical significance.

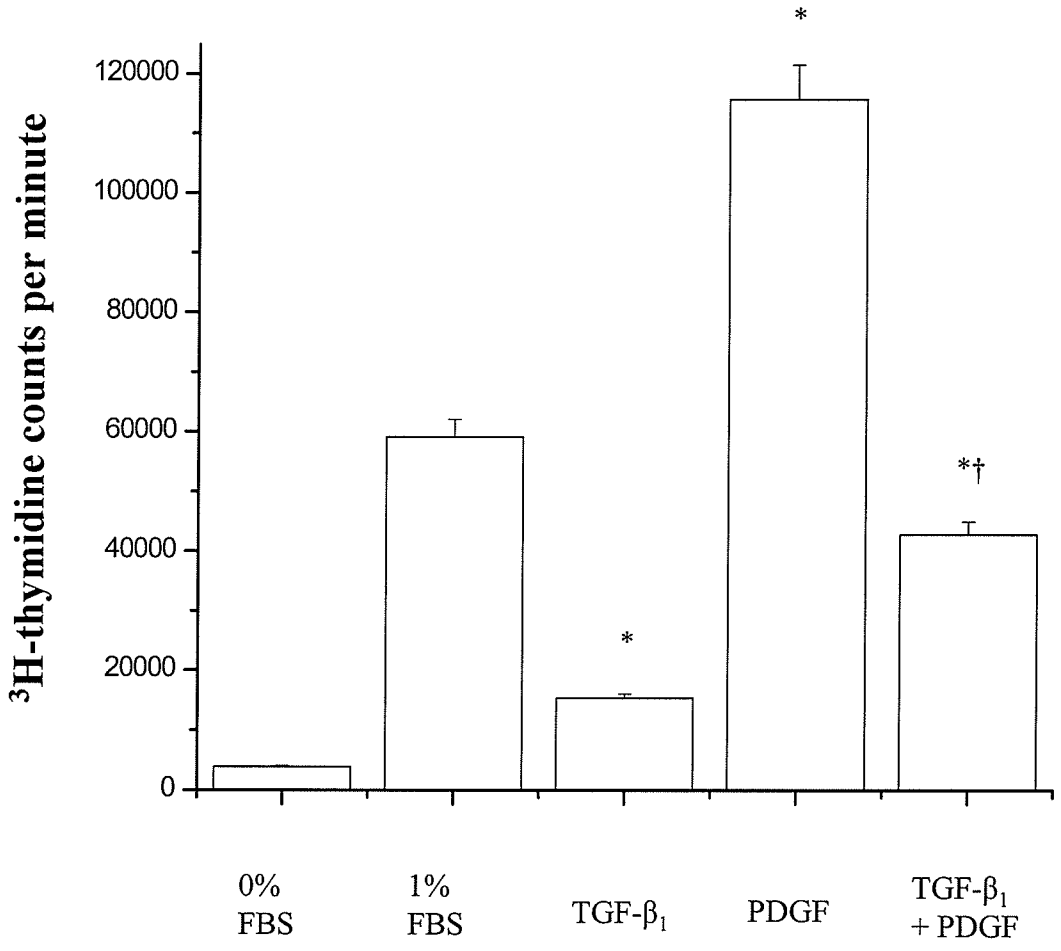


Figure 7. Incorporation of ^{3}H-thymidine into DNA of cardiac myofibroblasts. Cells were grown for 24 hours in 10% FBS DMEM and rendered quiescent by 24 hr incubation in 0%FBS DMEM. Cytokines (TGF β_1 10 ng/ml; PDGF 1ng/ml) were added in the presence of 1%FBS DMEM and incubated for 24 hours. *$p \leq 0.05$ vs control; † $p \leq 0.05$ vs PDGF. Data are expressed as mean + SEM, n=5. Student t-test was used to check for statistical significance.

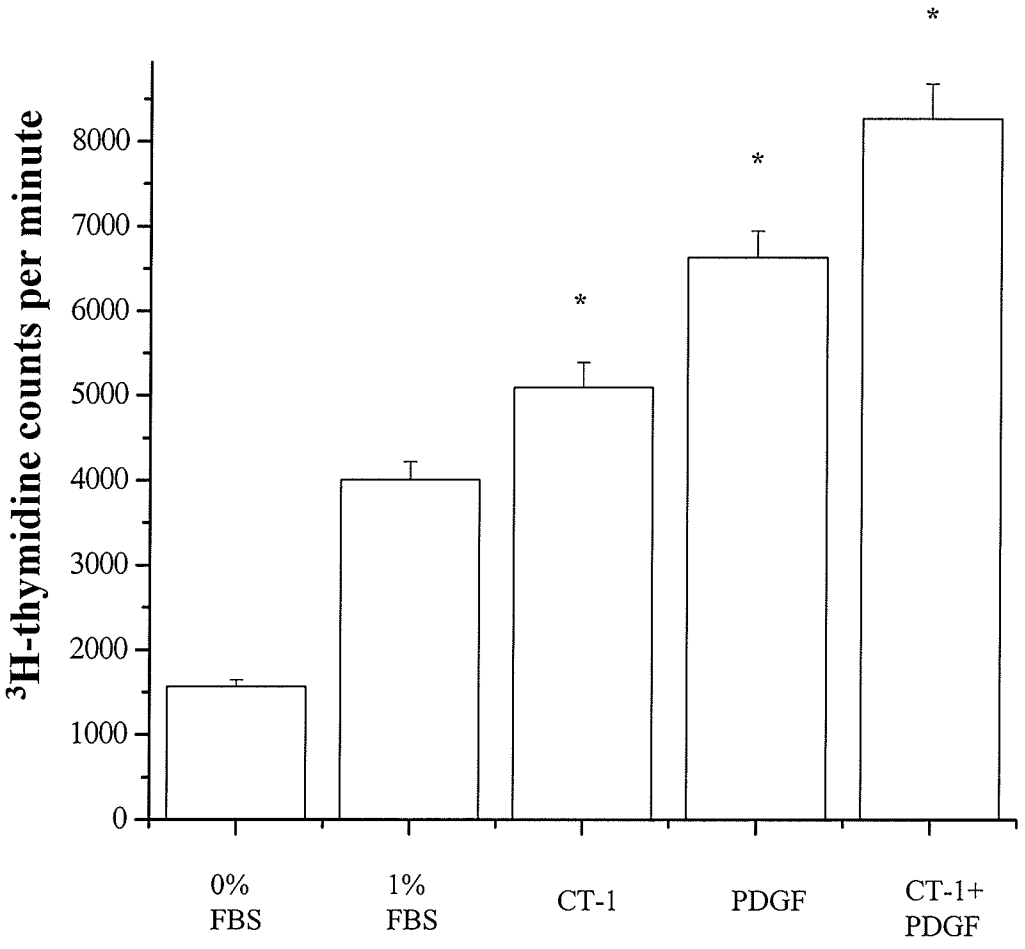
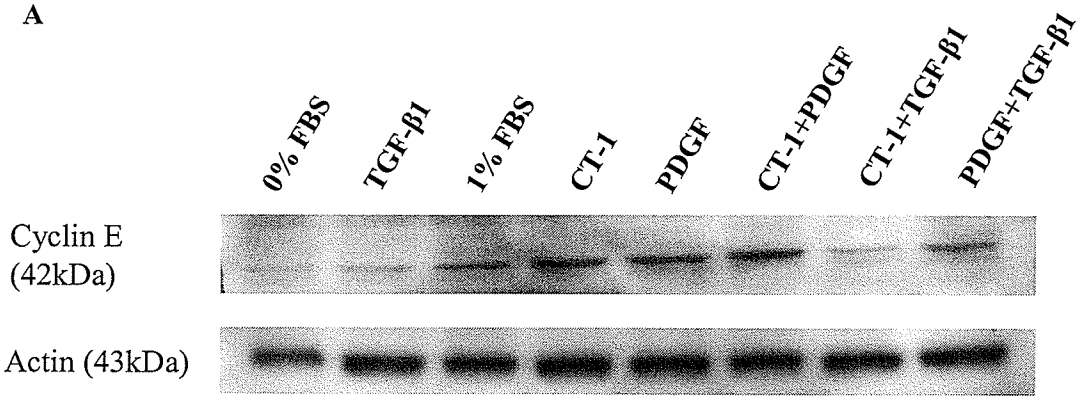


Figure 8. Incorporation of ^{3}H thymidine into DNA of cardiac myofibroblasts. Cells were grown for 24 hours in 10% FBS DMEM and rendered quiescent by 24 hr incubation in 0%FBS DMEM. Cytokines (CT-1 10ng/ml, PDGF 1ng/ml) were added in the presence of 1%FBS DMEM and incubated for 24 hr.*$p \leq 0.05$ vs control (1% FBS). Data are expressed as mean + SEM, n=5. Student t-test was used to check for statistical significance.

2. Effect of CT-1, TGF-β_1 and PDGF on the expression of cyclin E in p1 cardiac myofibroblasts

Expression of cyclin E varies throughout the cell cycle (181). Formation of the cyclin E/cdk2 complex is necessary for the progression of the cell cycle into S phase thereby increased expression of cyclin E is the marker of G1 to S phase transition (181). We have looked at the expression of cyclin E in p1 cardiac myofibroblasts that were grown to 40-50% cell confluency in 10% FBS DMEM/F12 and were rendered quiescent by incubation in 0% FBS DMEM/F12 for 24 hours. Subsequently, cells were stimulated with CT-1 (10ng/ml), TGF-β_1 (10ng/ml), PDGF (1ng/ml) which were added in the presence of 1% FBS DMEM/F12 for the time period of 24 hours.

Our results shown in Figure 9 reveal that TGF-β_1 induced a decrease in the relative expression level of cyclin E. However, treatments of the cells with CT-1 and PDGF alone and in combination have caused a decrease in the expression of cyclin E when compared to the control (1% FBS) but this change was not found to be at a statistically significant level. In addition, when TGF-β_1 was added to either CT-1 or PDGF a decrease in the expression of cyclin E was noted when compared to the control (1% FBS).



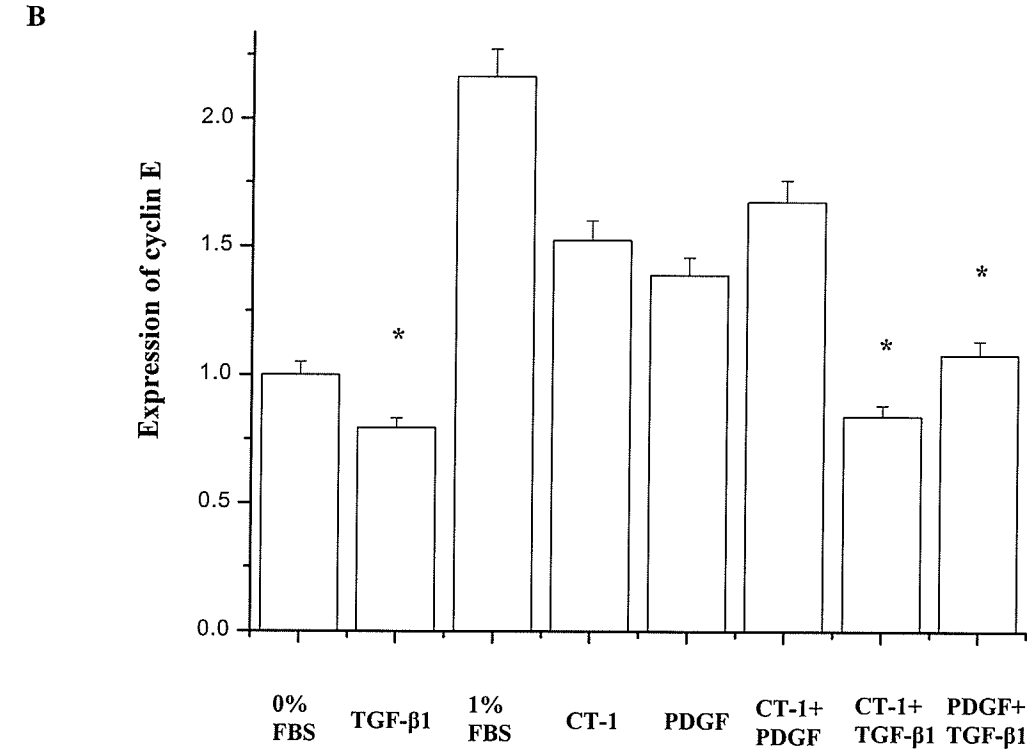


Figure 9. Cyclin E expression in primary cardiac myofibroblasts. **Panel A**: Western blot analysis of cyclin E expression in cultured primary cardiac myofibroblasts (0%FBS starvation for 48 hours, total cell lysates) treated with CT-1 (10ng/ml), TGF-β1 (10ng/ml) or PDGF(1ng/ml) for 24 hours in the presence of 1%FBS. Representative Western blots show expression of cyclin E (~49kDa) and actin (~43 kDa) protein indicating relatively even protein loading. **Panel B:** Histographic representation of quantified data of immunoreactive cyclin E from the groups of samples shown in the panel A (quantified by densitometric scanning); P*≤0.05 vs. control (1%FBS); data are expressed as mean + SEM (n=3).

3. Effect of CT-1, TGFβ_1 and PDGF on the expression of cdk2 in p1 cardiac myofibroblasts

Increased expression of cdk2 is observed during the cell's transition from G1 to S phase. Cdk2 is a crucial component of cyclin E/cdk2 complex which is needed for the cell proliferation process to occur (220). Cells were grown to 40-50% confluency in 10% FBS DMEM/F12 and rendered quiescent by 24 hour incubation in 0% FBS DMEM/F12. Stimulation of the cells with CT-1 (10ng/ml), TGF-β_1 (10ng/ml), PDGF (10ng/ml) in the presence of 1% FBS DMEM/F12 was performed for 24 hours.

Western blot analysis of the cdk2 protein expression in Figure 10 reveals that the CT-1 or PDGF added alone showed a trend towards increased expression of cdk2 but this did not reach a significant statistical level. Treatment of the cells with TGF-β_1 resulted in a decreased expression of cdk2 comparable to the levels in quiescent cells. Co-incubation of TGF-β_1 in combination with either CT-1 or PDGF failed to reverse the inhibitory effect of TGF-β_1 on the expression of cdk2. Co-incubation of PDGF together with CT-1 induced a significant increase in cdk2 levels.

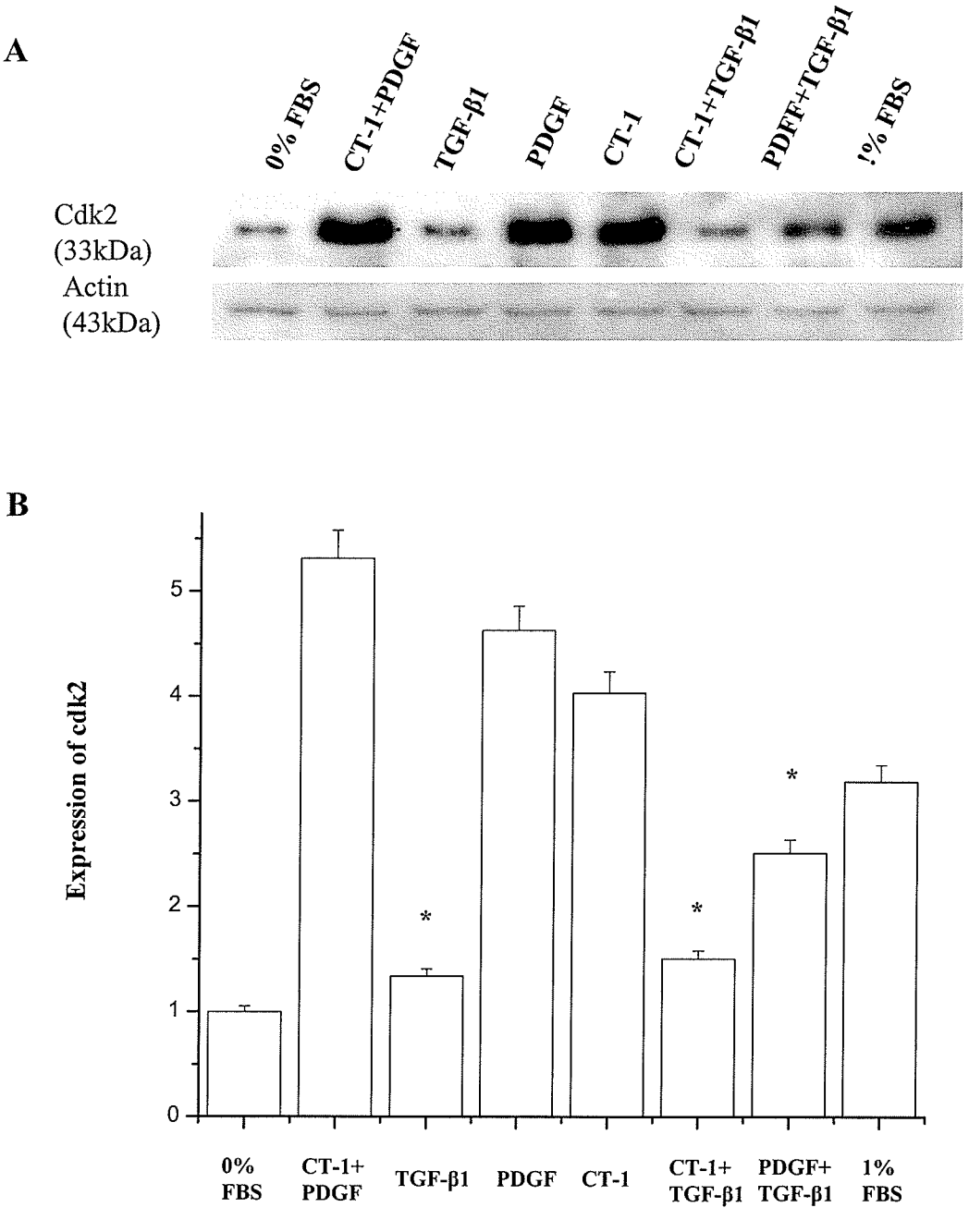


Figure 10. Expression of cdk2 in primary cardiac myofibroblasts. **Panel A**: Western blot analysis of cdk2 expression in cultured primary cardiac myofibroblasts (0%FBS starvation for 48 hours, total cell lysates) treated with CT-1 (10ng/ml), TGF-β1 (10ng/ml) or PDGF (1ng/ml) for 24 hours in the presence of 1%FBS. Representative Western blots show expression of cdk2 (~33 kDa) and actin (~43 kDa) protein indicating relatively even protein loading. **Panel B:** Histographic representation of quantified data of immunoreactive cdk2 from the groups of samples shown in the panel A (quantified by densitometric scanning);P*≤0.05 vs.. control (1%FBS); data are expressed as mean + SEM (n=3).

4. Effect of CT-1, TGF-β_1 and PDGF on the expression of p27 in p1 cardiac myofibroblasts

Accumulation of p27 prevents progression of the cell cycle into S stage thereby inhibiting cell proliferation. The function of p27 in the inhibition of the cell cycle is by inhibiting the cyclin E/cdk2 formation. Thus increased expression of p27 is a marker of non-proliferating cells (152). Therefore, we have looked at the expression of p27 in p1 cardiac myofibroblasts when they were grown in 10% FBS DMEM/F12 until they reached cell confluency of 40-50%. Cells were rendered quiescent by 24 hour incubation in 0% FBS DMEM/F12 and stimulated for the next 24 hours hours with CT-1 (10ng/ml), TGF-β_1 (10ng/ml), PDGF (1ng/ml) that were added in the presence of 1% FBS DMEM/F12.

Western blot analysis of the protein levels of p27 in Figure 11 reveal that TGF-β_1 induced a significant increase in the expression of p27 when compared to the control (1% FBS). Treatment with CT-1 and PDGF alone or in combination did not alter the expression of p27. Co-incubation of TGF-β_1 with CT-1 or PDGF did not affect the increased expression of p27 that was induced by TGF-β_1.

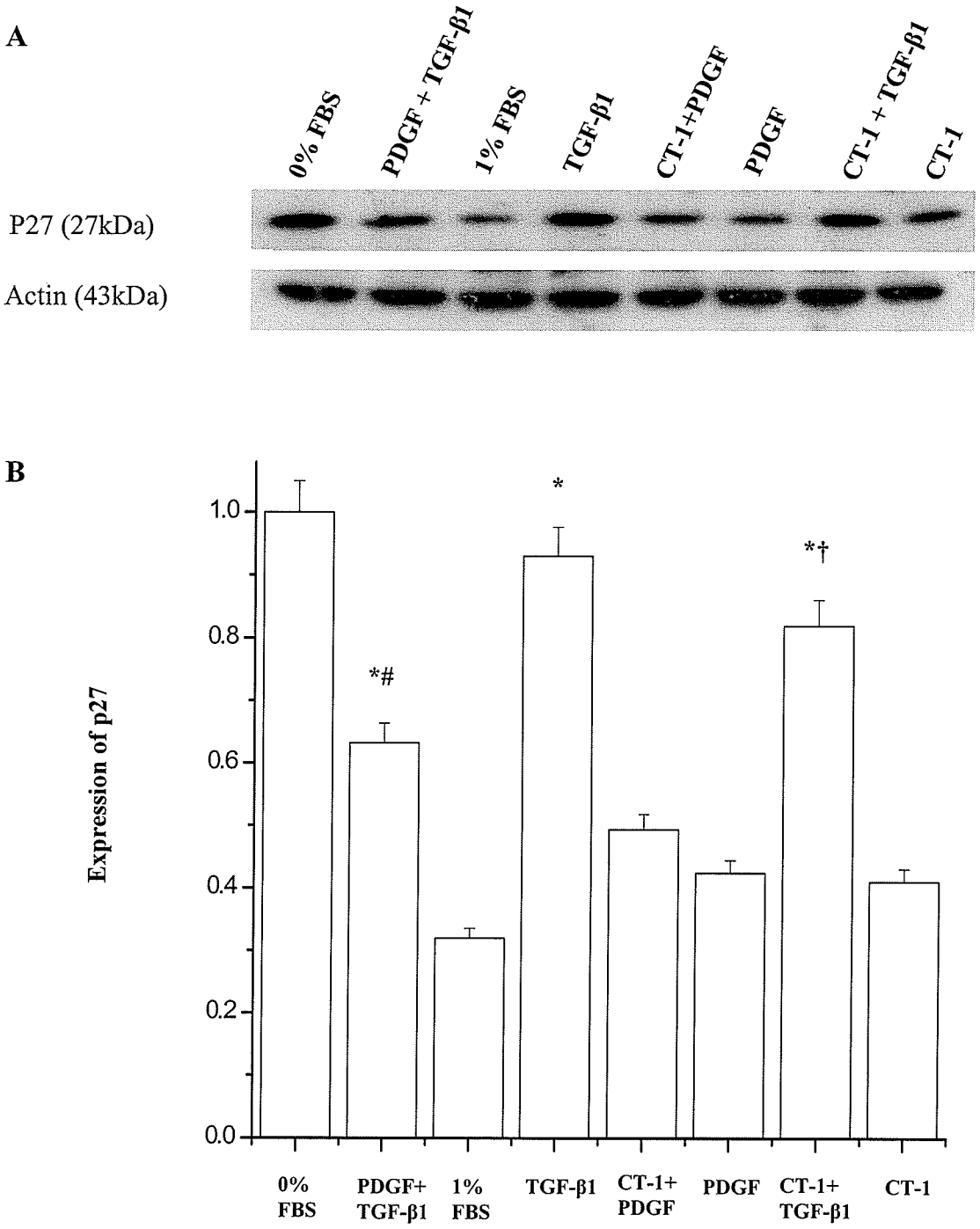


Figure 11. p27 expression in primary cardiac myofibroblasts. **Panel A**: Western blot analysis of p27 expression in cultured primary cardiac myofibroblasts (0%FBS starvation for 48 hours, total cell lysates) treated with CT-1 (10ng/ml), TGF-β1 (10ng/ml) or PDGF(1ng/ml) for 24 hours in the presence of 1%FBS. Representative Western blots show expression of p27 (~27kDa) and actin (~43 kDa) protein indicating relatively even protein loading. **Panel B:** Histographic representation of quantified data of immunoreactive p27 from the groups of samples shown in the panel A (quantified by densitometric scanning); P*≤0.05 vs..control (1%FBS); P† ≤0.05 vs. CT-1; P# ≤ 0.05 vs. PDGF data are expressed as mean + SEM (n=4).

5. Immunohistochemical analysis of PCNA localization in P1 cardiac myofibroblasts

PCNA is a DNA polymerase cofactor that is widely used as a marker for cell proliferation. Staining of PCNA in the nuclei is an indicator of an ongoing cell proliferation process (199). We determined the localization of PCNA in rat cardiac primary myofibroblasts that when reached desired confluency (50-60%) were rendered quiescent with 0% FBS DMEM/F12 for 24 hours. After starvation period was over quiescent cells were stimulated with CT-1 (10 ng/ml), TGF-β_1 (10 ng/ml), PDGF (10 ng/ml) for the time period of 24 hours. 1% FBS is used as positive control.

Our data revealed that CT-1 and PDGF treatments induced nuclear localization of PCNA indicating induction of cell proliferation in rat cardiac myofibroblasts (Figure 12) when compared to 0% FBS. However, treatment of the cells with TGFβ_1 did not induce nuclear localization of PCNA signifying cell proliferation was not induced (Figure 12). Moreover, we tested if TGF-β_1 would have an effect on CT-1 induced PCNA accumulation so we performed co-incubation with each of the cytokines. Our results revealed that TGF-β_1 did not prevent PCNA localization when added to CT-1 or PDGF. As expected co-incubation of CT-1 together with PDGF maintained the nuclear localization of PCNA indicating that cell proliferation is ongoing (Figure 12).

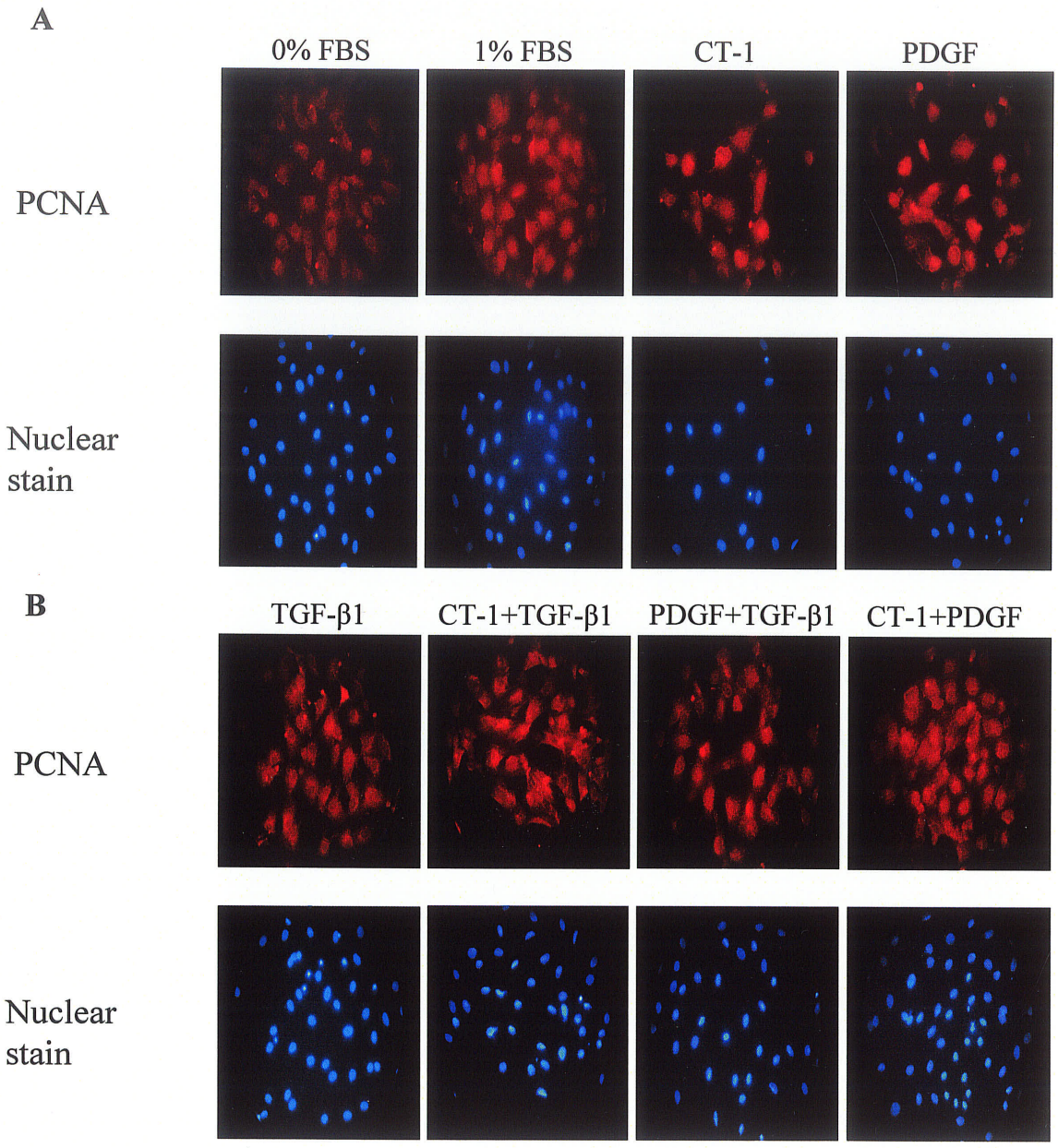


Figure 12. PCNA localization in primary cardiac myofibroblasts treated with CT-1, TGF-β1, and PDGF for 24 hours. **Panel A:** Immunofluorescent staining of PCNA in quiescent cells treatedwith CT-1 (10ng/ml) or PDGF (1ng/ml). **Panel B:** Immunofluorescent staining of PCNAin quiescent cells treated with TGF-β1 (10ng/ml), co-incubated with CT-1 (10ng/ml) and TGF-β1 (10ng/ml), PDGF (1ng/ml) and TGF-β1 (10ng/ml), and CT-1 (10ng/ml) and PDGF (1ng/ml) Nuclei of identical fields were- detected with Hoechst 33342 staining. Originalmagnification, x 400.

6. Effect of CT-1, TGβ_1 and PDGF on the collagen type I gel deformation in p1 cardiac myofibroblasts

Deformation of collagen type I gel is an *in vitro* model of wound contraction and has been previously used as a model for assessment of cellular contractility (200). We proceeded to examine the influence of CT-1, TGFβ_1 and PDGF on the contraction of cardiac myofibroblasts. We have seeded $1x10^5$ cells/ml on the top of the pre-formed gel (3 mm thick) in a 24 well dish and allowed cells to adhere and grow for the period of 24 hours in 10% FBS DMEM/F12. Afterwards, cells were rendered quiescent by incubation in 0% FBS DMEM/F12 for 24 hours and stimulated with CT-1 (10ng/ml), TGF-β_1 (10ng/ml), PDGF (10ng/ml). Photographs of the wells were taken at zero, 2, 4, 6, and 24 hours after stimulation. Gel surface area was analyzed with a custom made computer software (Measure Gel).

In order to establish the exact location of our cells we viewed our cells using a phase contrast microscope. Panel A of figure 13 shows a clear image of the cells located on top of the gel. To make sure that the cells are indeed on top of the gel, we put an eyelash (diagonally crosses the image) on top of the gel and photographed it. As we have focused toward the bottom of the culture dish there were no cells observed. In the panel B of the same figure we have marked the bottom of the culture dish to make sure that indeed we are viewing and photographing the bottom of the culture dish and thereby the bottom of the gel. Again, we have photographed the cells on top of the gel as they have been overcome with a shadow. As we focused toward the bottom of the culture dish there were no cells observed and the end photograph shows the marking on the bottom of the culture dish that actually caused the shadow in the first photograph.

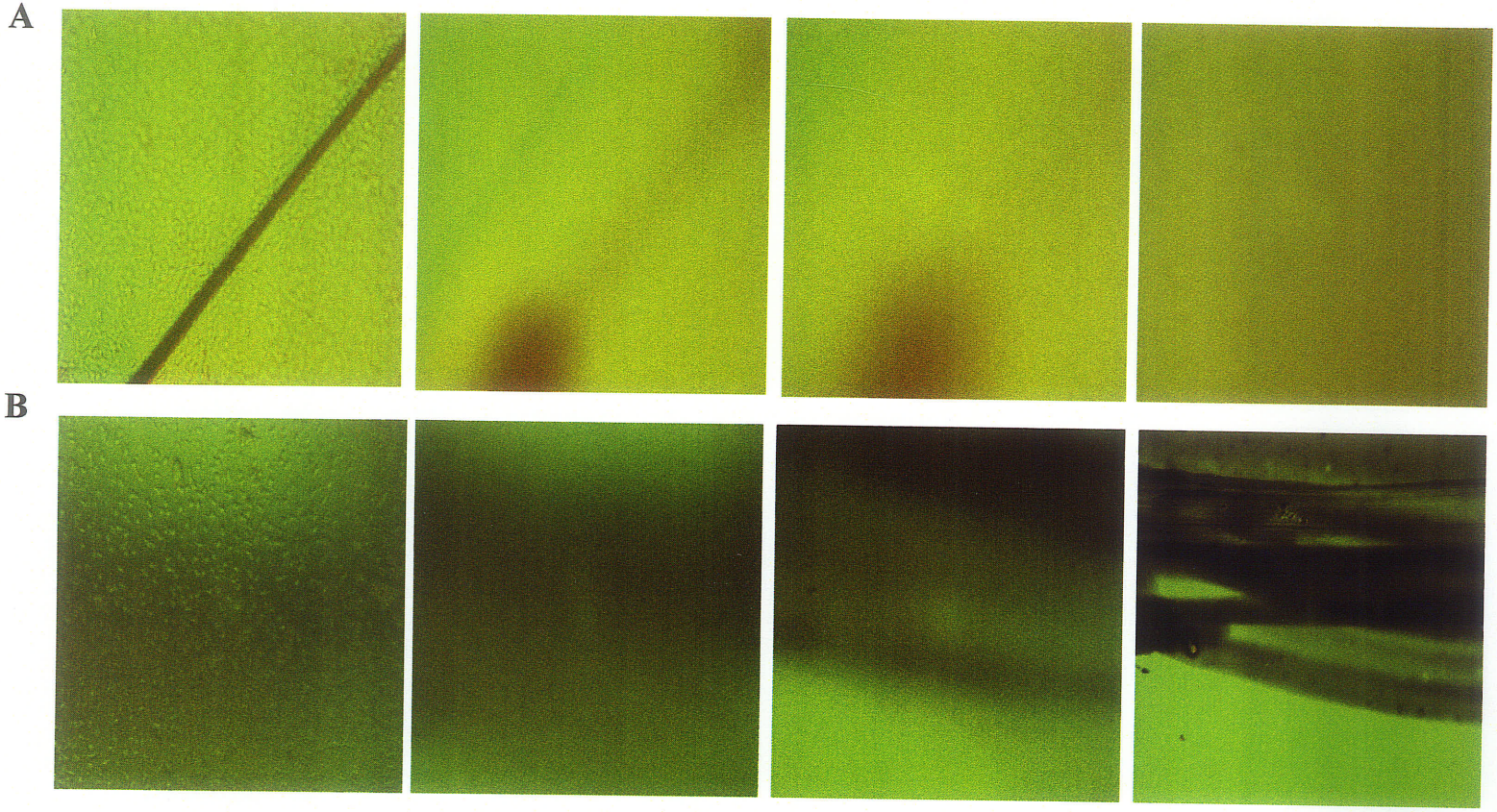


Figure 13. Phase contrast image of adult rat cardiac myofibroblasts seeded on top of a collagen type I gel matrix. **Panel A:** An eyelash was placed on top of the gel containing cells and photographs were taken while focusing through the gel towards the bottom of the culture dish. No cells have been observed anywhere else than on top of the gel. **Panel B:** Bottom of the culture dish was labeled with a marker. Photographs were taken again from the top (where cells are observed) and focusing through the gel toward the bottom of the culture dish. The shadow in the first photograph is the result of the marked bottom of the culture dish. Total magnification, x 100.

Upon analysis of the gel surface area we determined that CT-1 had no effect on the deformation of the collagen type I gel when compared to the deformation caused by untreated quiescent cells (control) (Figure 14). On the contrary, TGF-β_1 showed a very potent effect on gel contraction when compared to untreated cells (Figure 15). Significant contraction of the gel by TGF-β_1 was observed as early as 4 hours after the stimulation. PDGF also induced contraction of the collagen type I gel relative to control (Figure 16) as early as 2 hours after stimulation. When CT-1 was co-incubated together with TGF-β_1 the potency observed in TGF-β_1 contraction of the gel was maintained (Figure 17). The same result was observed when CT-1 and PDGF were co-incubated where CT-1 did not exert any effect on the ability of PDGF to induce contraction of the gel (Figure 18). When TGF-β_1 and PDGF were combined the significant gel contraction was observed as early as 2 hours resembling the potency of PDGF induced gel contraction (Figure 19).

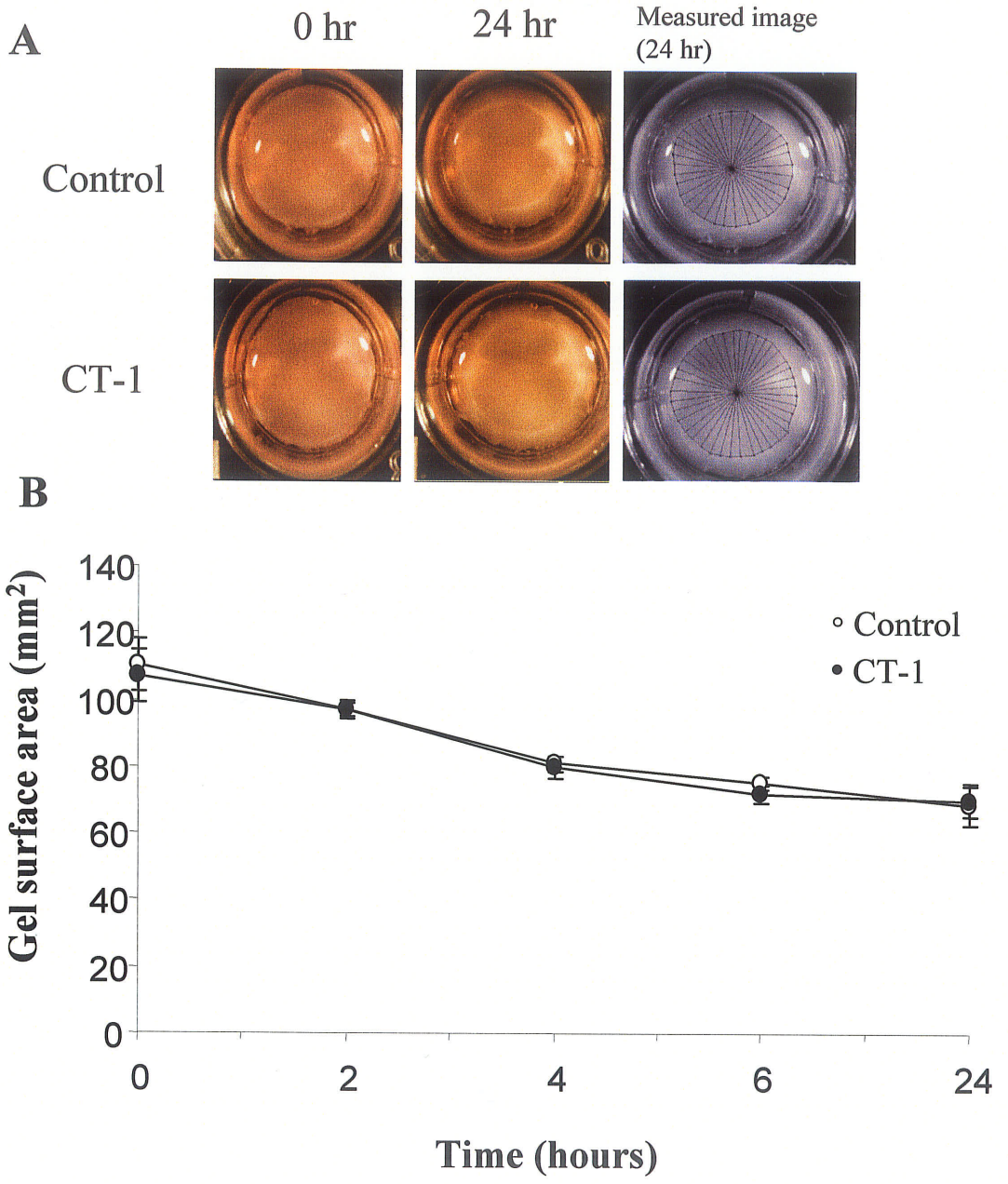


Figure 14. Collagen gel contraction assay. **Panel A:** Rat primary cardiac myofibroblasts were seeded on top of pre-formed collagen type I gel and grown to confluency in 10% FBS DMEM/F12. Cells were rendered quiescent in 0% FBS DMEM/F12 for 24 hours, and then were treated with CT-1 (10ng/ml). Representative photographs show control and CT-1 treated wells at 0 and 24 hour after stimulation, and the method used for measuring gel surface area. **Panel B:** Histographic representation of quantified data of control and CT-1 treatment ranging from time zero, 2, 4, 6 and 24 hours after the treatment with CT-1. Images were measured using Measure Gel computer software. Surface area was measured and plotted as mean ± SEM, n=5. One way ANOVA was used to check for statistical significance.

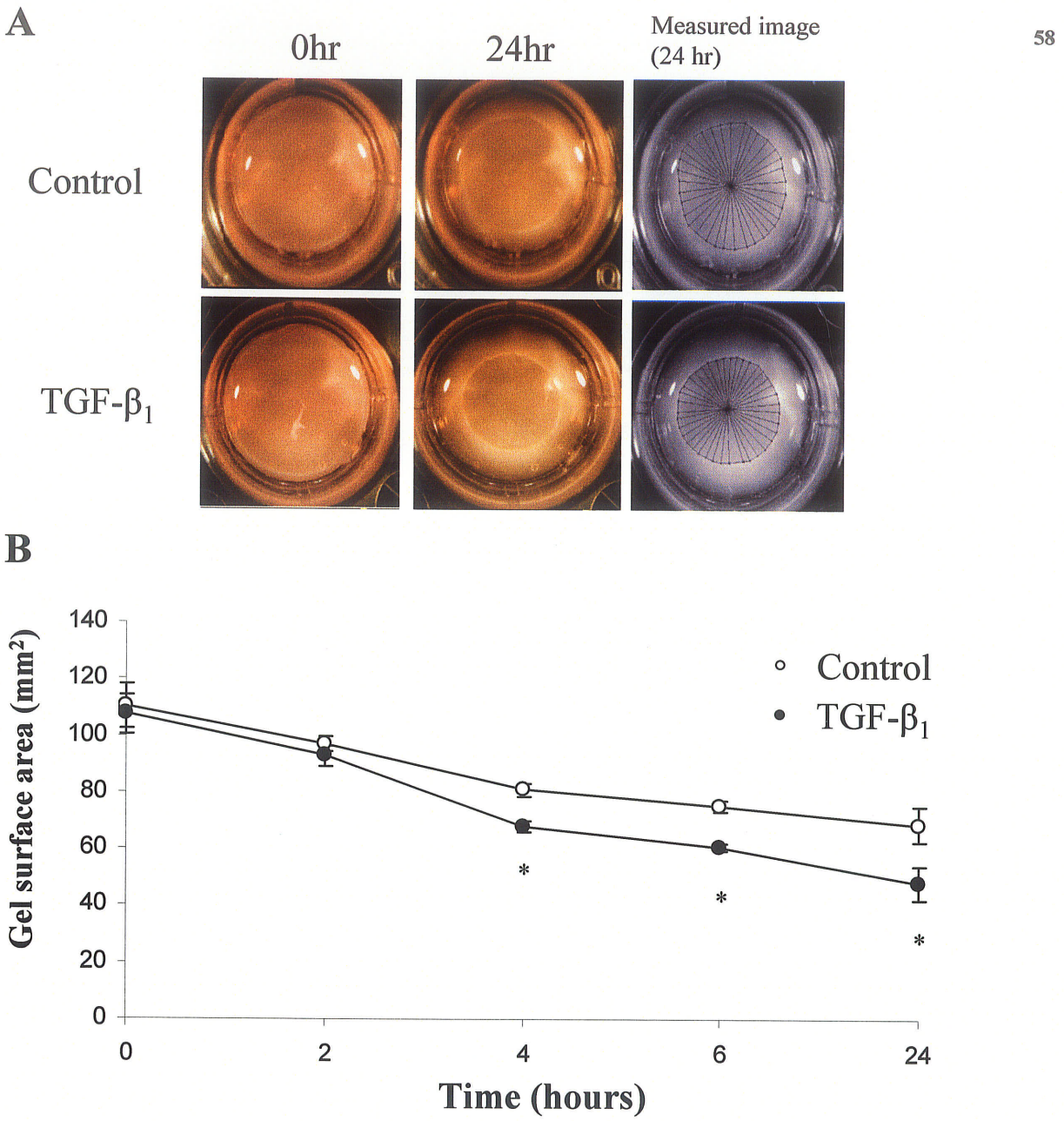


Figure 15. Collagen gel contraction assay. **Panel A:** Rat primary cardiac myofibroblasts were seeded on top of pre-formed collagen type I gel and grown to confluency in 10% FBS DMEM/F12. Cells were rendered quiescent in 0% FBS DMEM/F12 for 24 hours, and then were treated with TGF β_1 (10ng/ml). Representative photographs show control and TGF-β_1 treated wells at 0 and 24 hour after stimulation, and the method for measuring gel surface area. **Panel B:** Histographic representation of quantified data of control and TGF β_1 treatment ranging from time zero, 2, 4, 6 and 24 hours after the treatment with TGF β_1. Images were measured using Measure Gel computer software. Surface area was measured and plotted as mean ± SEM, n=5. One way ANOVA was used to check for statistical significance.

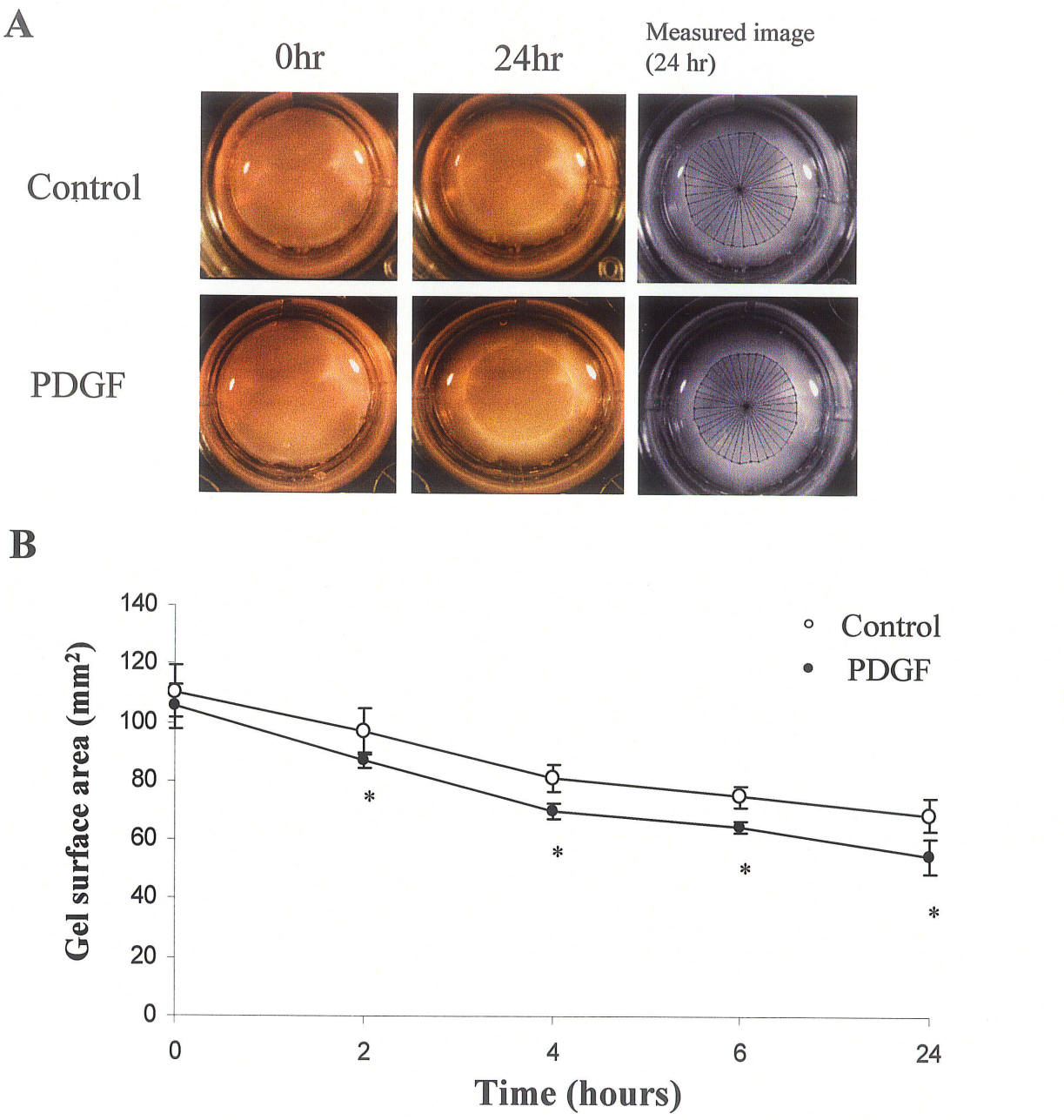


Figure 16. Collagen gel contraction assay. **Panel A:** Rat primary cardiac myofibroblasts were seeded on top of pre-formed collagen type I gel and grown to confluency in 10% FBS DMEM/F12. Cells were rendered quiescent in 0% FBS DMEM/F12 for 24 hours, and then were treated with PDGF (10ng/ml). Representative photographs show control and PDGF treated wells at 0 and 24 hour after stimulation, and the method used for measuring gel surface area. **Panel B:** Histographic representation of quantified data of control and PDGF treatment ranging from time zero, 2, 4, 6 and 24 hours after the treatment with PDGF. Images were measured using Measure Gel computer software. Surface area was measured and plotted as mean ± SEM, n=5. One way ANOVA was used to check for statistical significance.

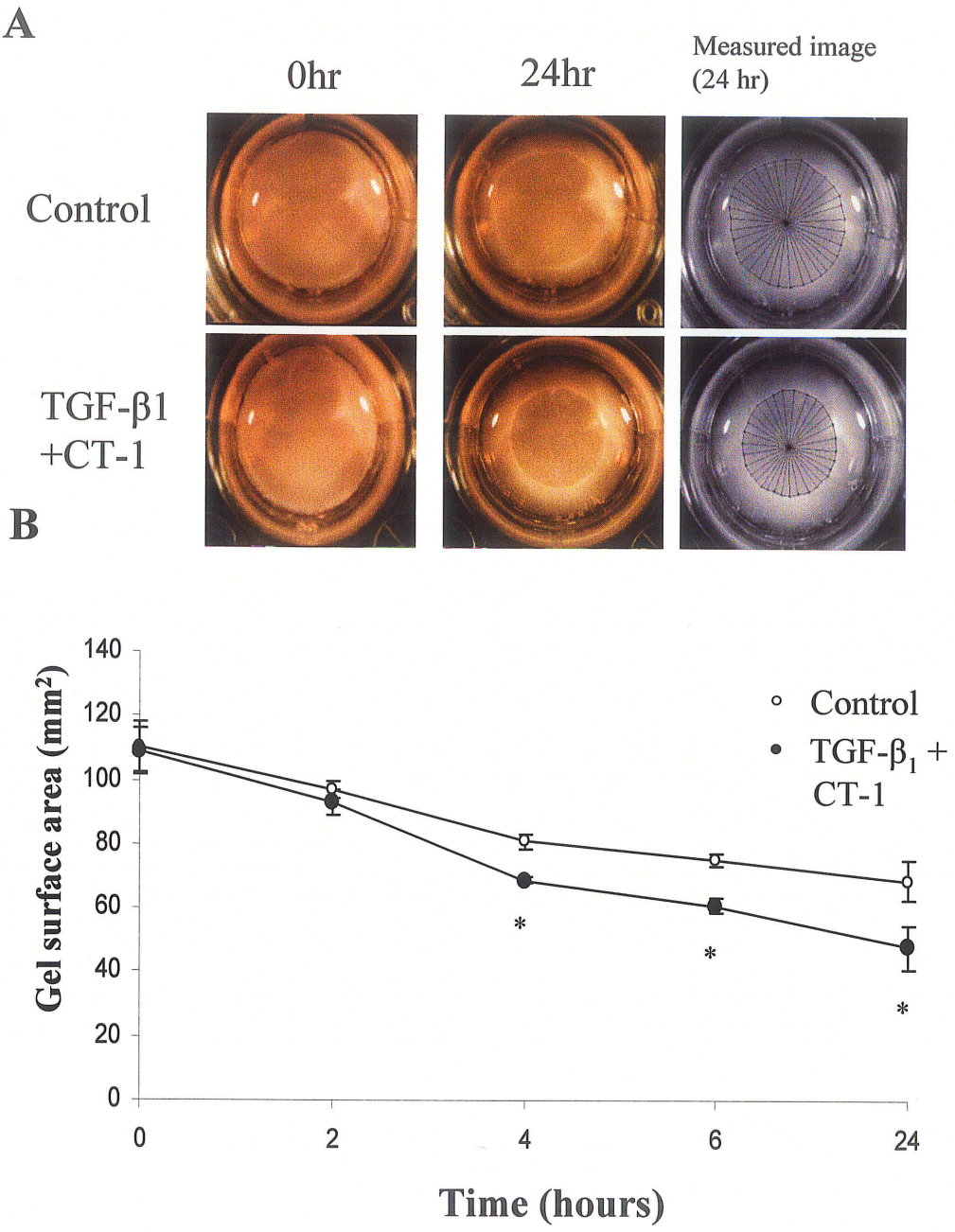


Figure 17. Collagen gel contraction assay. **Panel A:** Rat primary cardiac myofibroblasts were seeded on top of pre-formed collagen type I gel and grown to confluency in 10% FBS DMEM/F12. Cells were rendered quiescent in 0% FBS DMEM/F12 for 24 hours, and then were treated with CT-1 (10ng/ml) and TGF β1 (10ng/ml). Representative photographs show control and the treated wells at 0 and 24 hour after stimulation, and the method for measurement of gel surface area. **Panel B:** Histographic representation of quantified data of control and CT-1+TGF β1 treatment ranging from time zero, 2, 4, 6 and 24 hours after the treatment with CT-1+TGF-β1. Images were measured using Measure Gel computer software. Surface area was measured and plotted as mean ± SEM, n=5. One way ANOVA was used to checkfor statistical significance.

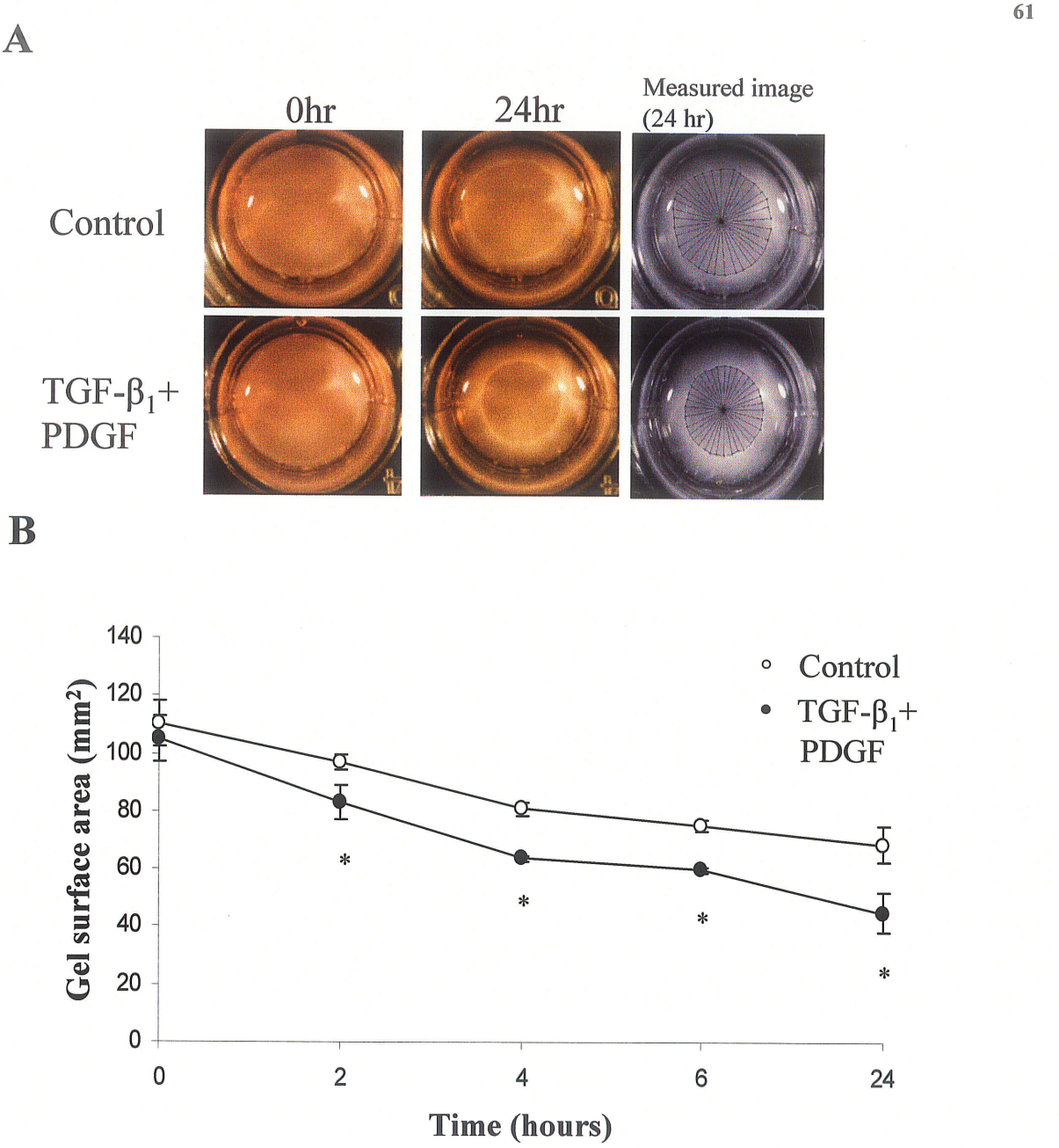


Figure 18. Collagen gel contraction assay. **Panel A:** Rat primary cardiac myofibroblasts were seeded on top of pre-formed collagen type I gel and grown to confluency in 10% FBS DMEM/F12. Cells were rendered quiescent in 0% FBS DMEM/F12 for 24 hours, and then were treated with TGF β1 (10ng/ml) and PDGF (10ng/ml). Representative photographs show control and TGF-β1+PDGF treated wells at 0 and 24 hour after stimulation, and the method of gel surface measurement. **Panel B:** Histographic representation of quantified data of control and TGF-β1+PDGF treatment ranging from time zero, 2, 4, 6 and 24 hours after stimulation. Images were measured using Measure Gel computer software. Surface area was measured and plotted as mean ± SEM, n=5. One way ANOVA was used to check for statistical significance.

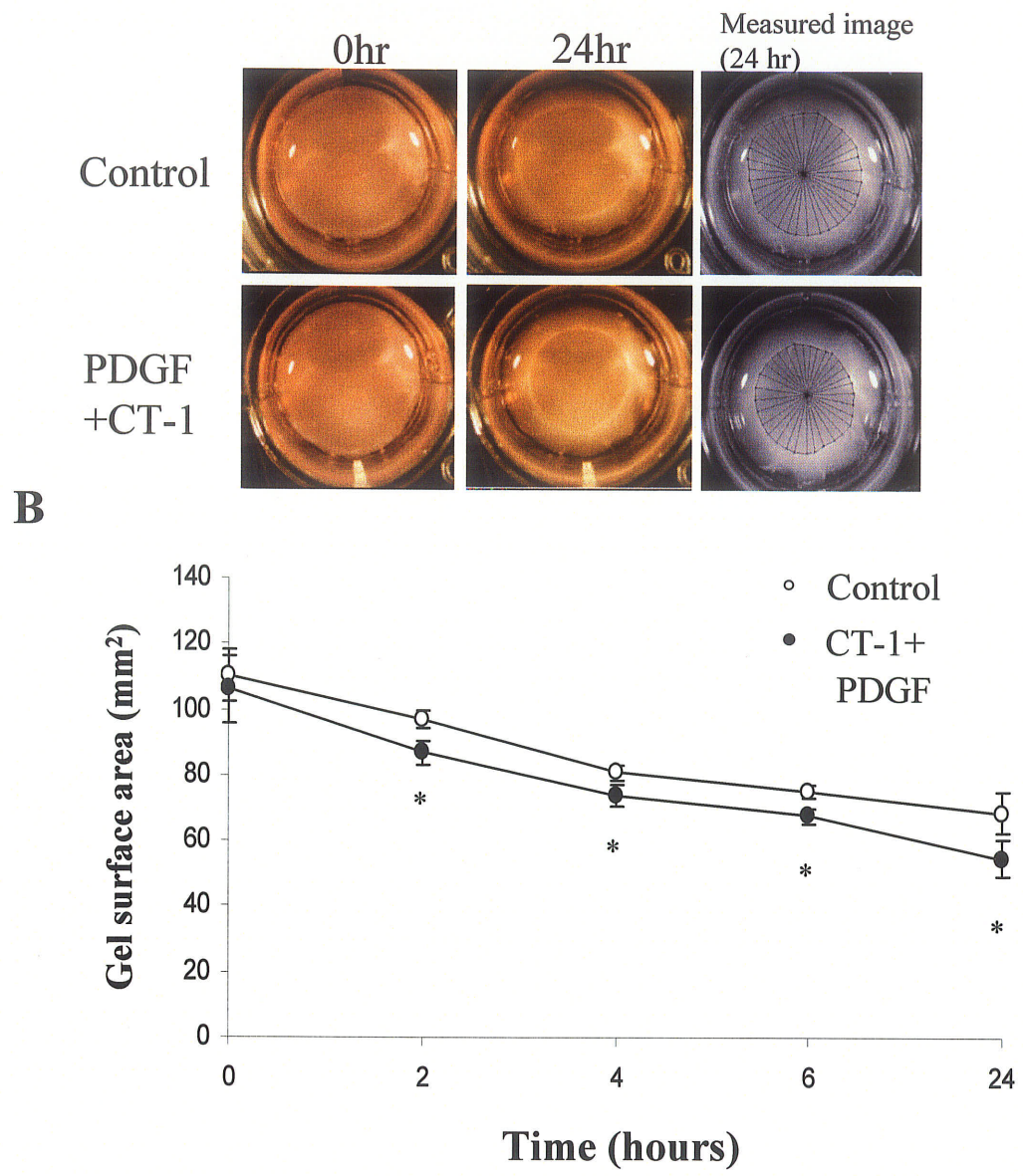


Figure 19. Collagen gel contraction assay. **Panel A:** Rat primary cardiac myofibroblasts were seeded on top of pre-formed collagen type I gel and grown to confluency in 10% FBS DMEM/F12. Cells were rendered quiescent in 0% FBS DMEM/F12 for 24 hours, and then were treated with CT-1 (10ng/ml) and PDGF (10ng/ml). Representative photographs show control and CT-1+PDGF treated wells at 0 and 24 hour after stimulation, and the method of gel surface area measurement. **Panel B:** Histographic representation of quantified data of control and treatment ranging from time zero, 2, 4, 6 and 24 hours after stimulation. Images were measured using Measure Gel computer software. Surface area was measured and plotted as mean ± SEM, n=5. One way ANOVA was used to check for statistical significance.

VII Discussion

In the current study we have investigated the effects of three different growth factors/cytokines eg, CT-1, TGF-β_1, and PDGF, on i)DNA synthesis as an indicator of proliferation of rat cardiac primary myofibroblasts and ii) contraction of collagen type I gel – this end-point is an *in vitro* model of wound contraction. Further, we tested combinations of these cytokines under the general premise that they appear and exert their effects in a simultaneous and coordinated manner in a myofibroblast-rich environment during the pathogenesis of heart disease eg, during wound healing and myocardial infarct scar formation in post-MI hearts. The main finding of the current work on both end-points is that the individual effects of CT-1, TGF-β_1 or PDGF are not similar to the net effect observed when co-incubation is performed. Specifically, we found that both 10 ng/ml CT-1 and 1 ng/ml PDGF-BB treatment of P1 cardiac myofibroblasts is associated with induction of proliferation. On the other hand, we have shown that TGF-β1 is a potent inhibitor of P1 myofibroblast proliferation under the current experimental conditions. When either culture of CT-1 - or PDGF-treated myofibroblasts are co-incubated with TGF-β_1, DNA synthesis is significantly ablated vs the individual treatment with CT-1 or PDGF. Finally, in the case of CT-1 co-incubation with TGF-β_1, its proliferative effect is completely ablated. Stated in the context of broad terms of cardiac-derived cells, the current results are in accordance with the previous work identifying TGF-β_1 as a negative regulator of cell proliferation of neonatal cardiac myocytes (6) and in cardiac fibroblasts (7). However, our work is the first to describe the effect of TGF-β_1 on the proliferation of fully differentiated adult rat primary cardiac myofibroblasts.

In the second major sub-set of experiments we have investigated the effect of CT-1, TGF-β_1 and PDGF (separately and in combination) on the deformation of myofibroblast-seeded collagen type I gels. There are three models of collagen gel deformation currently used to examine the ECM re-organization by fibroblasts: floating collagen matrix, anchored collagen matrix and stress relaxed collagen matrix (75). We have chosen anchored collagen type I matrix because this model facilitates development of mechanical tension that influences fibroblast to myofibroblasts phenotype switching. In addition, this experimental model is convenient insofar as it represents an *in vitro* model of wound contraction, which is an important component of the last stage in wound healing after MI (23). Cardiac myofibroblasts are responsible for the re-population of the infarcted site and their ability to express α-SM actin contributes to their role in wound contraction (201). The effects of TGF-β_1 (202, 204) and PDGF (53, 204, 205) as potent inducers of deformation (contraction) of collagen gels has already been described. The effect of CT-1 on the contraction of collagen gel is unexplored as well as its effects in combination with the other cytokines listed were unknown and thus they became the focus of the current study. Our data confirmed that TGF-β_1 and PDGF induced contraction of the gel indicating their potency as pro-fibrotic factors and in re-organization of the ECM. Conversely, CT-1 stimulation of the cells was not associated with the induction of the gel deformation. Although, we do not know of the effect of CT-1 on net gel relaxation *per se* (as our model system is limited to the assessment of gel contraction based on its design), our data support the suggestion that CT-1 may serve as a factor that in some ways seems to oppose the usual effects of pro-fibrotic growth factors *in vivo* (57). Freed *et al.* have shown that net collagen secretion by cardiac

myofibroblasts, normalized for 10,000 cells and compared to control cultures, is significantly decreased in the presence of CT-1 (57) versus unstimulated control cultures. Stated another way; despite the proliferative effect of these cells, net collagen secretion per cell is diminished in the presence of CT-1 and these authors claim that this cytokine may serve as an "internal brake" for net collagen secretion by myofibroblasts in the infarct scar, and thereby serve to balance the overtly pro-fibrotic signals supported by TGF-β1 and PDGF. Further support for this claim could be derived from the fact that the LIF/gp 130 receptor that is activated by CT-1. That is, this dimer receptor complex is shared with another member of IL-6 superfamily, namely leukemia inhibitory factor (LIF). While the most notable function of LIF is prevention of stem cell differentiation (206), recent evidence suggests that LIF is able to induce proliferation of adult cardiac fibroblasts, and also to inhibit phenotypic switching of cardiac fibroblasts to myofibroblasts, and finally LIF is associated with decreased MMP function, which highlights its possible role in modulation of excessive ECM remodeling (207). As Weiss *et al.* have shown that CT-1 as well as LIF has no effect on the expression of MMPs and their inhibitors in adult cardiac myocytes and fibroblasts, TIMPs (208), the mechanism for reduced MMP function is unclear and requires further study.

Implications of the current results - post-MI cardiac wound healing Concomitant with the reduction of the number of deaths attributed to acute MI, the number of post-MI survivors is increasing, and those that go on to develop congestive heart failure after myocardial infarction is also rising (209). Thus heart failure is not only a major cause of death in the aged population (210) but is becoming a widespread problem that typically begins at a relatively young age. As the number of people projected to develop heart

failure rises each year it is crucial to place emphasis on understanding and restricting processes that lead to heart failure. In this light, understanding of the molecules that directly contribute to each of the processes in wound healing of post-MI myocardium deserves continued attention. Post-MI induction of the inflammatory cascade is linked to the *de novo* synthesis and release of a host of cytokines that have an impact on cardiac fibrosis and dysfunction (211). Some cytokines eg, TGF-β1 act as pleiotropic factors and their precise mode of function seems to depend upon the cell type (even within a given organ), state of differentiation and status of growth development of a given cell type. Within the admixture of cytokines and growth factors present during tissue inflammation and wound healing, it has been suggested that specific cytokines may exert either synergistic or antagonistic properties on each other's function (212). While this concept is certainly not new, it is far less clear as to when a particular cytokine becomes regulated in cardiac wound healing; even the players in this process are unknown. Attempts to target the inflammatory response to reduce infarct size was largely unsuccessful in clinical trials despite initial experimental success (23). This problem spurs the need for a better understanding of the exact mediators and the time-dependent effects that they have on the processes following MI.

Putative roles of TGF-β1, CT-1 and PDGF in post-MI wound healing Due to its powerful pro-fibrotic and hypertrophic effect on cardiac fibroblasts and myofibroblasts, TGF-β1 is a major contributor to initiation and development of cardiac fibrosis (141) leading to heart failure . TGF-β1 is a pleiotropic protein that is well characterized as a potent regulator of collagen synthesis (7, 213). TGF-β_1 is crucial early in wound healing as it serves as a stimulus for migration of specific cells eg, leukocytes but not

myofibroblasts, to the site of inflammation (24). Further, as wound healing progresses, a number of TGF-β_1-mediated events occur in each stage. Functions of TGF-β_1 range from chemotaxis of leukocytes (23) to cardiac myofibroblast differentiation (8) to induction of excess synthesis of collagen (213). However, TGF-β1 does not operate alone in mediation of wound healing in the post-MI heart, but is part of a milieu of hormones, cytokines and growth factors that contribute to coordinated cell movement and function in the infarct scar. In this respect, CT-1 has been identified as a cytokine that may confer cytoprotection (129) and whose expression is elevated in the infarct scar for weeks after MI (10). However, while the hypertrophic effect of CT-1 on cardiomyocytes is well characterized, its influence on the non-myocytes cells has just been recently addressed. While, CT-1 is known to induce migration, DNA and protein synthesis in cardiac myofibroblasts *in vitro* (214) its influence on synthesis and secretion of collagen in these cells is weak (11).

Recently, Gabbiani *et al.* summarized the role of PDGF in modulation of myofibroblast function (141). PDGF is a well characterized effector of restenosis and atherosclerosis where it induces cell proliferation of smooth muscle cells (161). PDGF is also known to play a role in cardiac fibroblast/myofibroblasts phenotypic switching (17), as well as in the induction of collagen synthesis (53) and proliferation of cardiac fibroblasts (18, 215). In addition, PDGF is also a potent stimulator of ECM reorganization which may implicate this cytokine as a modulator of wound contraction.

To investigate the potential mechanism for TGF-β_1 inhibition of myofibroblast proliferation, we determined the expression of cell cycle proteins responsible for S phase entry. The finding that TGF-β_1 significantly reduces expression of cyclin E and cdk2

supports the suggestion that TGF-b1 may cause a reduction in the formation of the cyclin E/cdk2 complex and thus may effect a reduction of proliferation. As formation of cyclin E/cdk 2 complex is necessary for transition from late G1 into S phase (216, 217), and ectopic expression of cyclin E is known to accelerate progression through G1 phase it seems that these proteins may well be involved in the slowing of myofibroblast proliferation. This supposition is supported by evidence that the activity of cyclinE/cdk2 complex is absent in the cells that have exited the cell cycle and became differentiated or entered a quiescent stage (218). For the purpose of comparison, we also studied the effect of CT-1 and PDGF on the expression of cyclin E and cdk2. Our approach was somewhat unorthodox in that each cytokine was added in the presence of 1% FBS. As we postulated that TGF-β_1 will act to down-regulate proliferation of cardiac myofibroblasts we had to provide conditions for the initial proliferation to occur by adding cytokines in the presence of 1% FBS. We noted that low serum-mediated induction of cyclin E is relatively strong eg, stronger than the weak induction of cyclin E by either subsequent addition of CT-1 or PDGF to low-serum containing media. This effect could be attributed to the fact that 1% FBS is already a sufficient stimulus for myofibroblast proliferation, and as such, would mask or simply supercede the effects CT-1 and PDGF. It is also possible that low serum incubation of myofibroblasts may act in modulation of CT-1 and PDGF receptor expression and/or function resulting in diminished response to CT-1 and PDGF ligands. We have observed that CT-1 alone in serum-free media is a stimulus for cyclin E protein expression (unpublished observation). As opposed to cyclin E responses, we found that the combination of CT-1 and PDGF co-incubation elicited a significant increase in cdk2 expression, while treatment of cells with

CT-1 and PDGF alone was associated with only a mild increase in cdk2 protein levels. Finally, we have shown that TGF-β_1 dramatically down-regulates expression of cdk2 under low-serum conditions, and we noted that the TGF-β_1 was strong enough to dominate any other effects by either CT-1 or PDGF. We also investigated the expression of p27 as a putative mechanism of cell cycle control under our culture conditions, as this protein is a known cdk inhibitor. For example, it is known that ectopic expression of p27 impairs formation of the cyclin E/cdk2 complex resulting in the inhibition of oligodendrocytes proliferation (219, 220). In addition, it is known that TGF-β_1 exerts its down-regulatory effect on cell proliferation via accumulation of p27 (221). Our data revealed that TGF-β_1 treatment of myofibroblasts stimulated an increase in the expression of p27 in association with ablation of cell proliferation, and thereby supports earlier work cited. While CT-1 and PDGF treatments of myofibroblasts were not associated with significant alteration of p27 expression, we took this as evidence that their effects may oppose or at least not add to those of TGF-β_1 in this cell type. Again, co-incubation of TGF-β_1 with either CT-1 or PDGF maintained significantly increased expression of p27 suggesting that CT-1 and PDGF did not impair the ability of TGF-β_1 to inhibit cell proliferation.

In addition, we have examined nuclear staining of PCNA, a commonly used DNA polymerase cofactor which serves as a proliferative marker eg, nuclear staining of PCNA is an indicator of cell proliferation (199). In myofibroblasts, TGF-β_1 treatment did not result in an increased abundance of PCNA nuclear staining as CT-1 and PDGF treatments did, and our results showed that CT-1- and PDGF -induced nuclear localization of TGF-β_1 PCNA was not impaired by co-incubation of each cytokine with TGF-β_1. The lack of

TGF-β_1's effect in the examination of PCNA localization may be explained through the response of p21, another cdk inhibitor. While p21 is believed to function through an association with PCNA in inhibiting cell proliferation (220, 222, 223), unpublished data from our lab indicates that p21 expression was not altered by overexpression of I-Smad7, an endogenous TGF-β_1 inhibitor, meaning that the mechanism of TGF-β_1 inhibition of proliferation is likely not to employ p21.

VIII Conclusion

In conclusion, we show that TGF-β_1 acts in opposition to the induction of cell proliferation by CT-1 and PDGF. Furthermore, our study is the first one to show that addition of TGF-β_1 to CT-1 and PDGF results in complete ablation and significant reduction of induction of cell proliferation by each of the cytokines respectively. In addition, we have implicated alteration of the cell cycle protein expression as a possible mechanism in the down-regulation of cell proliferation by TGF-β_1. We have shown that TGF-β_1 increases expression of cell cycle inhibitor, p27, and at the same time causes a decrease in expression of cyclin E and cdk2 contributing to inhibition of cell proliferation. Furthermore, our study is first to examine the role of CT-1 in wound contraction in cell culture. We have found that CT-1 does not cause a change in the surface area of collagen type I gel indicating that CT-1 does not function in wound contraction and re-organization of ECM.

IX Future Directions

Our study elucidates the effect of TGF-β_1 on CT-1 and PDGF induced cell proliferation. However, future work needs to address whether TGF-β_1 affects the expression of CT-1 and PDGF receptors. Furthermore, we found that TGF-β_1 did not function to prevent of PCNA nuclear localization that was induced by CT-1 and PDGF. This result together with our finding that TGF-β_1 actually increases expression of p27 contributes to the idea that TGF-β_1 may not function through p21to down-regulation of cell proliferation. However, CT-1 and PDGF might exert their effects perhaps through diminishing the expression of p21 which would be another target of future work. Moreover, based on the wound contraction experiments from which we derived suggestion that CT-1 might act as an anti-fibrotic factor *in vivo,* future work should include the studies with loss of CT-1 function as well as with the overexpression of CT-1 in animal models to expand on the actual role of CT-1 in prevention/delay of cardiac fibrosis.

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