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Cardiotrophin-1 as an *Ex Vivo* Activator of a Stem-cell like Population in the Murine Heart

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Cardiotrophin-1 as An *Ex Vivo* Activator of a Stem-cell like Population In the Murine Heart

by

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This thesis is submitted to the School of Graduate Studies and Research in partial fulfillment of the requirements of the degree of Masters of Science in Growth and Development

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University of Ottawa

January 11, 2005

Your file Votre référence
ISBN: 0-494-11316-2
Our file Notre référence
ISBN: 0-494-11316-2

Abstract

The identification of a multi-potent stem cell-like population (SP) within the adult murine heart infers new methods of cardiac repair, i.e. stem cells compensate for damage by generating new cardiomyocytes. Several studies have emphasized the dominance of the tissue microenvironment on the differentiation & functional properties of stem cells. Of particular interest was Cardiotrophin-1 (CT-1), a member of the Interleukin-6 (IL-6) family of cytokines. To assess whether CT-1 functioned as an activator of cardiac SP cells in the murine heart, an adenovirus containing the full length CT-1 driven by a ubiquitous promoter was generated. The adenovirus was administered via intra-cardiac injections and the effects of CT-1 were primarily assessed using fluorescence-activated cell sorting (FACS) analysis. Analysis showed a temporary increase in the cardiac SP of CT-1 injected hearts. A closer look at the SP cells in other tissues (liver, skeletal muscle and bone marrow) demonstrated that this phenomenon was cardiac specific. Interestingly the cardiac SP expressed the Leukemia Inhibitory Factor (LIF) receptor, which is required for CT-1 signaling through the gp130 pathway. Protein analysis also showed that STAT3, a downstream member of the gp130 pathway becomes activated in the heart during CT-1 injections. Preliminary results from co-culture experiments suggested that this increase was also accompanied by SP cell differentiation. These results proposed that CT-1 not only increased the cardiac SP size but may have also activated a cardiac differentiation program. The existence of a potential biologic stimulant (CT-1) for cardiac stem cells is an exciting prospect and offers support to the notion that cardiac repair may become a viable therapeutic option in the not too distant future.

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List of Abbreviations

Ad-CT-1	cardiotrophin-1 adenovirus
Ad-CTRL	control adenovirus (LacZ)
ALS	amyotrophic lateral sclerosis
ANF	atrial natriuretic factor
ANP	atrial natriuretic peptide
BSA	bovine serum albumin
CMV	cytomegalovirus
CNTF	ciliary neutrophic factor
CRE	cyclic AMP-responsive element
CTRL	control adenovirus
CT-1	cardiotrophin-1
DAPI	4,6-Diamidino-2-Pheylindole
DMEM	Dulbecco's Modified Eagle Medium
DMEM+	Dulbecco's Modified Eagle Medium + hepes
DMEM-F12	Dulbecco's Modified Eagle Medium Nutrient mixture F-12
FACS	Fluorescence Activated Cell Sorting
FBS	fetal bovine serum
GFP	green fluorescent protein
gp130	glycoprotein 130
GCSF	granulocyte-colony-stimulating factor
HBSS	Hank's balanced salt solution

HBSS+	Hank's balanced salt solution plus hepes
HSC	hematopoietic stem cell
hsp	heat shock protein
IL-6	interleukin-6
IL-11	interleukin-11
ITS	insulin/transerrin/selenium
JAK	Janus Kinases
JM-MEM	Joklik's modified Eagle's medium
LIF	leukemia inhibitory factor
MDR-1	multi-drug resistance 1
MP	main population
NDS	normal donkey serum
NF-IL-6	nuclear factor-IL-6 transcription factor
NGF	nerve growth factor
PBS	phosphate buffered saline
PE	phycoerythrin
PFA	parafomaldehyde
PFU	plaque-forming units
pmn	progressive motor neuropathy
P/S	penicillin/streptomycin
RSV	Rous Sarcoma virus
RT-PCR	reverse transcription-polymerase chain reaction

Sca-1	stem cell antigen-1
SCF	stem cell factor
SDS	sodium dodecyl sulfate
SP	side population
TBS-T	Tris-Buffered Saline Tween-20

Acknowledgements

I would like to say a very special thank you to my supervisor, Dr. Lynn Megeney, for not only providing support, guidance and encouragement but for also believing in my abilities to pursue a Masters degree. I would also like to thank all the members of the Megeney lab, past and present; to the post-docs for their leadership; to my fellow grad students who know all too well the ups and downs of grad school; to the technicians for making sure the lab always ran smoothly and that we never ran out of supplies. A big thank you goes out to Dr. Pasan Fernando for his words of wisdom and helpful science talks, as well as for translating my CT-1 plasmid. I would also like to thank Dr. Axel Kahn for generously donating the CT-1 adenovirus and Dr. Robin Parks for amplifying it. Also thank you to my thesis committee, Dr. Michael Rudnicki and Dr. David Parry, for their valuable input.

I owe a tremendous amount of thanks to my parents, without your love and support I would never been able to complete this journey in my life. And to Little K, my sister, for the plethora of pep talks, the long and often dull proof reading you did for me, for watching me practice my presentations and for being a great friend. To all my other friends, I thank you for enduring my endless hours of whining about my trials, tribulations and successes, and for always providing me with encouragement.

"I am part of all that I have met;

Yet all experience is an arch wherethro'

Gleams that untravell'd world, whose margin fades

For ever and for ever when I move."

~~*~*Alfred Lord Tennyson*~*~*~*

Chapter 1

Introduction

1.1 Heart Development and Post-natal Cardiac Growth

In the developing embryo, the heart is the first organ to form (Harvey, 2002). The mesodermal tissues that give rise to the heart first become evident when the embryo is undergoing the process of gastrulation. In the mouse, this occurs around seven days post-fertilization (Moorman; 2003).

Development of the fetal heart, involves a series of intricate steps. First, the primordium of the heart forms into the cardiogenic plate. Angiogenic cell clusters in the plate grow together to form two endocardial tubes. These tubes are then forced into the thoracic region where they fuse together forming a single endocardial tube. The tube can be subdivided into primordial heart chambers: the sinus venosus, primitive atria, ventricle, and bulbus cordis.

The heart tube begins to grow rapidly forcing it to bend upon itself. The result is the bulboventricular loop. Septa begin to grow in the atria, ventricle and bulbus cordis to form right and left atria, right and left ventricles and the two major blood vessels- the pulmonary artery and the aorta. Finally partitioning occurs and the fetal heart has formed (reviewed in Meilhac, 2003).

Post-natally, the heart undergoes extensive growth. This growth is believed to be primarily due to the hypertrophy of individual heart cells. In postnatal life, myocytes permanently exit the cell cycle, preventing or limiting any further increases in cell number (MacLellan; 2000). Therefore, growth after birth is attributed to an increase in cell size and not cell numbers. A number of changes occur to facilitate hypertrophy

including sarcomeric reorganization, myofibril assembly and enhanced contractile activity (reviewed in Chien, 1991).

1.2 Challenging the Dogma: Evidence of Cardiomyocyte proliferation

A growing body of evidence suggests that hypertrophy is not the only mechanism responsible for post-natal cardiac growth, and that cardiomyocyte replication may occur in the adult myocardium (Kajstura, 1998; Limana, 2002; Beltrami, 2001) For example, early research reported that nuclear mitotic divisions in myocytes were occasionally observed (Quaini, 1994; Kajstura, 1994). These studies revealed that in non-diseased human hearts, 14 myocytes per million were undergoing mitosis. In diseased hearts, a dramatic increase in mitosis was documented, with 152 myocytes per million in end-stage ischemic heart disease and 131 myocytes per million in idiopathic dilated cardiomyopathy (Kajstura, 1998). The report concluded that a 10 fold increase in mitosis occurred in the diseased hearts, providing evidence that the heart may be capable of generating new muscle cells to repair damage.

Similarly the extent of cardiomyocyte mitosis in the human heart after myocardial infarction has been shown to be as high as 4-5% of the total cardiomyocyte population in areas surrounding the infarct site (Beltrami, 2001). Moreover additional studies have reported that aortic stenosis induced hypertrophy may result from a combination of myocyte hypertrophy and hyperplasia (Urbanek, 2003). This study found that the differentiation of cardiomyocyte precursor cells accounted for the new cardiomyocyte

formation. In addition, it was noted that these cells expressed cell surface markers that were enriched in stem cell populations including: c-kit, the receptor of stem cell factor (Ogawa, 1991); MDR1 (multi-drug resistance 1), a protein extruding toxic substances and Hoechst dye (Goodell, 1996); and Sca-1 (stem cell antigen-1) protein (Spangrude, 1988), which is involved in the self-renewal of hematopoietic stem cells (Osawa, 1996). As such, these investigators concluded that cardiomyocyte hyperplasia significantly contributed to cardiac hypertrophy and that the presence of a stem-like cell population accounted for the subpopulation of cycling cardiomyocytes (Urbanek, 2003).

Taken together, these concepts challenge the traditional hypothesis that all post-natal cardiac adaptation or growth is managed by cell hypertrophy alone. This research provided evidence to suggest that cardiomyocytes in the adult heart were capable of cell proliferation and alluded to the possibility of a resident stem cell population.

1.3 Adult Stem Cells

Adult stem cells are defined by two key characteristics: 1) they are capable of continuous self-renewal and 2) they can differentiate into a multiple of cell lineages. The biological role of adult stems cells is not well understood. Stem cells from the adult bone marrow are capable of repopulating the entire hematopoietic system (Szilvassy, 1990), while other stem cell populations are only involved in minor repair or the general maintenance of tissue homeostasis (Hennessy, 2004). There is a common notion that stem cells are highly sensitive to their immediate surroundings and are capable of responding to a variety of signals. Thus, the microenvironment, including contact with

surrounding cells, the extracellular matrix (Hay, 1990), the local chemical milieu (Studer, 2000) as well as growth and differentiation factors, are likely to play a key role in determining stem cell function. This concept is supported by several reports, which have illustrated that in addition to contributing to specialized cells within their host tissue, adult stem cells also display a surprising degree of plasticity (Blau, 2001; Poulsom, 2002).

1.4 Hematopoietic Stem Cells

Hematopoietic stem cells (HSCs) are the most widely studied and well characterized of the adult stem cell populations (Sutherland, 1989; Bhatia, 1997; Thomas, 1999). The principle function of HSCs is to maintain the hematopoietic system with a constant supply of new blood cells. HSCs meet the classic definition of a stem cell in that 1) they have the capacity for self-renewal and 2) are multipotent.

As HSCs occur at a very low frequency, approximately 1 in 10 000 (Szilvassy, 1990), the identification and use of numerous cell surface markers have eased study of HSC. These markers include CD34, a transmembrane glycoprotein (Krause, 1996); stem cell antigen-1 (Sca-1) a member of the ly-6 family (Spangrude, 1988); and c-kit, a tyrosine kinase receptor for stem cell factor (Ogawa, 1991). In addition to these stem cell markers, HSC purification/identification is also assisted by excluding non-HSC cells or cells that have undergone lineage specification. These lineage specific markers include CD2, CD3, CD4, CD5, CD8, NK1.1, B220, Ter119, GR-1 and Mac-1. These markers are collectively referred to as lineage specific markers and are not expressed by HSCs

(Okada, 1992). Typically HSC identification involves a combination of cell surface marker staining and analysis by fluorescent activated cell sorting (FACS). This allows for the exact identification of the HSC subset within the bone marrow.

1.5 Hematopoietic Stem Cells – Characterization of the Side Population Phenotype

More recently an additional strategy for isolating and enriching bone marrow stem cells has been successfully utilized. This technique exploits the efflux property of the Hoechst 33342 dye. Typically, bone marrow cells are stained with Hoechst 33342, a fluorescent DNA binding dye, and then analyzed by FACS (Goodell, 1996). However, the Hoechst 33342 is efficiently effluxed from stem cell populations. This efflux can then be measured and used to collect various subpopulations within the bone marrow. The ability to detect stem cell populations using this protocol is dependent on the unique property of stem cells, i.e. the presence of multi-drug resistant proteins that exclude dyes and chemicals. As such further analysis of SP populations have shown a highly enriched stem cell activity (up to 1000 fold) (Goodell, 1996). The positive identification of this population is then confirmed with verapamil, i.e. verapamil prevents the efflux of the dye by blocking the multi drug resistant proteins on the cell surface, in time causing the disappearance of the SP profile (Goodell, 1996; Goodell, 1997). In addition, the SP population is also enriched for stem cell surface markers (Goodell, 1996).

This technique has been an important discovery for the field of adult stem cell biology. New adult stem cell populations are not well characterized and therefore lack the cell

surface markers used for identification of these sub populations. As such, several adult stem cell populations have been initially isolated on the basis of Hoechst dye exclusion, such as populations from skeletal muscle, nervous tissue, and pancreas (Gussoni, 1999; Jackson, 1999; Lechner, 2002; Murayama, 2002).

1.6 Challenging the Dogma: Evidence Of A Cardiac Stem Cell Pool

Adult stem cells have been found in a variety of tissues including skeletal muscle, skin, brain and fat, (Gussoni, 1999; Reynolds, 1992; Toma, 2001; Zuk, 2001) as well as in the heart (Hierlihy, 2002). Given these observations, coupled with the reports of mitotic cell activity in the post-natal heart it is reasonable to hypothesize that the adult heart contains a resident stem cell population.

Indeed, a cardiac stem cell population was first identified using the Hoechst dye exclusion protocol (Hierlihy, 2002). Single cell suspensions derived from adult murine hearts stained with Hoechst 33342 dye and analysed by FACS, clearly defined a SP subpopulation. In addition, this SP population was not enriched for known hematopoietic stem cell markers or skeletal muscle specific lineage markers (CD34, c-kit, Sca-1, Flk-2 and Thy1.1) (Hierlihy, 2002). Further experimentation demonstrated that these cells retained stem cell like activity. For example, SP cells co-cultured with primary cardiomyocytes readily adopted characteristics of a cardiac phenotype, such as expression of proteins (myosin heavy chain) and cardiac enriched gap junction factors (connexin-43). Interestingly, these cardiac SP cells were also capable of fusing with

other cell types such as primary skeletal muscle myoblasts. However while fusion occurred, the cardiac SP cells retained their gene expression profile, suggesting that these cells may be restricted to a myocardial fate (Hierlihy, 2002).

Following this initial observation additional evidence has accumulated to suggest that cardiac stem cells exist both in the human and murine heart. Using the hearts from humans with aortic stenosis, a population of putative stem cells were identified (Urbanek, 2003). These cells were observed to be Sca1, MDR1, and c-kit positive. Furthermore these cells were demonstrated to be negative for hematopoietic lineage markers as well as transcription factors that are typical of cardiac and skeletal muscle cells. Upon further investigation, these cells had the capacity to become cardiogenic and ultimately terminally differentiated to form cardiomyocytes.

This same group reported the existence of a lineage negative, c-kit positive population of cells within the hearts of Fischer rats (Beltrami, 2003). These cells possessed the properties of stem cells, i.e. were capable of self-renewing, were clonogenic, and were multipotent. This population of cells also gave rise to a variety of cells types including cardiomyocytes, smooth muscle, and endothelial cells. When these cells were injected into ischemic hearts, they reconstituted a well-differentiated myocardium, forming both new blood vessels and cardiomyocytes. These newly differentiated cardiomyocytes contributed to approximately 70% of the reconstituted ventricle mass. As such, this study confirmed early observations and suggested that the heart contained a stem cell like population that was capable of contributing to cardiac repair.

A final study reported a Sca-1 positive, c-kit negative, lineage negative population of cells in mouse hearts (Oh, 2003), similar to the findings reported by Hierlihy et al. (2002). This cell population also exhibited the SP phenotype when stained with Hoechst 33342 and subjected to FACS analysis. Moreover the cells belonging to the SP population/fraction were >93% Sca-1 positive. Interestingly these cells possessed telomerase reverse transcriptase activity, which is associated with self-renewal potential. This activity was not present in the Sca-1 negative cells and occurred at the levels observed in the active neonatal myocardium. Using reverse transcription-polymerase chain reaction (RT-PCR) and microarray analysis they confirmed that these cells did not express cardiac structural genes (alpha and beta-MHC, atrial and ventricular myosin light chain-2, alpha actin), nor did they express hematopoietic stem cell transcription factors (Lmo2, GATA2, and Tal1/Scl). In addition, these authors employed the use of a cardiac specific cyclic AMP-responsive element (CRE) construct to denote donor cell identity and differentiation *in situ*. Briefly, animals that had under gone ischemia/perfusion were grafted with Sca-1 positive undifferentiated cells expressing a CRE induced marker. Two weeks after engraftment, a CRE protein was observed particularly in the infarcted zone, suggesting that injected cells migrated to the infarcted site and differentiated/fused in the damaged myocardium. This study reported a 3% contribution of donor cells to the damaged area, a 150-fold increase from what was previously reported for the engraftment of endogenous bone marrow derived SP cells (see section 1.8) (Jackson, 1999).

Together these studies provide compelling evidence that the post-natal heart does contain a resident cell population with stem cell like activity. Clearly, the concept of the

adult heart as a terminally differentiated organ without self-renewal potential has been undermined by the discovery of this cell population. However many questions remain unanswered such as the physiologic role of this native population of cells and whether there exists an innate ability (if any) to activate these cells to induce cardiac repair?

1.7 Potential of Stem Cell Therapies in the Adult Heart

Adult stem cell plasticity has been demonstrated in numerous ways and has been utilized as an approach to repair heart damage. The basic premise is that stem cells are isolated from a non-heart source and then administered to the heart, where in turn these stem cells react to cues in the microenvironment to become fully functioning cardiomyocytes. An effective example of this strategy is the use of adult bone marrow stem cells to regenerate infracted myocardium (Orlic, 2001). Bone marrow stem cells were isolated by FACS and transplanted into the infarcted hearts of mice. The study reported that a newly formed myocardium occupied 68% of the infarcted portion of the ventricles. However, a similar study reported the frequency of donor-derived cardiomyocytes to be only 0.02% (Jackson, 2001). Although the dramatic difference in frequency is disconcerting, the results do suggest that adult derived stem cells may contribute to the repair of damaged heart tissue. Another example of non-cardiac stem cell transplantation used the liver as a source of adult stem cells and similarly reported that these stem cells were capable of adopting a cardiomyocyte fate (Malouf, 2001).

It is important to note that the concept of adult stem cell "plasticity" has been challenged. Some researchers argue that transdifferentiation does not occur, but rather

that the stem cells adopt the phenotype of the existing cell via a cell to cell fusion event (Terada, 2002) For example, a recent study demonstrated that mouse bone marrow cells could fuse spontaneously with embryonic stem cells. In addition, similar reports of cell fusion have been reported with adult heart stem cells (Hierlihy, 2002). In this case, cardiac derived stem cells were co-cultured with primary skeletal muscle cells, yet the cardiac stem cells appeared to fuse with the skeletal muscle myoblasts while maintaining their own genotype (i.e. these fused stem cells did not express skeletal muscle specific factors such as myogenin).

1.8 Activation of Cardiac Stem Cells

The identification of a multi-potent stem cell-like population (SP) within the adult murine heart infers new methods of cardiac repair, i.e. stem cells compensate for damage by generating new cardiomyocytes. Several studies have emphasized the dominance of the tissue microenvironment on the differentiation & functional properties of stem cells (reviewed in Heinrich, 1998). These lines of evidence suggest that adult derived stem cells, like their embryonic counterparts possess a multi-potent differentiation capacity. In order to change stem cell fate, cells must respond to key growth & differentiation factors. These factors are released upon damage within the tissue & determine which genes will be activated.

One class of proteins that may influence stem cell activity is the cytokine family. Cytokines play a critical role in the communication between cells within an organism. These proteins act as cellular mediators that can regulate growth, differentiation, &

survival of cells (reviewed in Heinrich, 1998). Indeed cytokines have been employed to induce repair in infracted hearts. When stem cell factor (SCF) and granulocyte-colony-stimulating factor (GCSF) were injected into mice with infarcted heart damage a dramatic increase in survival and cardiac repair was observed (Orlic, 2001). These cytokines induced the immobilization of bone marrow cells, which increased the number of circulating stem cells and ultimately induced these cells to home to the infarcted site where they contributed to tissue repair. However these cytokines are likely to elicit numerous non-cardiac responses and effects.

Of particular interest is Cardiotrophin-1 (CT-1), a member of the IL-6 family of cytokines. Briefly, CT-1 is expressed in a relatively cardiac restricted manner. It is predominant in the early mouse embryonic heart tube, with low level expression in skeletal muscle, liver, lung & brain (Pennica, 1996; Sheng, 1996). CT-1 is responsible for a multiple of biological activities in a variety of tissues. In the heart, CT-1 has been observed to induce an increase in cardiomyocyte size during hypertrophy. CT-1 signals via the gp130 pathway, which utilizes the sequential activation of the JAK family of tyrosine kinases, which in turn activate the STAT family of transcription factors (Kuwahara, 1999). This pathway is responsible for a variety of complex cellular processes such as proliferation, and differentiation.

1.9 Cardiotrophin-1

Cardiotrophin-1 (CT-1) was initially characterized as a hypertrophic agent (Pennica, 1995). For example, it was observed that the media from differentiated embryoid bodies

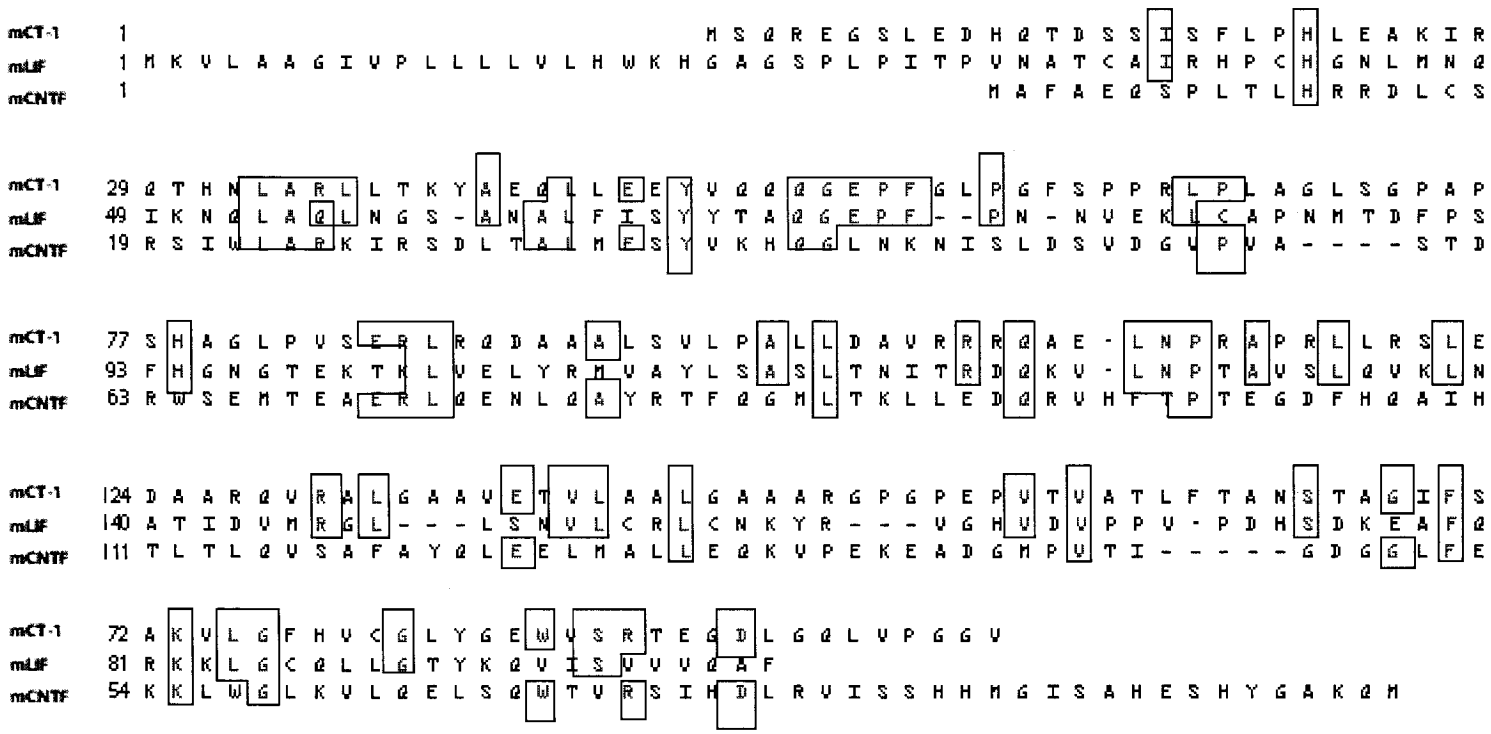
derived from mouse embryonic stem cells were capable of inducing a hypertrophic response in cardiomyocytes. Screening of these embryoid bodies using a subtractive expression cDNA library approach led to the identification of a novel factor, CT-1. Subsequently a number of studies have gone on to characterize the activity and function of this cytokine.

As noted above, CT-1 is a member of the IL-6 family of cytokines (Pennica, 1995). This family includes leukemia inhibitory factor (LIF), ciliary neutrophic factor (CNTF), oncostatin M, interleukin-6 (IL-6) and interleukin-11 (IL-11). These cytokines are distantly related with regards to their primary amino acid sequence (14-24%) (refer to figure 1), however they all share a common tertiary structure, the four alpha-helix bundle motif (reviewed in Wollert, 1997). Receptor binding and functional studies, confirmed CT-1 as a member of the IL-6 family, all of which signal through the gp130 receptor. CT-1 shares the signal transducing receptor components gp130 and LIF receptor beta subunit. Interestingly, CT-1 also maintains the greatest sequence homology (24%) with LIF and both cytokines share the LIF receptor beta subunit as a part of their signaling apparatus (Jin, 1996).

1.10 Cardiotrophin-1 Expression

CT-1 is a unique member of the IL-6 family of cytokines in that this protein is expressed primarily in the heart. However lower levels of CT-1 expression is also found in a variety of other tissues such as skeletal muscle, liver, lung & brain (Pennica, 1996; Sheng, 1996). During embryonic development, CT-1 is first detectable in the primitive

Figure 1: Comparison of amino acid sequences of IL-6 cytokine family members. Encoded amino acid sequence of mouse CT-1 (mCT-1, GenBank accession number Ay007567) aligned with that of mouse LIF (mLIF, GenBank accession number NM_008501) and mouse CNTF (mCNTF, GenBank accession number NM_053007). The light blue boxes indicate regions of sequence homology.


mCT-1
mLIF
mCNTF

heart tube at E8.5, while it remains undetectable in other tissues (Sheng, 1996). The heart continues to be the main site of CT-1 expression until E10.5, at which point other organs (i.e. skeletal muscle, liver and dorsal root ganglia) begin to express CT-1. A closer examination of the CT-1 expression pattern revealed that it is present in the atrial and ventricular muscle but not in the endocardium. In addition, CT-1 continues to be expressed in the adult heart, suggesting that CT-1's role is not limited to cardiac development alone (Sheng, 1996).

While CT-1 is normally found in healthy hearts, it is also expressed in diseased hearts. In several instances CT-1 has been shown to be up regulated in a variety of pathological states. For example the over expression of CT-1 and gp130 has been reported in the ventricles of myocardial infarcted rat hearts (Takimoto, 2002). Similarly, increased expression of CT-1 has been observed in the ventricles of hypertensive rats (Ishikawa, 1996). These observations suggest that CT-1 may play a role in both heart development and cardiac disease.

1.11 Cardiotrophin-1 Signalling

As noted previously, CT-1, like the other members of the IL-6 family, signals through the gp130/Jak/Stat3 pathway (reviewed in Heinrich, 1998). The first event in CT-1 activation of this pathway involves a ligand-induced hetero-dimerization. The two components involved in this step are gp130 and the LIF receptor beta subunit. The formation of the signalling complex induces the phosphorylation/activation of gp130 associated Janus Kinases (JAKs). Upon activation, the JAKs then phosphorylate

tyrosine residues located on the cytoplasmic tails of the gp130 protein. These phosphorylated tyrosine residues are docking sites for the STAT family of transcription factors. The STAT factors, mainly STAT3 and STAT1 bind to matching SH2 domains of the docking sites, and subsequently become phosphorylated. The phosphorylated STATs form dimers and translocate to the nucleus where they function as a bona fide transcription factor, binding to specific enhancer sequences to stimulate or repress transcription of target genes (reviewed in Heinrich, 1998).

1.12.0 The Role of Cardiotrophin-1

1.12.1 Induction of Cardiac Hypertrophy

Although the expression of CT-1 indicates a role during embryogenesis, characterization of CT-1 function to date suggests that it is primarily a mediator of cardiomyocyte hypertrophy (Pennica, 1995). In particular, studies have demonstrated that CT-1 promoted cell growth and sarcomeric organization as well as inducing gene activation and secretion of atrial natriuretic peptide (ANP). *In vitro* studies, indicated that this was a distinct form of hypertrophy, when compared with other hypertrophic agents such as alpha-adrengenic stimulation (Wollert, 1996). Specifically, CT-1 induced hypertrophy led to an increase in cardiomyocyte size, specifically an increase in cell length and not a change in cell width. In addition, the cells taken from CT-1 induced hypertrophic hearts show an assembly of sarcomeric units in series as opposed to the parallel addition as observed with alpha-adrenergic stimulation. Clearly, CT-1 induces a very specific form of cardiac hypertrophy (Wollert, 1996).

These *in vitro* CT-1 studies were also supported by *in vivo* investigation of CT-1 function. A study was performed in which CT-1 was administered to mice via repetitive interperitoneal injections (Jin, 1996). The results indicated a dose-dependent increase in both the total heart and ventricular weights relative to the total body weights. Similarly, chronic stimulation of the gp130 receptor, a member of the CT-1 signalling pathway, has also been demonstrated to induce cardiac hypertrophy (Hirota, 1995; Ancey, 2003).

1.12.2 Cardio-protective Effects of Cardiotrophin-1

The up regulation of CT-1 during cardiac disease/stress may also suggested a protective role for this cytokine in the heart. For example, neonatal rat cardiomyocytes treated with CT-1 displayed increased survival and proliferation (Sheng, 1996). The increased proliferation was not unexpected as CT-1 is highly expressed in the developing heart tube, when early cardiomyocytes are rapidly proliferating.

Moreover, it was shown that pre-treatment with CT-1 prior to damage could enhance cell survival (Stephanou, 1998). Rat cardiomyocytes pre-treated with CT-1 and subsequently exposed to severe thermal or ischemic stress displayed a significant increase in cell survival. Further investigation found that CT-1 induced an increase in the levels of heat shock proteins (hsp), hsp 70 and hsp 90. Previous studies have shown that these heat shock proteins protect cardiomyoctyes from stress (reviewed in Latchman, 2000). This anti-apoptotic effect was also observed when CT-1 was administered following the period of ischemia (Brar, 2001).

1.12.3 Role of Cardiotrophin-1 in Neuromuscular Disease

In addition to cardiogenic effects, CT-1 also appears to moderate the pathology of specific neuromuscular diseases. For example, adenoviral CT-1 gene transfer has been shown to protect progressive motor neuropathy (pmn) mice from disease progression (Bordet, 1999; Lesbordes, 2002). These mice suffer from motorneuron degeneration, muscle paralysis and premature death. When CT-1 was administered to pmn mice they exhibited prolonged survival and improved motor functions.

A second study looked at the benefits of CT-1 in a mouse model of spinal muscle atrophy (Lesbordes, 2003). These mice are characterized by degeneration of the lower motor neurons and animals that received CT-1 intra-muscular injections had improved survival, delayed motor neuron defects and decreased loss of motor axons.

Similarly, CT-1 administration has been demonstrated to modify the effects on neuromuscular degeneration in amyotrophic lateral sclerosis (ALS) (Bordet, 2001). Mice expressing a mutation in the superoxidase dismutase exhibit an autosomal dominant adult onset of ALS with a loss of cortical and spinal motorneurons. As with other neurologic disease models, ALS mice treated with CT-1 showed delayed onset of motor impairment and slowed axonal degeneration. Taken together there is strong evidence that CT-1 also has a protective role in skeletal muscle as well as a role in promoting motorneuron survival.

1.13 Experimental Rationale

The emerging evidence that an endogenous population of stem cells may exist in the adult heart opens the door for therapeutic applications and suggests new mechanisms of cardiac repair. In particular it has been shown that CT-1 improves cardiomyocyte survival and has a cardioprotective effect when administered prior to damage. Therefore, given the profile of CT-1, it is reasonable to hypothesize that this cytokine could activate this endogenous stem cell population. In an attempt to address this hypothesis, the previously described method of differential Hoechst dye efflux/FACS was employed to identify the impact of CT-1 administration on the cardiac stem cell population (SP).

CT-1 is also a member of the IL-6 family of cytokines. Cytokines play an important role in the communication between cells. These proteins regulate a variety of critical biological functions, such as survival, growth and differentiation (reviewed in Heinrich, 1998). Cytokines are not stored, they are synthesized and secreted upon stimulation. As a result cytokines generally are effective at very low concentrations and often have a very short half-life (minutes to hours). To overcome these issues, a CT-1 adenovirus was used to administer the cytokine in a systemic fashion thereby avoiding the limitations that would typically accompany the use of recombinant cytokine protein.

1.14 Objectives

1) To determine if adenoviral administered CT-1 has an effect on the endogenous population of SP cells in the adult murine heart

2) To determine if CT-1 effects are specific to the cardiac lineage

3) To evaluate if the CT-1 effect on the cardiac SP population is concurrent to an activation of the gp130 signalling cascade.

1.15 Hypothesis

It is proposed that CT-1 acts as a regulator of stem cell expansion in the adult murine heart, and that this expansion is specific to the cardiac lineage.

Chapter 2

Materials and Methods

2.1 The CT-1 adenovirus

The cardiotrophin-1 adenovirus was obtained as a gift from Dr. Axel Kahn at Institut National de la Santé et de la Recherche Médicale in Paris, France. To generate the adenovirus a full length murine CT-1 cDNA was isolated by screening an adult mouse muscle cDNA library with a PCR probe corresponding to nucleotides 504-785 (GenBank accession no. U18366). The CT-1 reading frame was fused with a 60 base pair pre-Nerve Growth Factor (NGF) leader sequence to promote secretion of the CT-1 protein. It was also fitted with the long terminal repeat of the Rous Sarcoma virus (RSV), a ubiquitous promotor, which acted to drive the CT-1 expression. Infection with the adenovirus resulted in the production of a secreted 23.5-kDa protein (Bordet, 1999).

A LacZ adenovirus (CTRL) was used as a control for all injection experiments. This adenovirus was kindly provided by Dr. Robin Park at the Ottawa Health Research Institute, Ottawa, Canada. The adenovirus contained the full length LacZ coding region and was driven by the cytomegalovirus (CMV) promoter.

2.2 Animal model

The main strain of mouse used in experiments was the B6C3F1 mouse strain. This model was employed in all injection time courses, cardiac SP characterization experiments and histological analyses, as well as for the isolation of protein, RNA and primary cardiomyocytes.

The green fluorescent protein (GFP) - expressing mouse strain, STOCK TgN (GFPu) 5Nagy was used in the co-culture experiment. These mice were obtained through Jackson Laboratories. This transgenic strain carried the green fluorescent protein driven by chicken beta-actin promoter and CMV intermediate early enhancer. Mice, and the cells derived from them, are distinguished from wild type on the basis of green fluorescence.

2.3 Primary Cardiomyocyte Isolation

Primary cardiomyocytes were isolated as previously published (Argentin et al., 1994). In summary, the hearts were removed from neo-natal pups (4 –12 days old); the ventricles were separated and rinsed in Joklik's modified Eagle's medium (JM-MEM) (Gibco). The ventricles were minced in a petri dish using two scalpel blades and rinsed in JM-MEM. The minced tissue was then transferred to a 50 mL Falcon tube and digested with 1% collagenase B (Roche) for 15 minutes at 37 °C. This digestion was repeated a total of four times. After each digestion step, the undigested tissue was dispensed back into the 50mL falcon tube, while the supernatant was collected and pooled in a Falcon tube containing 10 mL of cold Dulbecco's Modified Eagle Medium (DMEM). The digestion process was terminated by the addition of 10% fetal bovine serum (FBS)(Gibco) to the DMEM. Tissue was rinsed with JM-MEM and triturated to further dissociate cells between each digestion. The supernatant from each wash was transferred to the same DMEM + 10% FBS collection tubes. Following the last incubation, the remaining tissue was washed twice in 10 mL DMEM + 10% FBS, the supernatant was added to the DMEM + 10% FBS containing pools. Pooled cells were

centrifuged and the cell pellets were re-suspended in DMEM + 10% FBS. Cells were then filtered through a 74 μm nylon filter (Fisher Scientific) to remove any undigested tissue. To remove any contaminating fibroblasts, cells were pre-plated twice for 15 minutes at 37 °C on a 10 cm petri dish (Fisher Scientific). After the final pre-plating, cardiomyocytes that did not adhere were collected and counted using a hemocytometer (VWR Canlab). Cells were then plated in DMEM + 10% FBS at a density of 10^5 cells per cm^2. Cells were plated on either collagen type one pre-coated cover slips (Fisher Scientific) in petri dishes or on collagen type one pre-coated glass chamber slides (BD Biosciences). The day following the final plating step, media was removed and replaced with serum free Dulbecco's Modified Eagle Medium Nutrient mixture F-12 (DMEM-F12) (Invitrogen) media containing 1X insulin/transerrin/selenium (ITS) (VWR) and penicillin/streptomycin (100U/mL/100ug/mL) (P/S) (Gibco). The ITS was added to prevent the proliferation of non-cardiomyocytes. Cardiomyocytes were maintained at 37°C in a CO_2 enriched (5%) humid atmosphere. Media (DMEM-F12 + ITS +P/S) was changed every 48 hours.

2.4 Adenovirus Injections

All injections were administered by a trained animal care technician according to the standards of the Animal Care Committee at the University of Ottawa, Ottawa, Canada. Briefly, animals were anaesthetized with halothane. The injections were administered via intra-cardiac delivery using a 29-gauge insulin needle (VWR). A maximum volume of 50 μL of adenovirus was injected per mouse. Post-recovery mice were monitored closely for weight loss, dehydration and cardiac distress.

2.5.0 Characterization of the CT-1 Adenovirus

2.5.1 CT-1 Western Blot Analysis

Primary cardiomyocytes were isolated and plated in a 10 cm petri dish (VWR) that contained collagen type one coated glass cover slips. The day following the initial plating, primary cardiomyocytes were infected with CT-1 adenovirus at 10^8 plaque-forming units (PFU) per mL of media. Untransfected primary cardiomyocytes served as a control. At 72 hours post-infection, the media from the treated and untreated cells was collected. Protein concentrations were determined using Bradford assay (Bio-Rad), with bovine serum albumin (BSA) (VWR) as a standard. The protein standard was then used to determine CT-1 production via western blot analysis. A total of 50 μg of protein was boiled in 1X Laemmli buffer and separated by 15% SDS-polyacrylamide gel electrophoresis. Protein lysates were then transferred to an Immobilon-P membrane (Millipore Corporation) at 6 volts for 180 minutes. A commercially produced recombinant CT-1 protein (100 ng) was used as a positive control. The efficiency of protein transfer and the verification of equal protein loading were verified by western blot analysis using a tubulin antibody. Membranes were blocked at room temperature for one hour in 5% BSA in 0.1% Tris-Buffered Saline Tween-20 (TBS-T), then incubated in anti-CT-1 antibody (1:1000) (R&D systems) in 0.1% TBS-T overnight at 4 °C. The membrane was rinsed in 0.1% TBS-T three times for 5 minutes at room temperature. Then the membrane was incubated one hour at room temperature in secondary antibody horseradish peroxidase-conjugated goat-anti-mouse IgG (1:7500) (Bio-Rad Laboratories), then washed five times for 10 minutes in 0.1% TBS-T at room temperature with vigorous shaking. Enhanced chemiluminescence was then performed

using ECL™ Western Blotting Detection Reagents (Amersham). The blots were developed on Kodak x-ray film (Amersham).

2.5.2 *In vitro* translation of CT-1 Plasmid

In vitro translation was performed on the bluescript plasmid used to generate the CT-1 adenovirus, courtesy of Dr. Pasan Fernando, Ottawa Health Research Institute, Ottawa, Canada. Briefly, the plasmid was translated using [S^{35}]methionine. The resulting protein was then separated on a 12% SDS gel. A previously characterized pro-caspase 3 expression plasmid was used as a translation control.

2.6 Adenoviral Injection Protocol

A total of twelve mice were administered intracardiac injections. Mice were injected with 50 μL of Ad-CT-1 at concentrations of 6.0 X 10^9, 3.0 X 10^8 or 3.0 X 10^7 PFU/mL. Three mice were injected per group. A control group of 3 B6C3F1 mice were injected with 50 μL Ad-CTRL at 1.5 X 10^9 PFU/mL. At 72 hours post injection, the mice were sacrificed and the heart ventricles were excised. The cells were stained with Hoechst dye 33342 (Sigma-Aldrich) according to the protocol and then analysed by fluorescence activated cell sorting (FACS). The side population (SP), the verapamil sensitive population from each of the groups were compared to the SP from the control group.

2.7.0 FACS analysis

2.7.1 Isolation of single cells from heart tissue for FACS analyses

Single cell cardiac suspensions were derived from isolated ventricles excised from B6C3F1 mice. The mice were approximately 2 months of age with the exception of one experiment in which FACS analysis was performed on mice that were between 2 – 52 weeks old. The ventricles were digested in collagenase B (10 mg/mL) (Roche) plus dispase II (2 units/mL) (Roche) for 35 minutes at 37 °C. During digestion the tissue was triturated every 10 minutes. Digested cells were filtered through a 74 μm nylon filter (Costar) into a 50 mL falcon tube. Filtered cells were centrifuged at 1000 rpm for 10 minutes. The supernatant was aspirated and the cell pellet was washed with cold Dubecco's Modified Eagle's Medium (DMEM) (Invitrogen) containing 2% FBS (Invitrogen) and 10 mM Hepes (Invitrogen) (DMEM+). The re-suspended cells were re-centrifuged (1000 RPM, 10 minutes) and the supernatant was aspirated leaving a cell pellet. The pellet was re-suspended in DMEM+ at a concentration of 10^6 cells/mL.

2.7.2 FACS: Hoechst dye 33342

Following the excision of hearts and the preparation of a single cell suspension in DMEM+, the cells were stained with Hoechst dye 33342 (5 ug/mL) (Sigma-Aldrich). The dye and cell suspensions were co-incubated at 37°C for 90 minutes. As an SP control, the drug verapamil (50 uM) (Sigma-Aldrich) was added to an aliquot of cells simultaneously stained with Hoechst 33342. Verapamil prevents the efflux of the dye by blocking the multi drug resistant proteins on the cell surface, in time causing the disappearance of the SP profile (Goodell, 1996; Goodell, 1997) Following the 90-

minute staining, cells were placed on ice for 10 minutes and were on ice for the duration of the experiment to limit any further biological activity. The cells were pelleted and washed in cold DMEM+ to remove any dead cells or cell debris. Cells were finally re-suspended in 500 uL of Hanks Balanced Salt solution with 2% FBS and 10mM Hepes (HBSS+). The cells were filtered through a 50μm Cell Tric® (disposable filters made of monofil nylon material) (Partec GmbH) and remained on ice until FACS analysis was performed or were subject to further processing.

2.7.3 FACS Immunolabeling – Sca-1

Following staining with Hoechst 33342 dye, cells were re-suspended in cold 1 X phosphate buffered saline (PBS) containing 5% BSA at a concentration of 10^6 cells/mL. The BSA was used to block any non-specific binding of the Sca-1 antibody. The cells were left on ice for 15 minutes. Then a Sca-1 fluorescent conjugated monoclonal antibody was added (1:100) (Pharmingen). As a control for background staining, PE-conjugated mouse monoclonal IgG2A isotype antibody (1:100) (Pharmingen) was used on a separate aliquot of cells. All cells were left on ice for 15 minutes. Once staining was complete, cells were washed in cold PBS with 2% FBS, pelleted and finally re-suspended in 500 μL of HBSS+. The cells were then passed through a 50μm Cell Tric® (Partec GmbH) and kept on ice for FACS analyses. Two sets of experiments were performed using the Sca-1 antibody:

1) Heart cells from mice were stained with Hoechst 33342 then labelled with the Sca-1 antibody. A comparison was made between the side population (SP) and the main population (MP) and the Sca-1 expression profiles.

2) First, mice were injected with either the CT-1 or CTRL adenovirus. At 48 hours post-injection, the hearts were excised and analyzed for Sca-1 expression. In this scenario a comparison was made between the SP cells from CT-1 and CTRL adenovirus injected hearts and their Sca-1 expression profiles.

2.7.4 FACS Immunolabeling – LIF receptor

Following staining with Hoechst 33342, cells were re-suspended in cold 1 X PBS containing 5% BSA and 5% normal donkey serum (NDS) (Invitrogen) at a concentration of 10^6 cells/mL. The BSA and NDS was used to block non-specific antibody binding. The cells were left on ice for 15 minutes, before adding the LIF receptor antibody (in 1X PBS + 5% BSA was added (1:100). As a control for background staining, Alexa Fluor 488 labelled Donkey anti-goat IgG antibody (1:100) in 1X PBS (Molecular Probes) was used on a separate aliquot of cells. Cells were pelleted and washed with cold PBS with 2% FBS, pelleted and re-suspended in cold PBS containing 5% BSA and 5% NDS with the secondary antibody, Alexa Fluor 488 labelled Donkey anti-goat IgG antibody (1:100) (Molecular Probes). Cells were left on ice for 15 minutes, followed by 2 washes in cold 1 X PBS with 2% FBS. Finally cells were re-suspended in 500uL of HBSS+, passed through a 50μm Cell Tric® (Partec GmbH) and kept on ice for FACS analyses. A comparison was made between the LIF receptor expression in the heart SP and the non-SP (main population, MP).

2.7.5 FACS – Other Tissues

Other tissues were also analyzed by FACS: bone marrow, liver and skeletal muscle. These tissues were subject to the single cell suspension procedures as described above. The bone marrow however did not require the digestion step, as it was already a single cell suspension upon collection. In order to obtain the bone marrow, the femur bones were removed from each mouse leg, and all muscles were carefully dissected away. A 28-gauge insulin syringe (VWR) was filled with 1 X PBS and the bone marrow was flushed out of the bone. Once the bone marrow cell suspension was collected it was washed with cold DMEM+, pelleted and resuspended in DMEM+. The cells were then stained with Hoechst 33342 dye or immunolabeled as already described above.

2.7.6 FACS- Specifications and Analysis Software

Cell sorting was performed using a DakoCytomation MoFlo high-speed cell sorter (DakoCytomation). Forward and side scatter was measured at 488 nm (Spectraphysic Argon Laser). The Hoechst dye was excited at 359 nm (I90C laser from Coherent). Blue emission was measured at 424 nm (424/44 band pass filter) and red emission was above 675 nm (675 AGLP long pass filter). All data was collected and analyzed with Summit^{TM} Data Acquisition and Analysis Software (DakoCytomation).

2.8 Injection Schedule – Tissue Retrieval

A total of 24 mice per time course were injected, 12 with the CT-1 adenovirus and 12 with the CTRL adenovirus. The mice received a one time intra-cardiac injection of 50 uL of virus at a concentration of $3.0X10^8$ PFU/mL. Following injections, animals were

sacrificed at 24, 48, 72 and 96 hours post-injection. The hearts, livers, bone marrow and skeletal muscles were excised and processed for FACS at each time point. Each time point was comprised of pooled tissue of three mice. The tissues were digested and stained with Hoechst dye 33342 as described above. The results were compared between each time point as well as between the CT-1 and CTRL adenovirus injected mice.

2.9 Methylcellulose Culture: Hematopoietic Activity Assay

The hematopoietic colony assay has been commonly used to test for stem cell/progenitor-like activity (Seale, 2000), and is based on colony growth in a semi-solid environment (i.e. methocult). Methocult is a commercial semi solid methylcellulose media that contains various cytokines and growth factors optimized to promote the proliferation and differentiation of hematopoietic activated stem cells and progenitor cells (Stem Cell Technologies). The prospective colonies that emerge under these conditions contain mature hematopoietic cell types such as granulocytes, macrophages and natural killer cells. As such, the assumption in the field is that emergence of these cells types reflects prior activation of a stem cell pool.

SP cells were sorted and 10^4 cells were re-suspended in 2.5 mL of Methocult media GF3434 (Stem Cell Technologies) using a 5 mL syringe and a 12 gauge needle. Cells were then plated on 2 cm plastic petri dishes and incubated in humidity chambers at 37°C and 5% CO2 for 14 days. At 14 days post plating, colonies were counted using a Zeiss inverted microscope.

2.10 Extended time course – Skeletal Muscle

The results from the skeletal muscle SP cells in the hematopoietic activity assay prompted further investigation. It remained possible that the effects of CT-1 on skeletal muscle SP cells were delayed and later time point analysis may reveal alterations in the SP population. To investigate this possibility, an extended time course of CT-1 administration was performed. A total of 6 mice received intra-cardiac injections with either the CT-1 or CTRL adenovirus (50 uL of 3.0 X 108 PFU/mL). Then at 10 days, 4 weeks and 6 weeks, the animals were culled, and skeletal muscle was removed. The tissue was processed, stained with Hoechst dye 33342 and analyzed using FACS.

2.11 The Effect of Age on the Cardiac SP

A time course was performed to look at the effect of the age of mice as related to stem cell response. A total of 30 mice were injected with either the CT-1 or CTRL adenovirus, via intra cardiac injections. A total of three mice per age group were injected per treatement. Mice were 3 weeks, 6 weeks, 2 months, 4 months and > 6 months old. The hearts were collected, stained with Hoechst 33342 and analyzed using FACS at 48 hours post injection.

2.12 Protein Isolation

Tissues (heart, liver, skeletal muscle, brain and kidney) were excised from mice immediately following euthanasia and flash frozen in liquid nitrogen. Frozen tissue was powdered and homogenized in cold PLC gamma lysis buffer containing various protease inhibitors (1mM PMSF, 1 mM Apoprotenin, 1 mM Leupeptin, 1 mM Sodium

Vanadate). Lysates were centrifuged and the supernatant containing the protein was transferred to a pre-chilled eppendorf tube. The protein concentrations were determined by Bradford analysis using BSA as a standard.

2.13.0 Western Blotting: CT-1 and STAT3

2.13.1 CT-1 Western Blot

Protein samples were collected from the hearts, livers and skeletal muscles of mice that had received either a CT-1 or CTRL adenovirus injection. A total of 300 μg of protein from each tissue sample was boiled with Laemmli buffer and separated by 15 % SDS-polyacrylamide gel electrophoresis. The lysates were then transferred to an Immobilon-P membrane (Millipore Corporation) at 6 volts for 300 minutes. The efficiency of protein transfer and equal protein loading was verified by western blot analysis an anti-α-tubulin antibody (Developmental Studies Hybridoma Bank, University of Iowa; refer to section 2.13.3). Membranes were blocked at room temperature for one hour in 5% BSA in 0.1% TBS-T, then at four degrees overnight. The extended blocking period was done to minimize background. The membrane was then incubated with polyclonal goat anti mouse CT-1 antibody (R&D systems) at a dilution of 1:1000 (in 0.1% TBS-T overnight at 4°C. After 3X 5 minute washes in 0.1% TBS-T, membranes were incubated in secondary antibody horseradish peroxidase-conjugated goat-anti-mouse IgG (Bio-Rad Laboratories) at a dilution of 1:5000 in 5% BSA + 0.1% TBS-T for one hour at room temperature. Enhanced chemiluminescence was performed using ECL ™ Western Blotting Detection Reagents (Amersham). A recombinant CT-1 protein (100 ng) (Peprotech) was used as a positive control, as well as media from CT-1 adenovirus

infected primary cardiomyocytes. Media from uninfected cells was used as a negative control.

2.13.2 Phospho-STAT3 Western Blot

To detect STAT3-phosphorylation, 500 μg of total protein from each tissue sample was boiled with 1 X Laemmli buffer and separated by 10 % SDS-polyacrylamide gel electrophoresis. The lysates were then transferred to an Immobilon-P membrane (Millipore) at 6 volts for 180 minutes. The efficiency of protein transfer and verification of equal protein loading was verified by western blot analysis using an anti-α-tubulin antibody (Developmental Studies Hybridoma Bank, University of Iowa; refer to section 2.13.3). Membranes were blocked at room temperature for one hour in 5% BSA in 0.1% TBS-T. The membrane was then incubated with monoclonal phospho-Stat3 (Ser 727) antibody (Cell Signalling) at a dilution of 1:1000 in 0.1% TBS-T overnight at 4°C. After 3 X 5 minute washes in 0.1% TBS-T, membranes were incubated in secondary horseradish peroxidase-conjugated goat-anti-mouse IgG antibody (Bio-Rad Laboratories) at a dilution of 1:5000 in 5% BSA in 0.1% TBS-T for one hour at room temperature. A total of 6 X 5 minutes washes with 0.1% TBS-T with vigorous shaking were performed to eliminate any non-specific binding. Enhanced chemiluminescence was performed using ECL^{TM} Western Blotting Detection Reagents (Amersham). Stat3 control lysate (Cell signalling) was used as a positive control.

2.13.3 Tubulin Western Blot

To detect equal loading of protein, western blot analysis was performed using an anti-α-tubulin antibody (Developmental Studies Hybridoma Bank, University of Iowa). Previously probed western blot membranes were stripped using Restore™ Western Blot Stripping Buffer (Pierce) according to the manufacturer's directions. Membranes were blocked at room temperature for one hour in 5% milk in 0.1% TBS-T. The membrane was then incubated with an α-tubulin antibody (Developmental Studies Hybridoma Bank, University of Iowa) at a dilution of 1:15 in 0.1% TBS-T overnight at 4°C. After 3 X 5 minute washes in 0.1% TBS-T, membranes were incubated in secondary horseradish peroxidase-conjugated goat-anti-mouse IgG antibody (Bio-Rad Laboratories) at a dilution of 1:5000 in 0.1% TBS-T for one hour at room temperature. A total of 6 X 5 minutes washes with 0.1% TBS-T with vigorous shaking was performed to eliminate any non-specific binding. Enhanced chemiluminescence was performed using ECL™ Western Blotting Detection Reagents (Amersham).

2.14 Co-Culture of Cardiac SP Cells and Primary Cardiomyocytes

Primary cardiomyocytes were isolated from wild type mice as previously described (refer to section 2.3). Isolated cardiomyocytes were plated on collagen type-1 coated, 2-well chamber slides (BD Biosciences) at a density of 10 000 cells per cm^2. The primary cardiomyocytes were cultured overnight. The following day, cardiac SP cells from 2-month-old GFP mice (described in section 2.2) were sorted and collected using FACS. A total of 5000 GFP positive cardiac SP cells were added to the previously plated primary cardiomyocytes. The co-cultures were maintained for 14 days and then fixed

and stained. Cells were fixed in 4% paraformaldehyde (PFA) for 10 minutes, washed in PBS and then permeabilized with 0.3% Triton-X 100 (Fisher Scientific) in PBS for 10 minutes. Cells were subsequently blocked in 3% BSA (Roche) in PBS for 30 minutes. Following blocking, cells were incubated in connexin-43 antibody (1:200) (Chemicon) overnight at 4°C. Cells were washed briefly using PBS and then incubated in Cy3-conjugated goat-anti-mouse secondary antibody (1:50) (Chemicon) at room temperature for 1 hour. The nuclei were then stained using 4, 6-Diamidino-2-Pheylindole (DAPI) (Sigma-Aldrich) diluted in PBS (1:10000). Cells were mounted using Dako fluorescent mounting medium (Dako Corporation) and were visualized/photographed using a Zeiss Axioscope microscope equipped with an ultraviolet light source.

2.15 RNA Extraction and Northern Blot Analysis

Mice were injected with either the CT-1 or CTRL adenovirus as previously described. At 72 hours post injection, total RNA was collected from hearts using the Trizol Reagent (Invitrogen) following the manufacturer's instructions. The RNA was quantified using spectrometry. A total of 15 ug of RNA from each sample was separated on a 1.0% agarose-formaldehyde gel and transferred to a Hybond-N+ nylon membrane (Amersham) as per manufacturer's instructions. The RNA was UV cross linked to the membrane and membranes were pre-hybridized for 1 hour in 10 mL of Church Buffer (0.5M Na_2HPO_4, 0.5M EDTA, 7% SDS) containing salmon sperm DNA (as a blocking agent) at 65°C. The membranes were then hybridized overnight at 65°C with denatured [alpha-32P] dCTP labelled probe spanning a portion of the murine atrial natriuretic factor (ANF) sequence. The sequence was labelled using the Rediprime II

prime labelling system (Amersham). The membranes were washed using various stringencies and then exposed to Kodak x-ray film (Amersham) at -80°C.

2.16 Heart to Body weight – hypertrophy assessment

Animals were injected with either the CT-1 or CTRL adenovirus, as previously described. At one week post injection the animals were culled and their hearts were excised and weighed. Prior to culling animals body weights were taken. As a means to assess hypertrophy, these weights were compared in the form of a ratio, heart weight in mg to body weight in g. The averages were taken from each group, CT-1 adenovirus hearts and CTRL adenovirus hearts and compared using a two tailed, unpaired Student's *t* test. Differences were considered statistically significant at a p value less than 0.05.

2.17 CT-1 treated H9C2 cells

The rat heart cell line (H9C2) (American Type Culture Collection) was cultured in DMEM with 10% FBS and penicillin/streptomycin (100 U/ml, 100 ug/mL) (growth media) on glass chamber slides. Cells were cultured to a confluence of 70% and then cells were infected with the CT-1 adenovirus. Two cell culture controls were also utilized, one with no adenovirus and the other with the adenovirus plus CT-1 antibody. 48 hours post treatment, media was removed and the cells were washed with 1 X PBS, fixed in 4% paraformaldehyde (PFA) in 1X PBS at room temperature for 15 minutes. The chamber slides were washed twice in 1 X PBS and then permeabilized for 10 minutes in 0.3% Triton X-100. The nuclei were stained with DAPI (Sigma-Aldrich)

diluted 1:10000 in 1 X PBS for 10 minutes at room temperature. Cells were mounted using Dako fluorescent mounting medium (Dako Corporation) and were visualized/photographed using a Zeiss Axioscope microscope equipped with an ultraviolet light source.

2.18 Histology Analysis of Hearts and Skeletal Muscle

Mice were injected intra-cardially with either CT-1 or CTRL adenovirus. At 5 days post-injection, hearts and skeletal muscle were excised and rinsed in cold 1 X PBS. The tissues were fixed in 4% PFA in 1 X PBS for 2 days. They were then embedded in paraffin, sectioned at 10 μm and counterstained with hematoxylin and eosin to visualize the nuclei and cytoplasm. Sections were dehydrated in a graded ethanol series ending in CitriSolv (Fisher Scientific). The slides were cover slipped prior to analysis using a Zeiss Inverted microscope. Sections were examined for gross morphological differences, such as changes in cell size, number of nuclei and number of total cells.

2.19 Statistical Analysis

Differences between Ad-CT-1 and Ad-CTRL injected tissues were evaluated for statistical significance using two tailed, unpaired Student's *t* tests. Differences were considered statistically significant at a p value less than 0.05. Prior to applying the *t* test numbers were normalized for each time point. Each number was divided by the CTRL value for that given time point, i.e. all CTRL values are equal to one. Morphological differences between Ad-CT-1 and Ad-CTRL injected hearts and skeletal muscle were

evaluated for statistical significance using two tailed, unpaired Student's *t* tests. Once again, differences were considered statistically significant at a p value less than 0.05.

Chapter 3

Results

3.1 Identification of Cardiac SP cells

Recently published work has identified a stem cell-like population in the heart (Hierlihy, 2002). In order to identify and isolate these cells, a combination of fluorescent staining with dye Hoechst 33342 and fluorescence activated cell sorting (FACS) was used. This technique was first employed to identify a multi-potent stem cell population within the bone marrow of adult mice (Goodell, 1996). Briefly, this specific bone marrow population had a unique weak Hoechst 33342 staining pattern that appeared as a distinct group of cells, also known as the side population or SP. Further experimentation showed that the SP was highly enriched for stem cell activity (Goodell, 1996). Using these previous findings as an experimental platform, Hierlihy et *al.* were able to isolate a subset of cardiac cells, the cardiac SP, which appeared to have stem cell like qualities. To confirm the existence of the SP in the heart this method was repeated. The ventricles from the hearts of adult mice were stained with Hoechst 33342 and analyzed by FACS. The current results confirmed that a distinct population of SP cells was indeed present in the heart (Figure 2A), and that this population was also verapamil sensitive (Figure 2B). The aim of this study was to investigate the responsiveness of this population to an exogenous challenge, i.e. cytokine exposure.

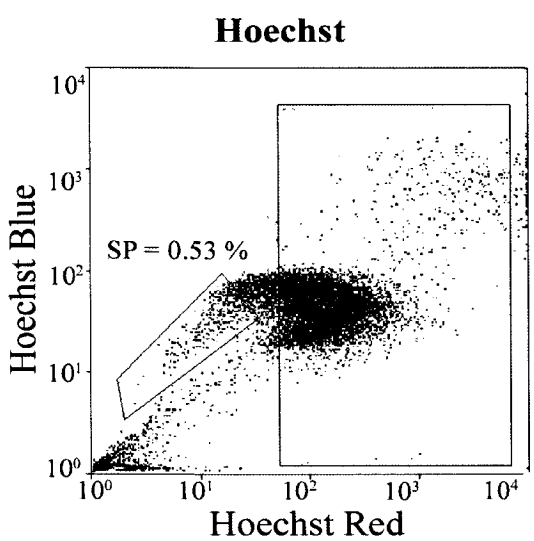
3.2 Characterization of the CT-1 Adenovirus

The CT-1 adenovirus was received as a gift from Dr. Axel Kahn at the National Institute of Health and Medical Research in Paris, France. The construct used to generate the adenovirus was also provided. The construct contained the full-length

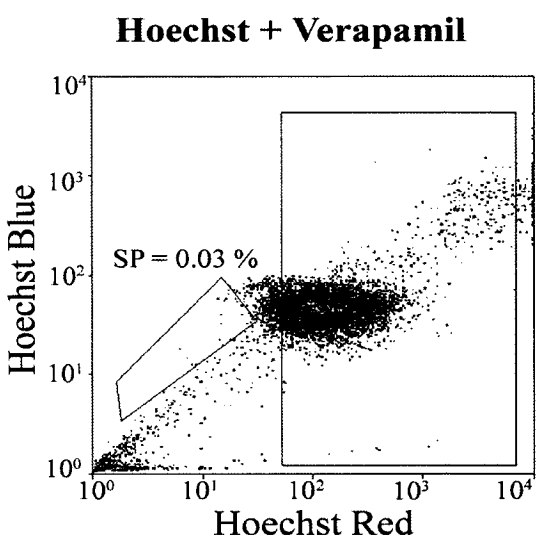
Figure 2: Identification of a Verapamil-Sensitive Side Population from Murine Heart Cell Suspensions.

FACS analyses of hearts from 2 month-old wildtype mice. Cells were stained with Hoechst dye 33342 (A) or Hoechst dye 33342 plus verapamil (B). The results revealed ~ 0.5 % Hoechst dye excluding fraction (i.e. SP) which was verapamil sensitive.

A


Hoechst
SP = 0.53 %
Hoechst Blue
Hoechst Red
10^0
10^1
10^2
10^3
10^4

B


Hoechst + Verapamil
SP = 0.03 %
Hoechst Blue
Hoechst Red
10^0
10^1
10^2
10^3
10^4

murine CT-1 sequence fused to the pre-Nerve Growth Factor leader sequence, driven by the ubiquitous Rous Sarcoma Virus (RSV) promoter in a bluescript plasmid (Figure 3A). This was confirmed by DNA sequencing. In order to confirm that a secreted full length CT-1 protein was generated, two experiments were performed. In the first experiment, primary cardiomyocytes were treated with the CT-1 adenovirus. The media from the infected cardiomyocytes was collected and analyzed by western blot analysis (Figure 3B). The blot compared the media of treated and untreated cells and confirmed that a full length CT-1 protein was being produced by the adenovirus. The CT-1 was being secreted into the media, and was of the expected size, 23.5 kDa (Bordet, 1999). The size is slightly larger than the endogenous CT-1 protein, 21.5 kDa (Pennica, 1995) owing to the fusion with the pre-nerve growth factor (NGF) amino acid sequence.

Secondly, the bluescript construct was then *in vitro* translated using S^{35} labeled methionine. The results also indicated that a 23.5 kDa protein was produced (Figure 3C). Both of these experiments confirmed that the adenovirus did produce a secreted protein of the expected size. This provided confidence that upon *in vivo* injection of the adenovirus diffusible CT-1 protein would be produced.

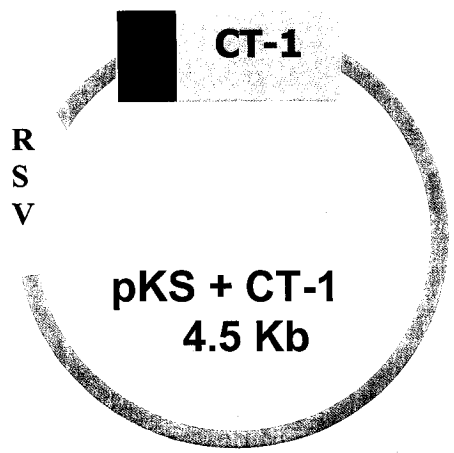
3.3 CT-1 Adenovirus Injection Protocol

Following the expression characterization of the CT-1 adenovirus, experiments were undertaken to determine whether CT-1 adenovirus administration would effect on the

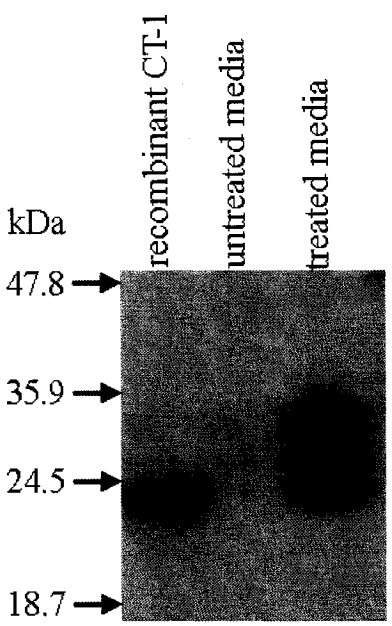
Figure 3: Characterization of the CT-1 Adenovirus (Ad-CT-1)

The CT-1 adenovirus was obtained from Dr. Axel Kahn. The virus contained the full-length CT-1 sequence fused to the nerve growth signal driven by the RSV promoter (A). Western blot analysis confirmed that the CT-1 adenovirus produced a CT-1 protein (B). Analysis was performed using 50 μg of protein from the media of Ad-CT-1 treated or untreated primary cardiomyocytes. The size of the recombinant CT-1 protein is 21.5 kDa and the size of the adenovirus produced CT-1 is 23.5 kDa. To confirm these findings, the plasmid used to generate the CT-1 adenovirus was *in vitro* translated using [S^{35}]methionine (C). As a control pro-caspase 3 was also translated. The S^{35} labelled CT-1 protein generated was the appropriate size, 23.5 kDa.

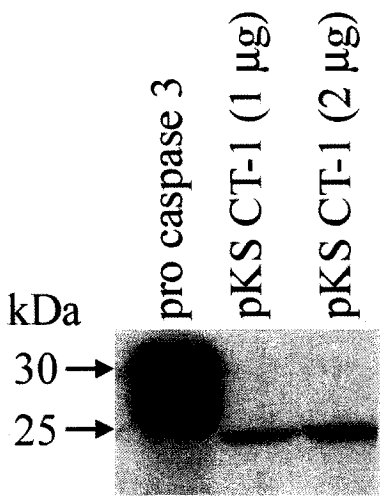
A


CT-1
R
S
V
pKS + CT-1
4.5 Kb

B


recombinant CT-1
untreated media
treated media
kDa
47.8
35.9
24.5
18.7

C


pro caspase 3
pKS CT-1 (1 µg)
pKS CT-1 (2 µg)
kDa
30
25

SP population in murine hearts. Previous studies had been published that used the same adenovirus (Bordet, 1999; Lesbordes, 2003). However these studies looked at the use of the CT-1 adenovirus in models of skeletal muscle and neural disease. For example, the amount of adenovirus injected intramuscularly (gastrocnemius, triceps brachii or dorsal muscle of the thoracic trunk) was in the range of 1.0×10^7 to 3.0×10^8 PFU in 100 uL of PBS (Bordet, 1999). To determine the effects of CT-1 on cardiac SP cells a systemic delivery option was employed. The injection protocol consisted of direct cardiac injection in the ventricle chambers with a volume of 50uL at one of three concentrations: a low dose (3.0×10^6 PFU/100 uL), a medium dose (3.0×10^7 PFU/ 100 uL) or a high dose (6.0×10^8 PFU/100 uL). A control group was also injected with 50 uL of the LacZ adenovirus (CTRL) at a concentration of 1.5×10^8 PFU/ 100 uL. A total of 12 mice were injected, 3 mice per group. At 72 hours post injection the hearts were collected and their side populations (SP) were analyzed using Hoechst dye 33342 and FACS. It is important to note that the high dose resulted in the death of all three animals within the first 24 hours post injection. A post mortem examination showed inflammation of the liver, while all other organs appeared "normal". As a result only the low, medium and control injected animals were utilized for analysis.

The results indicated that the CT-1 adenovirus injections caused an increase in the percentage of SP cells (Table 1). The control adenovirus had an average SP of 1.26 %, while the low dose had an average SP of 1.93% and the medium dose an average SP of 2.16%. Both low and medium CT-1 adenovirus concentrations had an increasing effect on the cardiac SP, however the greatest effect without death was seen at the medium

Table 1: Effective Dose Range for Ad-CT-1 *In Vivo* Administration

Various concentrations of Ad-CT-1 were administered via intra-cardiac injection. A total of three mice per group were injected with a volume of 50 μL. The SP fraction was analysed at 72 hours post injection. The results showed a maximum increase in the cardiac SP at the medium dose. As a result it was this dosage that was used in all future injections, 50 uL of 3.0 X 10^7 PFU/ 100 uL (for both the CT-1 and CTRL adenoviruses).

Adenovirus	Dose (PFU/mL)	SP (t=72 h)
CTRL Ad (LacZ)	1.5×10^9	1.26 %
CT-1 Ad (low)	3.0×10^7	1.93 %
CT-1 Ad (medium)	3.0×10^8	2.16 %
CT-1 Ad (high)	6.0×10^9	All animals died

3.4 Effect of CT-1 Adenovirus on the Murine Heart

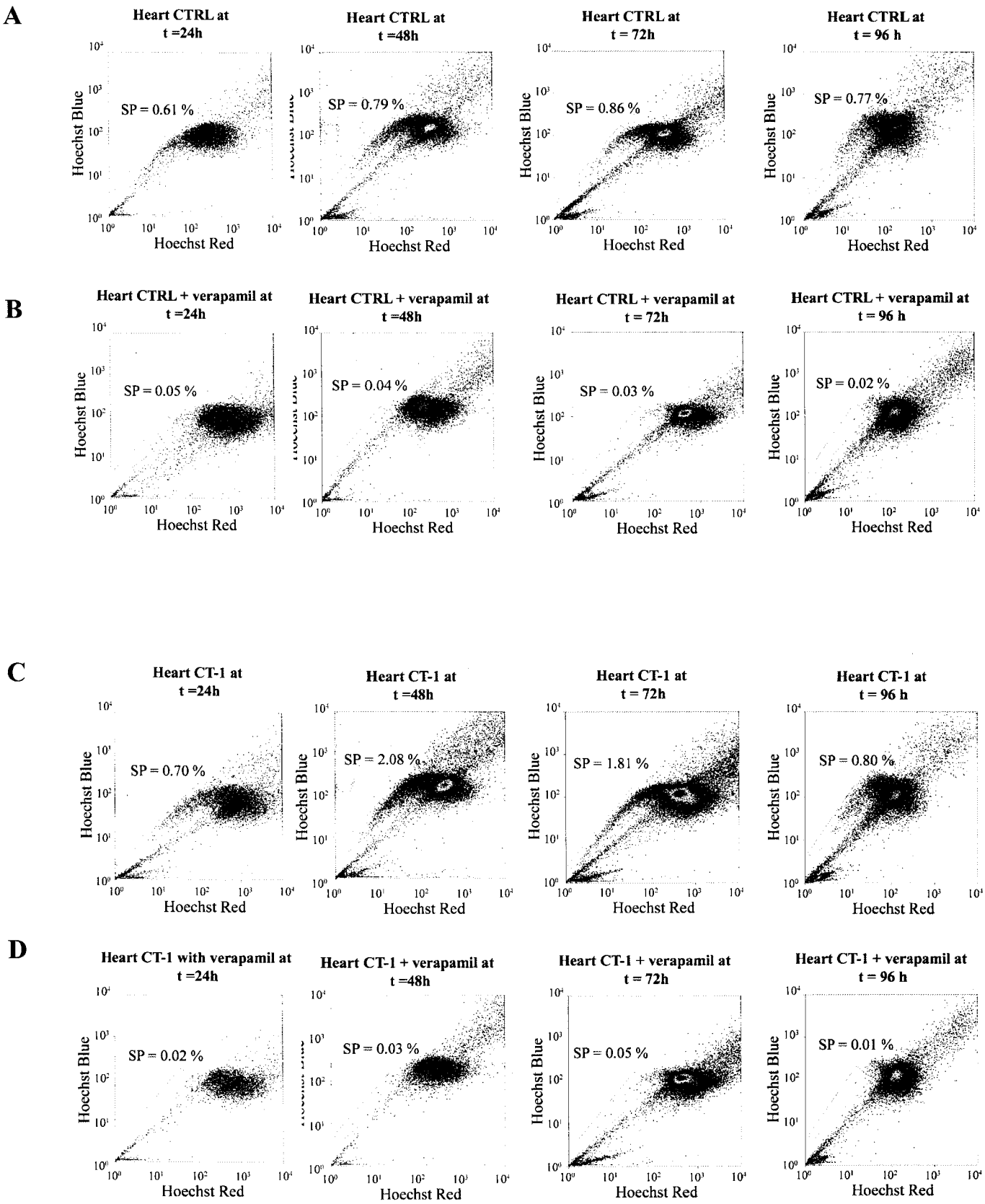
The next experiment examined the time-dependent effect of the CT-1 adenovirus on the cardiac SP population and whether longer exposure to the adenovirus would elicit a greater increase in the cardiac SP? To address this question, animals were injected with either the CT-1 or CTRL adenovirus. Then at 24, 48, 72 or 96 hours post injection, the hearts were excised, processed into single cell suspensions, stained with Hoechst dye 33342 and subject to FACS analysis. At each of the time points that SP populations were measured, the results indicated that Ad-CT-1 exposure led to a short- term increase in the cardiac SP population (Figure 4, Graph 1). The increase in the percentage of SP cells was between two and three fold and occurred only during the first 72 hours (at 24, 48, 72 hours $P < 0.05$, $n=4$; at 96 hours $P > 0.05$, $n=4$). After 72 hours the SP fraction returned to its pre-injection levels. No increase in the percentage of SP cells was observed in animals that were injected with the CTRL adenovirus. These results indicated that: 1) the CT-1 adenovirus caused an increase in the percentage of cardiac SP cells and 2) that this increase was short-term, lasting only 72 hours.

3.5 Effects of CT-1 Adenovirus on Other Tissues

Exposure to the CT-1 adenovirus may have enhanced the cardiac SP population through one of two mechanisms. First the SP increase may have originated from an activation of a resident population. Alternatively CT-1 cardiac administration may have promoted the recruitment of non-cardiac stem cell populations. To address this issue, cell populations

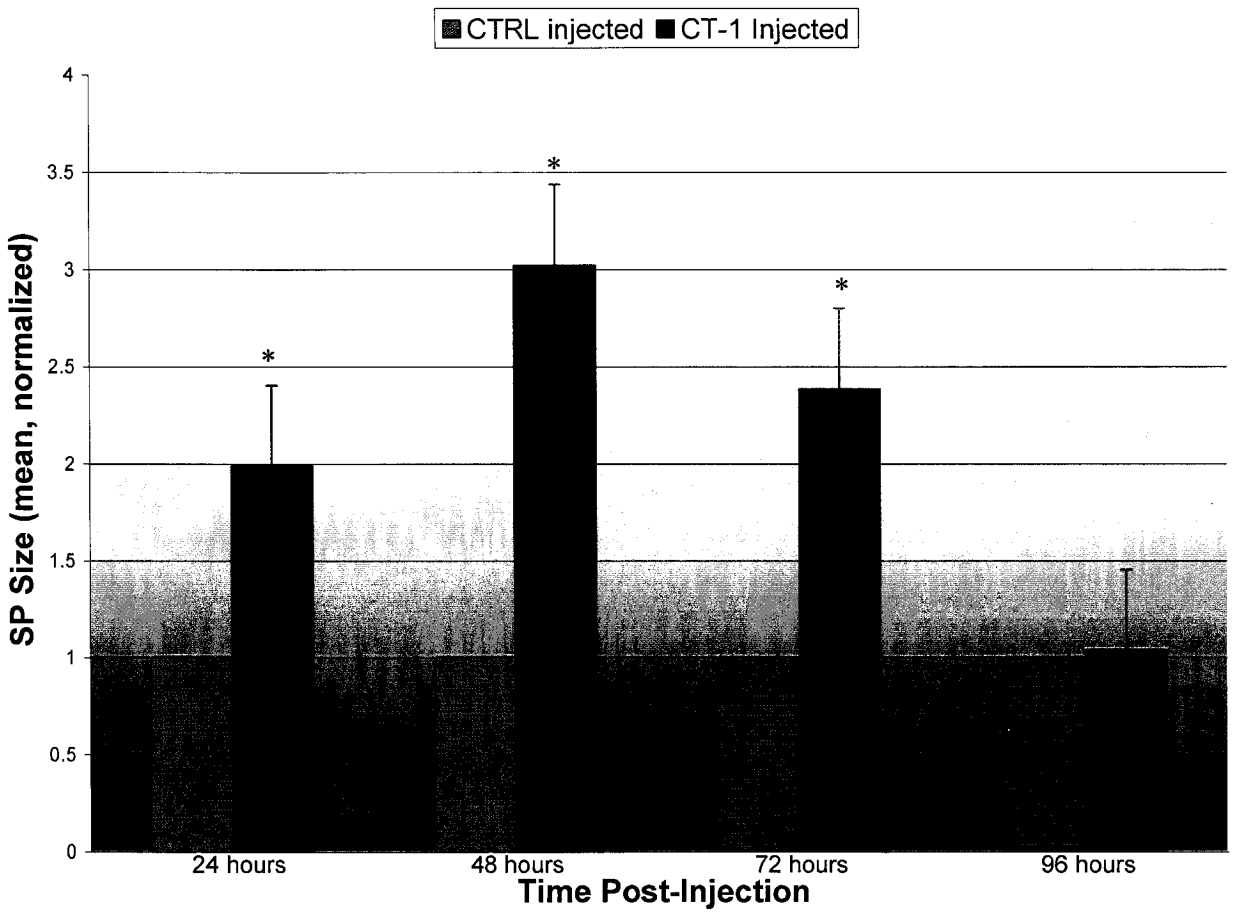
Figure 4: Effects of Ad-CT-1 on the Murine Heart.

FACS analyses of cardiac cells from ~2 month old wild type mice that had been injected with either Ad-CTRL (A & B) or Ad-CT-1 (C & D). Cell suspensions were stained with either Hoechst dye 33342 (A & C) or Hoechst dye 33342 plus verapamil (B & D). Cells were analysed at 24, 48, 72 or 96 hours post injection. The results revealed a short-term increase in the cardiac SP within the first 72 hours in mice injected with Ad-CT-1 (A compared to C).


A
Heart CTRL at t =24h
SP = 0.61 %
Heart CTRL at t =48h
SP = 0.79 %
Heart CTRL at t = 72h
SP = 0.86 %
Heart CTRL at t = 96 h
SP = 0.77 %
B
Heart CTRL + verapamil at t =24h
SP = 0.05 %
Heart CTRL + verapamil at t =48h
SP = 0.04 %
Heart CTRL + verapamil at t = 72h
SP = 0.03 %
Heart CTRL + verapamil at t = 96 h
SP = 0.02 %
C
Heart CT-1 at t =24h
SP = 0.70 %
Heart CT-1 at t =48h
SP = 2.08 %
Heart CT-1 at t = 72h
SP = 1.81 %
Heart CT-1 at t = 96 h
SP = 0.80 %
D
Heart CT-1 with verapamil at t =24h
SP = 0.02 %
Heart CT-1 + verapamil at t =48h
SP = 0.03 %
Heart CT-1 + verapamil at t = 72h
SP = 0.05 %
Heart CT-1 + verapamil at t = 96 h
SP = 0.01 %
Hoechst Blue
Hoechst Red

Graph 1: Effects of Ad-CT-1 on the Cardiac SP over time.

A bar graph illustrating the means obtained by FACS analyses of cardiac cells from ~2 month old wild type mice that had been injected with either Ad-CTRL or Ad-CT-1. Cells were analysed at 24, 48, 72 or 96 hours post injection. The results represent the mean SP size at each time point. The means were calculated using normalized data ($P<0.05$ by student's t-test, n=4, error bars represent the standard error)

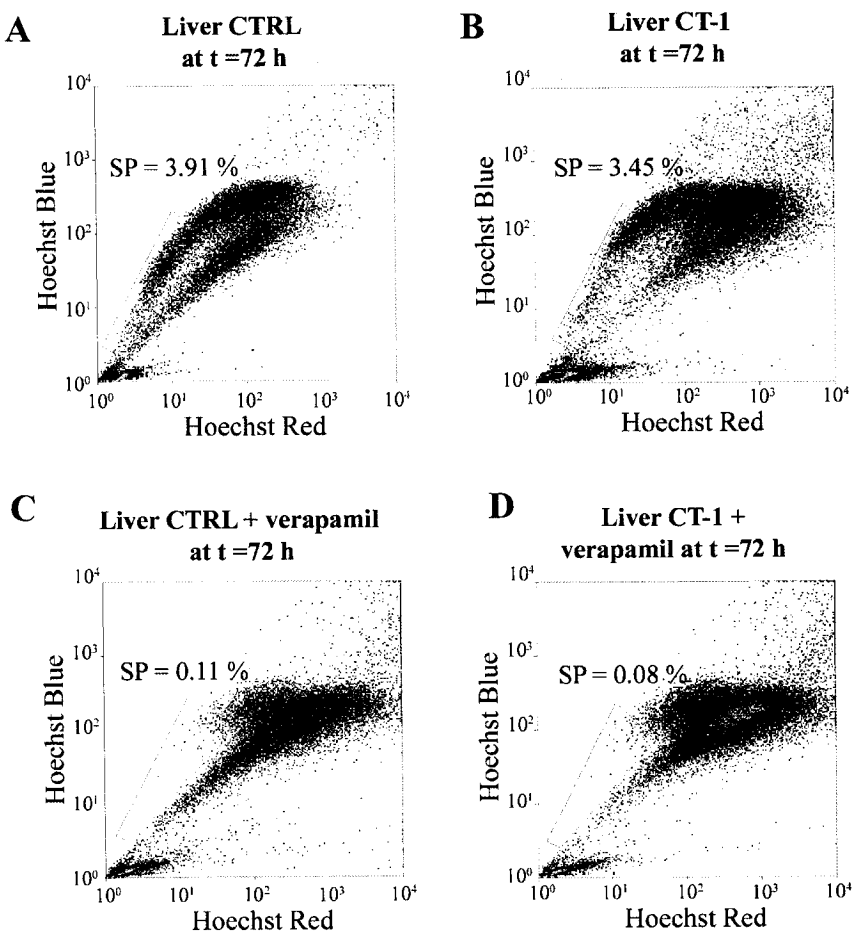


(* denotes a statistical difference between control and CT-1 injected hearts, where $P<0.05$ by student's t-test, $n = 4$)

from other tissues were examined following CT-1 injection. The tissues chosen for analysis included: liver, skeletal muscle and bone marrow. It is known that the majority of an injected adenovirus (~80%) reaches the liver (Paielli, 2000; Alemany, 2001) necessitating examination of this tissue. Previous studies have looked at the effect of cytokines on bone marrow cells. It has been reported that in the presence of an acute myocardial infarct, administered cytokines (stem cell factor and granulocyte-colony stimulating factor) mediated the translocation of bone marrow cells to the infarct site (Orlic, 2001). The migrating bone marrow cells contributed to a significant degree of tissue regeneration (Orlic,2001[a,b]). As a result, bone marrow cells were analyzed to determine if CT-1 had a similar translocation effect. Skeletal muscle and heart are both muscle tissues. In order to determine if CT-1 had a cardiac or a muscle specific effect, skeletal muscle was also analyzed.

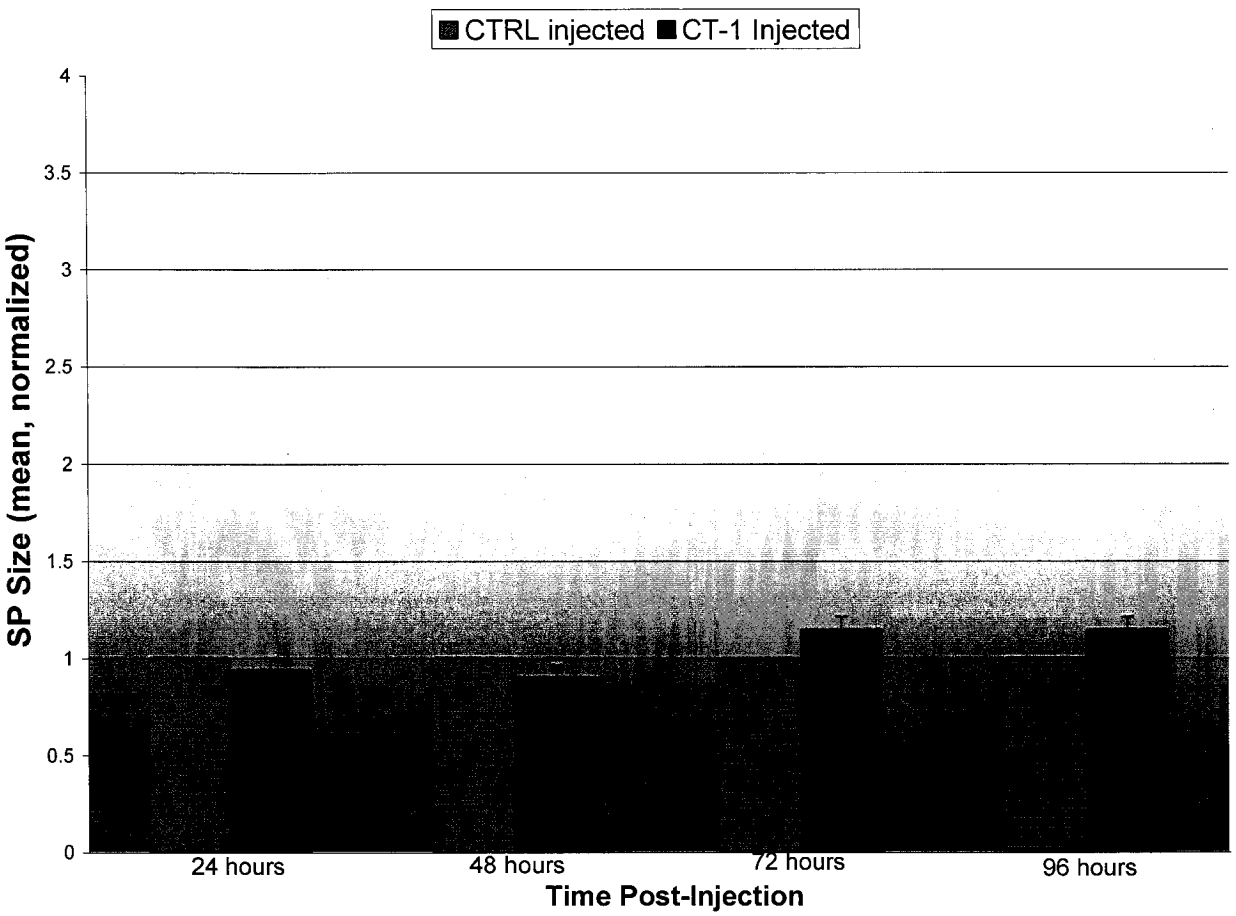
Subsequently, animals were injected with either the CT-1 or CTRL adenovirus. Then at 24, 48, 72 or 96 hours post injection, liver, skeletal muscle and bone marrow samples were processed, stained with Hoechst dye 33342 and analyzed by FACS. Interestingly, the percentage of SP cells did not increase over time in liver ($P<0.05$, $n=3$) (Figure 5, Graph 2), bone marrow ($P<0.05$, $n=4$) (Figure 6, Graph 3) or skeletal muscle ($P<0.05$, $n=4$) (Figure 7, Graph 4). These results led to the conclusion that the effect of the CT-1 adenovirus on the cardiac SP population was a specific response and did not originate from increased stem cell activity or recruitment from other locations.

Figure 5: Effects of Ad-CT-1 on the Murine Liver at t= 72 hours post-injection. FACS analyses of liver cells obtained from ~2 month old wild type mice that had been injected with either Ad-CTRL (A & C) or Ad-CT-1 (B & D). Cell suspensions were stained with either Hoechst dye 33342 (A & B) or Hoechst dye 33342 plus verapamil (C & D). Cells were analyzed at 24, 48, 72 or 96 hours post injection (72 hour time point shown here). The results revealed no changes in the liver SP following exposure to Ad-CT-1. ($P<0.05$ by student's t-test, n=3)


A
Liver CTRL
at t =72 h
SP = 3.91 %
Hoechst Blue
Hoechst Red
B
Liver CT-1
at t =72 h
SP = 3.45 %
Hoechst Blue
Hoechst Red
C
Liver CTRL + verapamil
at t =72 h
SP = 0.11 %
Hoechst Blue
Hoechst Red
D
Liver CT-1 +
verapamil at t =72 h
SP = 0.08 %
Hoechst Blue
Hoechst Red

Graph 2: Effects of Ad-CT-1 on the Liver SP over time.

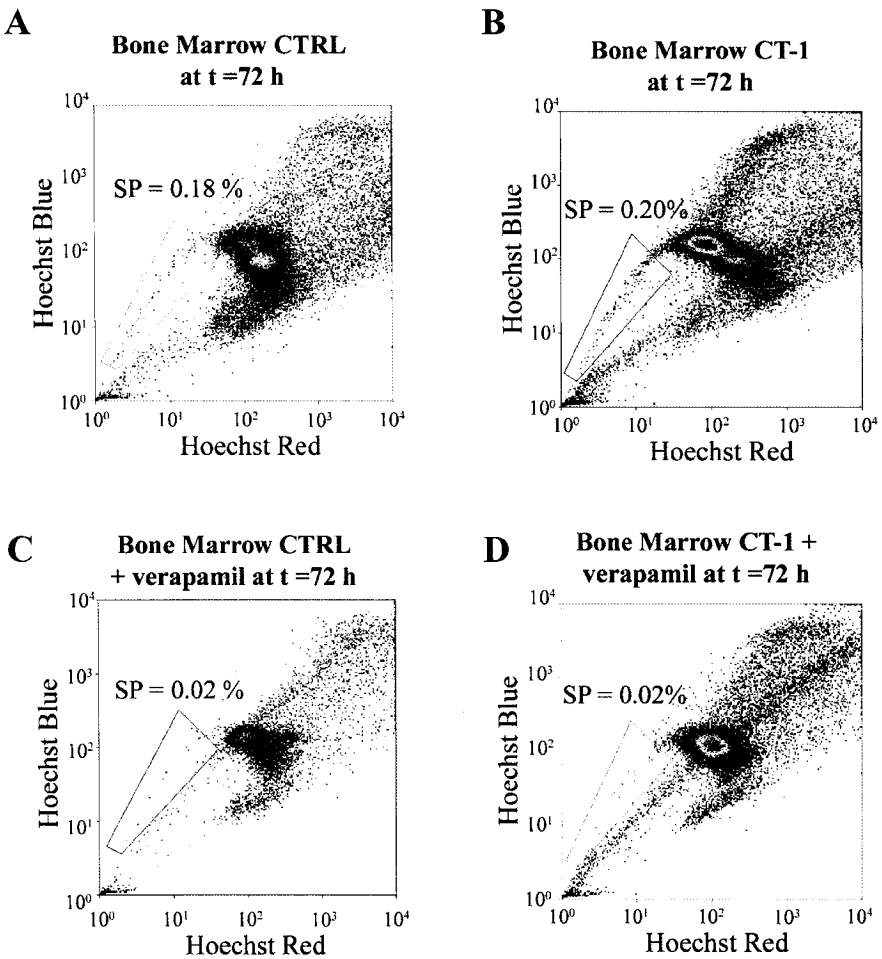
A bar graph illustrating the means obtained by FACS analyses of liver cells from ~2 month old wild type mice that had been injected with either Ad-CTRL or Ad-CT-1. Cells were analysed at 24, 48, 72 or 96 hours post injection. The results represent the mean SP size at each time point. The means were calculated using normalized data ($P<0.05$ by student's t-test, n=4, error bars represent the standard error).



(* denotes a statistical difference between control and CT-1 injected mice, where $P<0.05$ by student's t-test, $n = 4$)

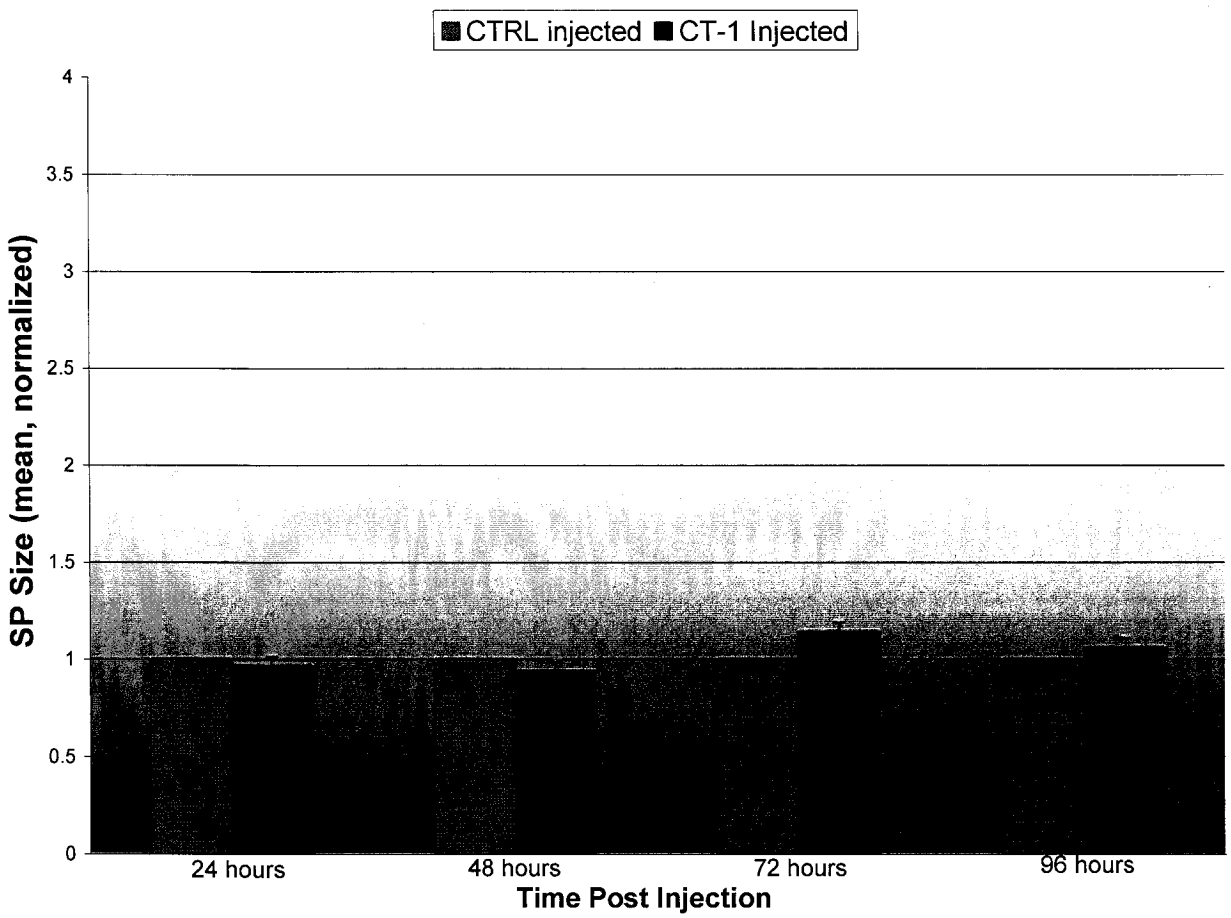
Figure 6: Effects of Ad-CT-1 on Murine Bone Marrow at t= 72 hours post injection.

FACS analyses of bone marrow cells obtained from ~2 month old wild type mice that had been injected with either Ad-CTRL (A & C) or Ad-CT-1 (B & D). Cell suspensions were stained with either Hoechst dye 33342 (A & B) or Hoechst dye 33342 plus verapamil (C & D). Cells were analysed at 24, 48, 72 or 96 hours post injection (72 hour time point shown here). The results revealed no changes in the bone marrow SP following exposure to Ad-CT-1. ($P<0.05$ by student's t-test, n=4).


A
Bone Marrow CTRL
at t =72 h
SP = 0.18 %
Hoechst Blue
Hoechst Red
B
Bone Marrow CT-1
at t =72 h
SP = 0.20%
Hoechst Blue
Hoechst Red
C
Bone Marrow CTRL
+ verapamil at t =72 h
SP = 0.02 %
Hoechst Blue
Hoechst Red
D
Bone Marrow CT-1 +
verapamil at t =72 h
SP = 0.02%
Hoechst Blue
Hoechst Red

Graph 3: Effects of Ad-CT-1 on the Bone Marrow SP over time.

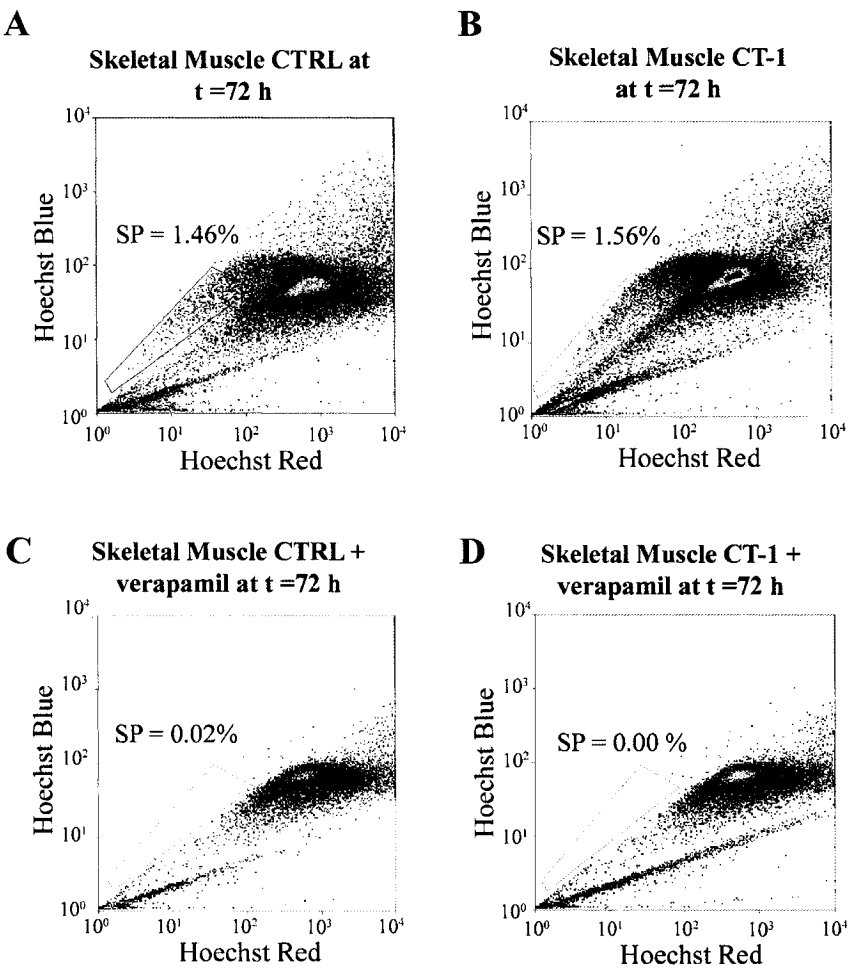
A bar graph illustrating the means obtained by FACS analyses of bone marrow cells from ~2 month old wild type mice that had been injected with either Ad-CTRL or Ad-CT-1. Cells were analysed at 24, 48, 72 or 96 hours post injection. The results represent the mean SP size at each time point. The means were calculated using normalized data ($P<0.05$ by student's t-test, n=4, error bars represent the standard error).



(* denotes a statistical difference between control and CT-1 injected mice, where $P<0.05$ by student's t-test, $n = 4$)

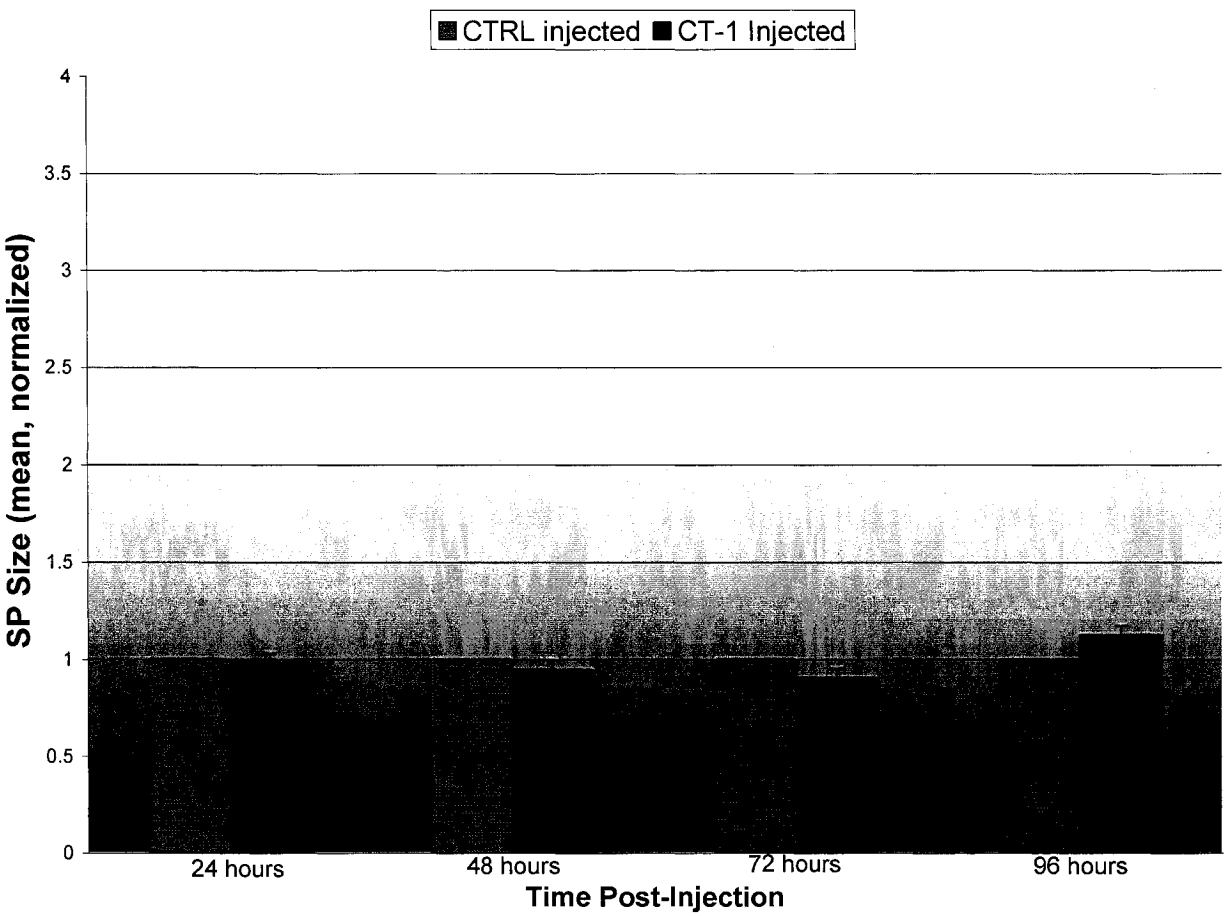
Figure 7: Effects of Ad-CT-1 on Murine Skeletal Muscle at t=72 hours post injection.

FACS analyses of skeletal muscle cells from ~2 month old wild type mice that had been injected with either Ad-CTRL (A & C) or Ad-CT-1 (B & D). Cell suspensions were stained with either Hoechst dye 33342 (A & B) or Hoechst dye 33342 plus verapamil (C & D). Cells were analysed at 24, 48, 72 or 96 hours post injection (72 hour time point shown here). The results revealed no changes in the skeletal muscle SP following exposure to Ad-CT-1. ($P<0.05$ by student's t-test, n=4)


A
Skeletal Muscle CTRL at t =72 h
SP = 1.46%
Hoechst Blue
Hoechst Red
B
Skeletal Muscle CT-1 at t =72 h
SP = 1.56%
Hoechst Blue
Hoechst Red
C
Skeletal Muscle CTRL + verapamil at t =72 h
SP = 0.02%
Hoechst Blue
Hoechst Red
D
Skeletal Muscle CT-1 + verapamil at t =72 h
SP = 0.00 %
Hoechst Blue
Hoechst Red

Graph 4: Effects of Ad-CT-1 on the Skeletal Muscle SP over time.

A bar graph illustrating the means obtained by FACS analyses of skeletal muscle cells from ~2 month old wild type mice that had been injected with either Ad-CTRL or Ad-CT-1. Cells were analysed at 24, 48, 72 or 96 hours post injection. The results represent the mean SP size at each time point. The means were calculated using normalized data ($P<0.05$ by student's t-test, n=4, error bars represent the standard error).



(* denotes a statistical difference between control and CT-1 injected mice, where $P<0.05$ by student's t-test, $n = 4$)

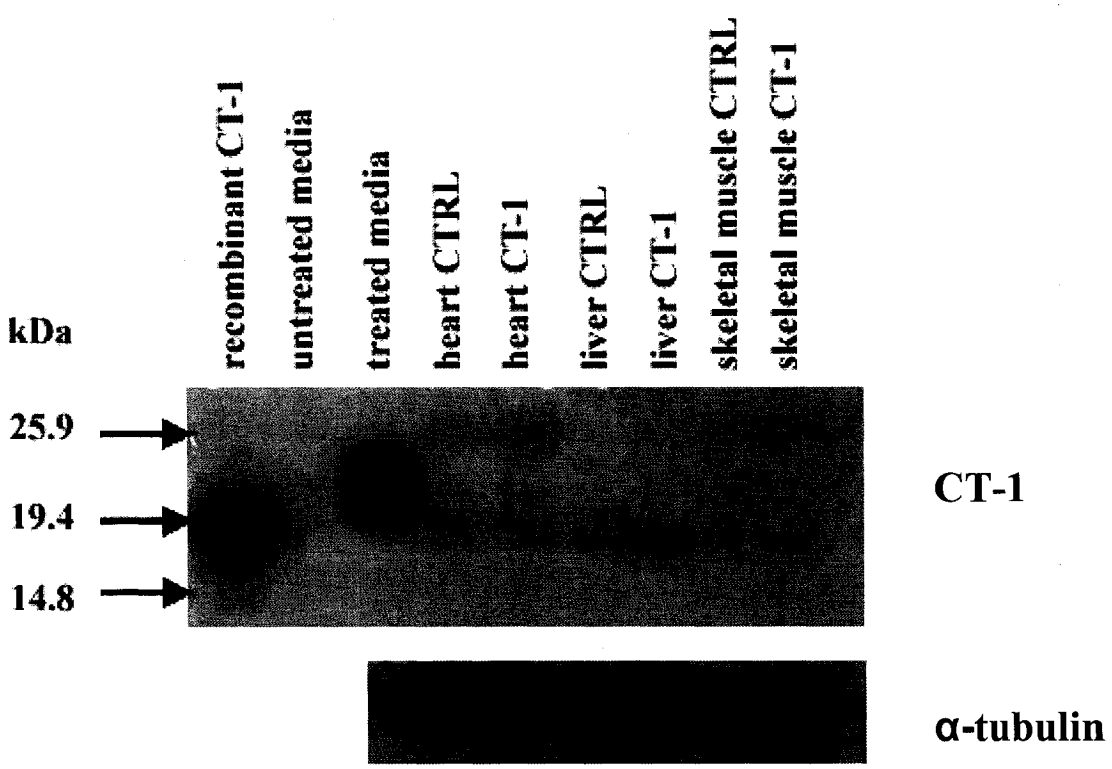
3.6 Level of CT-1 expression post Adenovirus Injections

Given these observations it was important to confirm that the CT-1 protein was being over expressed in mouse tissues. This would provide evidence that the CT-1 protein was responsible for the increase in the percentage of cardiac SP cells. To accomplish this, western blot analysis of tissue lysates was performed. Protein was isolated from the hearts, skeletal muscle and livers of adenovirus-injected mice (CT-1 or CTRL). Protein was then separated by electrophoresis, transferred to a membrane, and subject to incubation with CT-1 antibody. The results indicated that there was an increase in the amount of CT-1 protein in all three tissues (heart, skeletal muscle, liver) (Figure 8). However the liver showed the most dramatic increase in CT-1 protein levels. As previously mentioned up to 80% of administered Ad-CT-1 reaches the liver (Paielli, 2000; Alemany, 2001). Presumably the abundance of Ad-CT-1 in the liver would result in a large quantity of CT-1 protein being produced. No increase in Ad-CT-1 protein was observed in either the heart or skeletal muscle. The results showed that the CT-1 adenovirus was producing protein as an increase in the amount of CT-1 was observed in the liver. However the results could not confirm that CT-1 expression was being increased in the heart or skeletal muscle.

3.7 Stem Cell Activation: Assessment of Hematopoietic Colony Formation

The SP phenotype is not the only measure that can be used to evaluate the presence of stem cell populations. However, many stem cell validation assays are tissue specific. In particular, the adult bone marrow stem cell population is well characterized and several tests are available, such as hematopoietic colony formation assays or blood marrow

Figure 8: Western Blot Analysis for CT-1 Expression following Ad-CT-1 Injection. A total of 300 μg of protein from each tissue sample were separated by 15 % SDS-polyacrylamide gel electrophoresis. Protein samples were collected from the hearts, livers and skeletal muscles of mice that had received either CT-1 or CTRL adenovirus injections. The membrane was then incubated with CT-1 antibody (R&D systems) at a dilution of 1:1000 followed by incubation in secondary antibody at a dilution of 1:5000. Enhanced chemiluminescence was performed to visualize the proteins. A recombinant CT-1 protein (100 ng) (Peprotech) was used as a positive control, as well as media from CT-1 adenovirus infected primary cardiomyocytes. Media from uninfected cells was used as a negative control. (CT-1 size ~ 23.5 kDa). Tubulin was used to verify equal protein loading. The results indicated little or no change in CT-1 expression in the heart or skeletal muscle. There was however a large increase in CT-1 expression in the liver of Ad-CT-1 injected mice. To determine equal loading, blots were probed using an antibody for α-tubulin.


recombinant CT-1
untreated media
treated media
heart CTRL
heart CT-1
liver CTRL
liver CT-1
skeletal muscle CTRL
skeletal muscle CT-1
kDa
25.9
19.4
14.8
CT-1
α-tubulin

reconstitution experiments. With the recent discovery of adult stem cells in a variety of tissues (liver, kidney, skin, heart) (Bittner, 1999; Pereira, 1995; Lagasse, 2000; Hierlihy, 2002) this method is also being utilized to assess non-bone marrow stem cell populations.

Specifically, the hematopoietic colony assay has been commonly used to test for stem cell/progenitor-like activity (Seale, 2000), and is based on colony growth in a semi-solid environment (i.e. methocult). Methocult is a commercial semi solid methylcellulose media that contains various cytokines and growth factors optimized to promote the proliferation and differentiation of hematopoietic activated stem cells and progenitor cells (Stem Cell Technologies). The prospective colonies that emerge under these conditions contain mature hematopoietic cell types such as granulocytes, macrophages and natural killer cells. As such, the assumption in the field is that emergence of these cells types reflects prior activation of a stem cell pool.

In this experiment, FACS-sorted SP cells from adenovirus injected (CT-1 or CTRL) mice were cultured in Methocult media. The colony formation abilities of heart, bone marrow and skeletal muscle SP cells were compared (Table 2). Colony formation was observed as early as 10 days.

Analyses of bone marrow (n=3), skeletal muscle (n=7) and heart (n=7) methocult culture experiments revealed that there was significantly more colony formation in skeletal muscle injected from CT-1 adenovirus injected animals when compared to that

from CTRL injected mice (Table 2, $P<0.05$ by student's t-test). The number of colonies formed by the heart and bone marrow SP cells showed no variation between the CT-1 and CTRL adenovirus injections.

These results suggested that the increase in the percentage of cardiac SP cells was not accompanied by a change in the hematopoietic stem cell/progenitor like activity in this tissue. The bone marrow also showed no change in activity. However it is interesting to note that while the percentage of skeletal muscle SP cells were not altered, there was a dramatic increase associated with stem cell progenitor activity. This finding prompted further investigation.

3.8 Long term effects of CT-1 Adenovirus on Skeletal Muscle

The surprising results from the skeletal muscle SP cells in the hematopoietic activity assay prompted further investigation. It remained possible that the effects of CT-1 on skeletal muscle SP cells were delayed and later time point analysis may reveal alterations in the SP population. To investigate this possibility, an extended time course of CT-1 administration was undertaken. Mice were injected with either the CTRL or CT-1 adenovirus and then at 10 days, 4 weeks and 6 weeks post injection skeletal muscle SP cells were collected, stained with Hoechst 33342 and analyzed by FACS (Figure 9). These results demonstrated that the skeletal muscle SP from CT-1 adenovirus injected mice did not increase. This result confirmed the previous findings that the increase in the percentage of the SP population was a cardiac specific event.

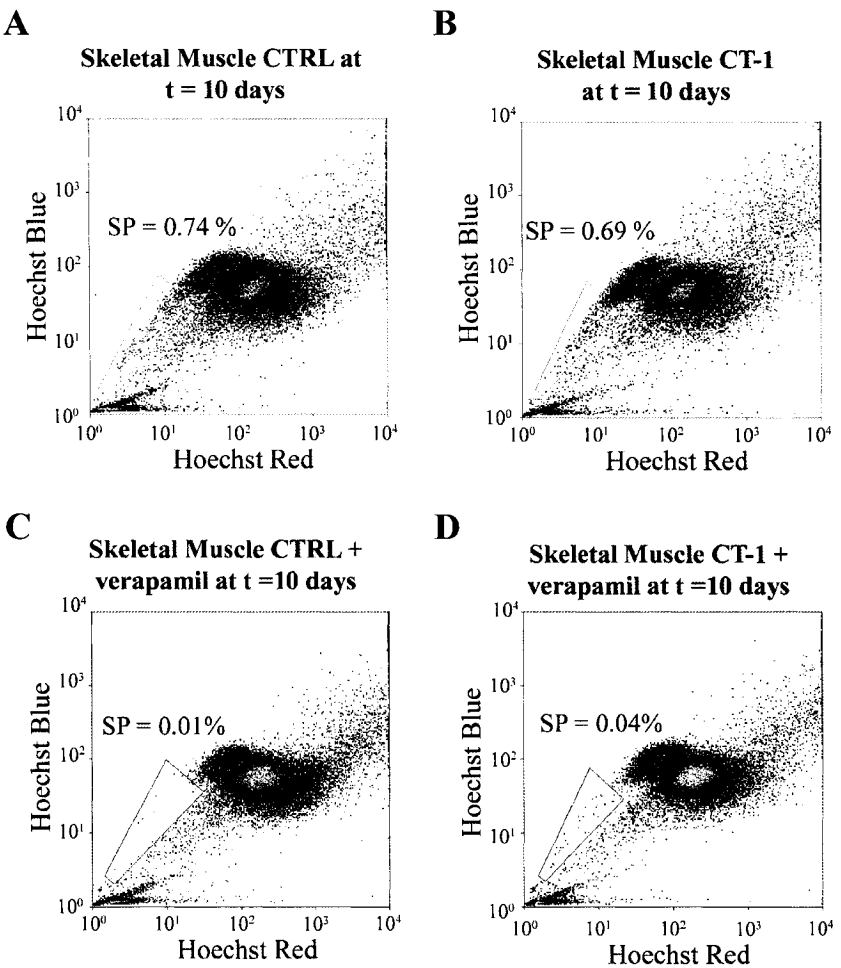
Table 2: Hematopoietic colony formation from SP and MP Cells following systemic injection of Ad-CT-1.

SP cell populations were derived from murine heart, bone marrow and skeletal muscle at 72 hours post injection using FACS. A total of 20000 cells were plated in Methocult and colonies were counted 14 days after initial plating. Each is represented as an average of the number of times each experiment was carried out (as indicated by the n value). The results indicated no significant differences in colonies formed with or without Ad-CT-1 in heart or bone marrow. However there was a significant increase in the number of hematopoietic colonies formed in Ad-CT-1 injected skeletal muscle when compared to the Ad-CTRL injected skeletal muscle. ($P<0.05$ by student's t-test)

14 days	SP (# of colonies)	MP (# of colonies)	# of experiments (n)
Heart CTRL	1.0	0	7
Heart CT-1	1.3	0.7	7
BM CTRL	35	7.0	3
BM CT-1	41	5.0	3
SkM CTRL	1.0	0.8	7
SkM CT-1	10.6	1.0	7

Figure 9: Long Term Effects of Ad-CT-1 on Murine Skeletal Muscle.

FACS analyses of skeletal muscle cells from ~2 month old wild type mice that had been injected with either Ad-CTRL (A & C) or Ad-CT-1 (B & D). Cell suspensions were stained with either Hoechst dye 33342 (A & B) or Hoechst dye 33342 plus verapamil (C & D). Cells were analysed at 10 days, 4 weeks and 6 weeks post injection (10 day time point shown here). The results revealed no changes in the skeletal muscle SP.


A
Skeletal Muscle CTRL at t = 10 days
SP = 0.74 %
B
Skeletal Muscle CT-1 at t = 10 days
SP = 0.69 %
C
Skeletal Muscle CTRL + verapamil at t =10 days
SP = 0.01%
D
Skeletal Muscle CT-1 + verapamil at t =10 days
SP = 0.04%
Hoechst Blue
Hoechst Red
10^0
10^1
10^2
10^3
10^4

However these results also suggested that CT-1 elicited a response in the skeletal muscle stem cell population.

3.9 Characterization of the Cardiac SP – Sca-1 Enrichment

As mentioned above, adult stem cells have been identified in a variety of tissues (liver, skin, heart). The most widely characterized population is that found in the adult bone marrow. In particular, these cells are identified not only by the SP phenotype but also by the expression of various cell surface markers such as Sca-1, CD34 or c-kit (Ogawa, 1991). In an attempt to find additional markers for sorting the cardiac SP and testing CT-1 responsiveness, cells were labeled with SCA-1 antibody and analyzed by FACS. The results showed that indeed the SP fraction (~ 75%) was enriched for the SCA-1 surface marker when compared with non-SP (main population, MP) (~15%) (Figure 10). This suggested that sorting Sca-1 positive cells that also exhibited the SP phenotype could further enrich the cardiac SP. It also provided additional support to the notion that the SP contained a "stem cell-like" population.

These results prompted an investigation of whether CT-1 had an effect on the Sca-1 population within the heart. Mice were injected with either the CT-1 or CTRL adenovirus, then at 48 hours post injection, the SP cells were harvested, stained with SCA-1 and analyzed by FACS. A comparison of the total cell Sca-1 expression and SP only Sca-1 expression yielded surprising results. The analyses indicated that there was no change in the total number of Sca-1 positive cells in the hearts of CT-1 adenovirus injected mice (Figure 11). Similarly, there was no change in the number of Sca-1

Figure 10: SCA-1 Expression in the Murine Heart.

FACS analyses of cardiac cell populations derived from ~2 month old wild type mice were stained with either Hoechst dye 33342 (A) or Hoechst dye 33342 plus verapamil (B) or PE only (C) or Sca-1 plus PE (D). The cardiac SP (F) and non-SP/MP (E) were individually analyzed for Sca-1 expression. These results revealed enrichment for Sca-1 expression in the cardiac SP (~75%) compared to Sca-1 expression in the non-SP/MP population (~25%). ($P<0.05$ by student t-test, n=3)

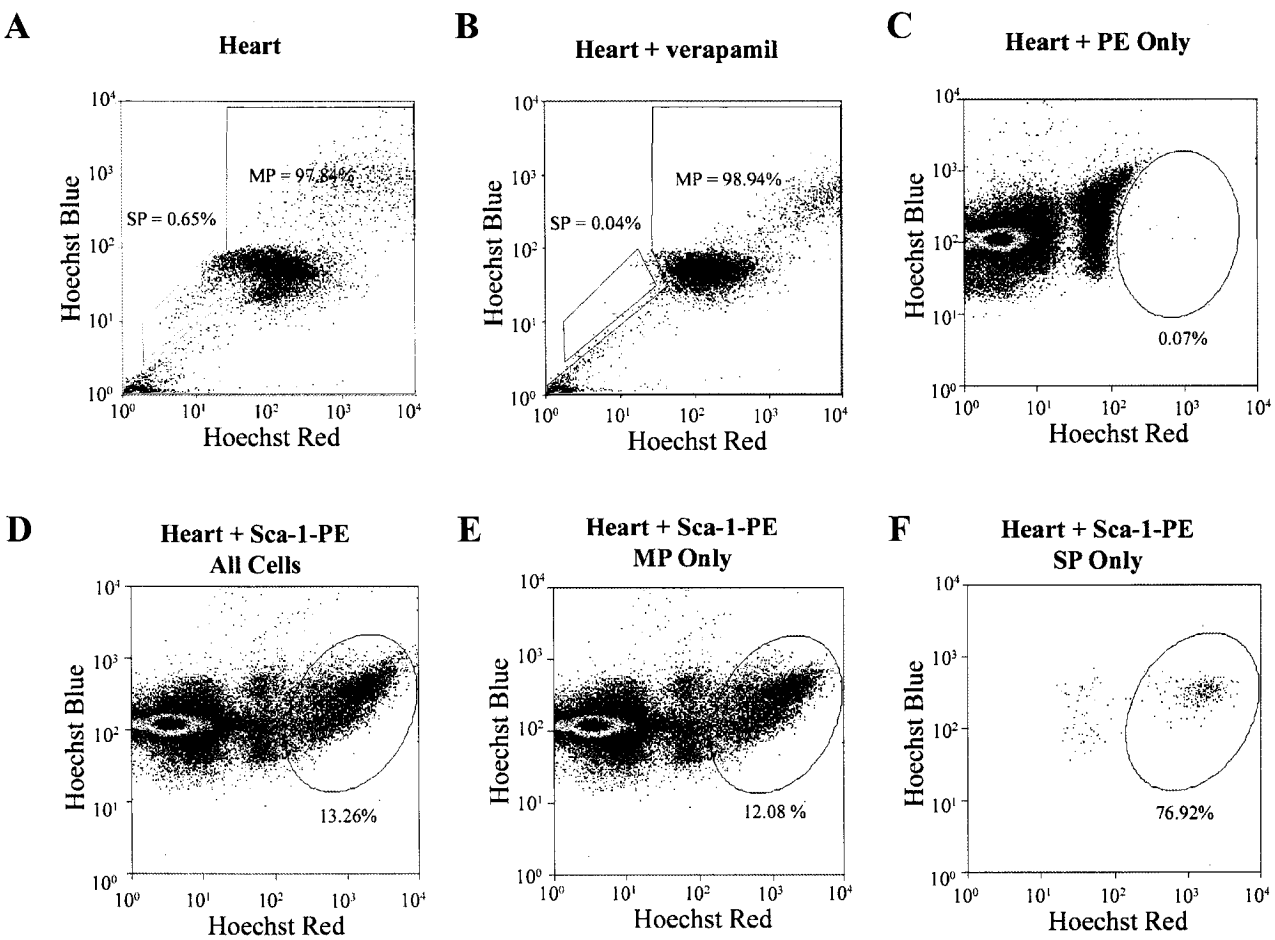
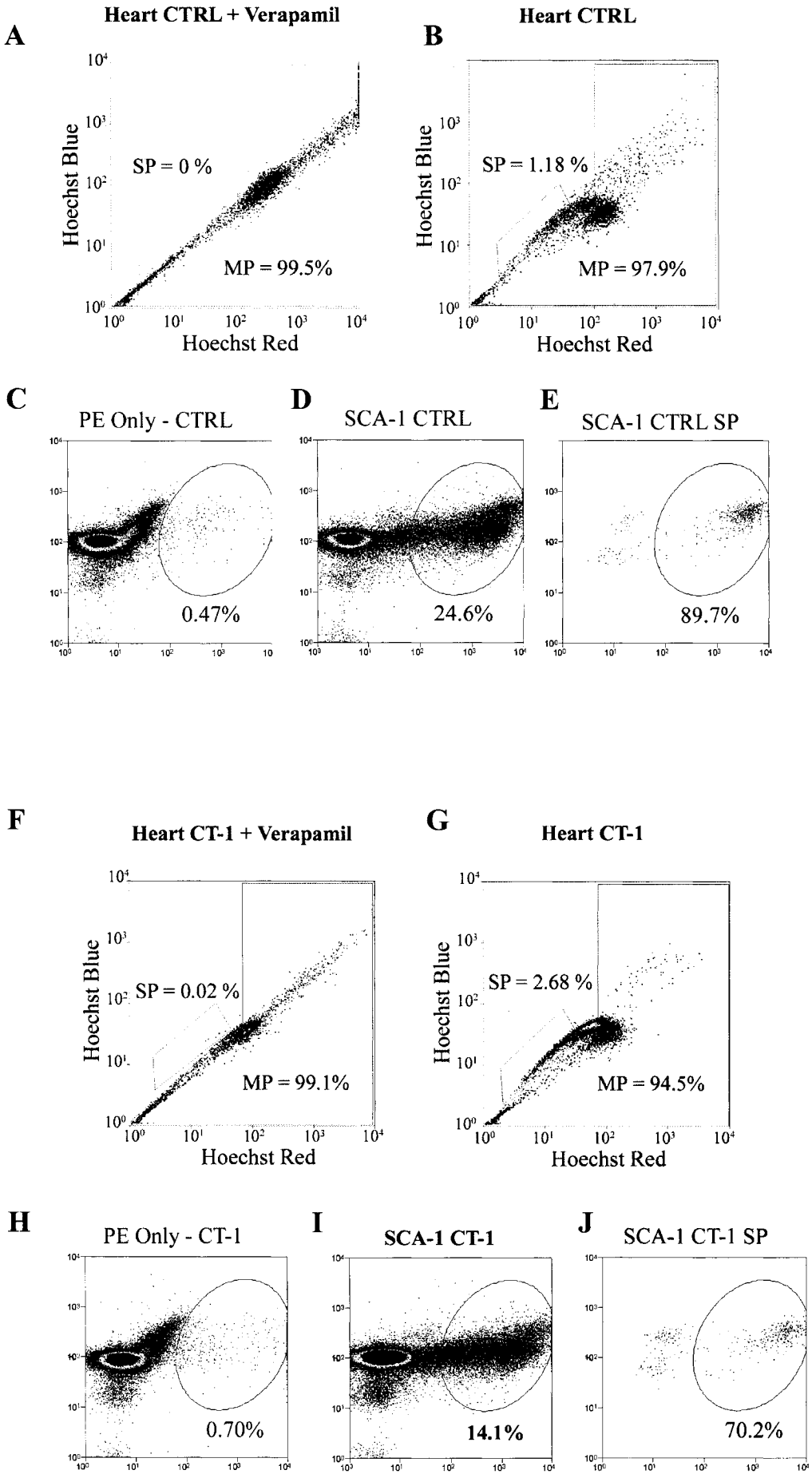

A
Heart
SP = 0.65%
MP = 97.84%
B
Heart + verapamil
SP = 0.04%
MP = 98.94%
C
Heart + PE Only
0.07%
D
Heart + Sca-1-PE
All Cells
13.26%
E
Heart + Sca-1-PE
MP Only
12.08 %
F
Heart + Sca-1-PE
SP Only
76.92%
Hoechst Blue
Hoechst Red

Figure 11: SCA-1 Expression in the Murine Heart Following Ad-CT-1 Injection. FACS analyses of cardiac cells from ~2 month old wild type mice that had been injected with either Ad-CTRL (A-E) or Ad-CT-1 (F-J). Cell suspensions were stained with either Hoechst dye 33342 (A & F) or Hoechst dye 33342 plus verapamil (B & G) or PE only (C & H) or Sca-1 plus PE (D & I). The cardiac SP (F & J) and non-SP/MP (E & G) were individually analysed for Sca-1 expression. The results revealed an enrichment for Sca-1 expression in the cardiac SP (~75%) compared to expression in the non-SP/MP (~25%), however Ad-CT-1 had no effect on Sca-1 expression. ($P<0.05$ using student's t-test, $n = 3$)


A
Heart CTRL + Verapamil
Hoechst Blue
Hoechst Red
SP = 0 %
MP = 99.5%
B
Heart CTRL
SP = 1.18 %
MP = 97.9%
C
PE Only - CTRL
0.47%
D
SCA-1 CTRL
24.6%
E
SCA-1 CTRL SP
89.7%
F
Heart CT-1 + Verapamil
SP = 0.02 %
MP = 99.1%
G
Heart CT-1
SP = 2.68 %
MP = 94.5%
H
PE Only - CT-1
0.70%
I
SCA-1 CT-1
14.1%
J
SCA-1 CT-1 SP
70.2%

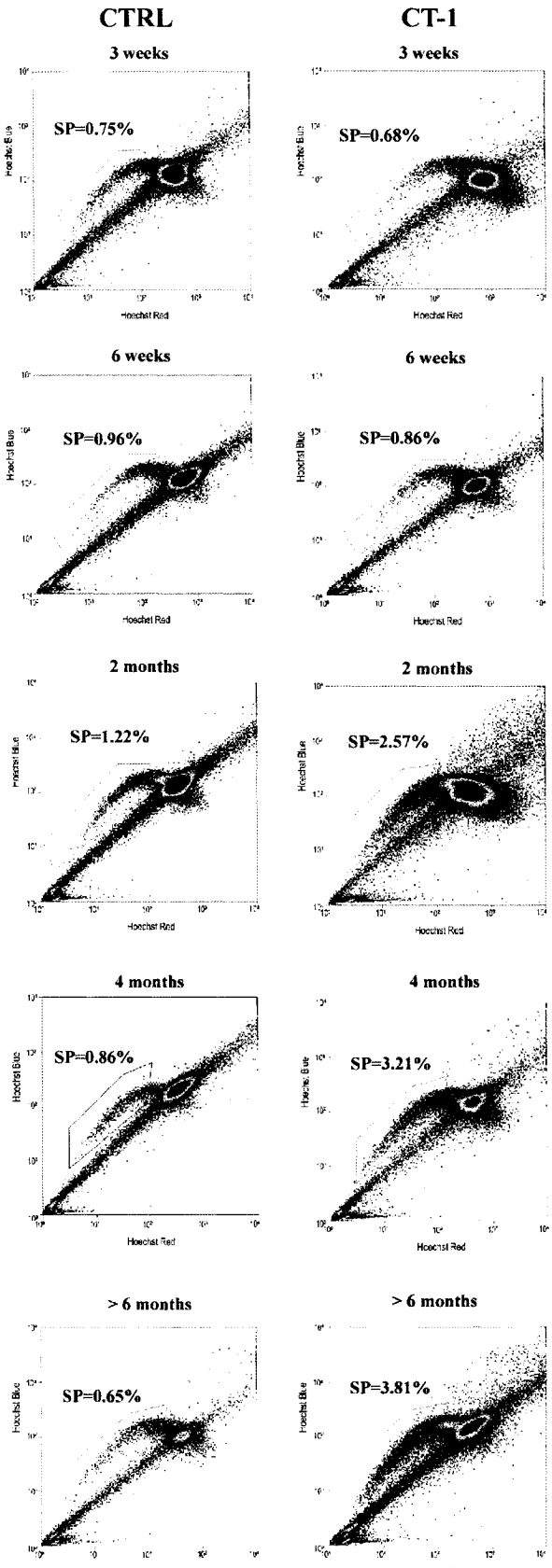
positive cells in the SP fraction (hearts, CT-1 adenovirus injected) (Figure 11). From this experiment it can be concluded that CT-1 did not have an effect on the Sca-1 positive fraction of the cardiac SP.

3.10 The Effects of Age and CT-1 Adenovirus on the Heart

It had previously been reported that with age the cardiac SP population diminishes with age, thereby suggesting that CT-1 may induce age related effects in the cardiac SP population (unpublished, correspondence with Hierlihy, et *al.*) Therefore an experiment was performed to evaluate the combinatorial effects of age and CT-1 cardiac SP cells. Animals were injected at various ages (3 weeks, 6 weeks, 2 months, 4 months, and >6 months) with either the CT-1 or CTRL adenovirus. At 48 hours post injection, the hearts were excised, stained with Hoechst 33342, and analyzed by FACS (Figure 12, Graph 5). The SP fractions were compared from each group, and a significant increase in the CT-1 effect was observed in the older animals ($P<0.05$, $n=3$). In older mice that had been administered Ad-CT-1 there was a greater increase in the percentage of cardiac SP cells (i.e. 6 month old Ad-CT-1 injected mice had a larger cardiac SP cell fraction compared to those at 2 months of age). A trend was also observed in which there was a steady decline in the percentage SP as the mouse aged. It is interesting to note that the mouse is considered as "adult" at two months of age – the same time point at which the SP in the heart starts to diminish.

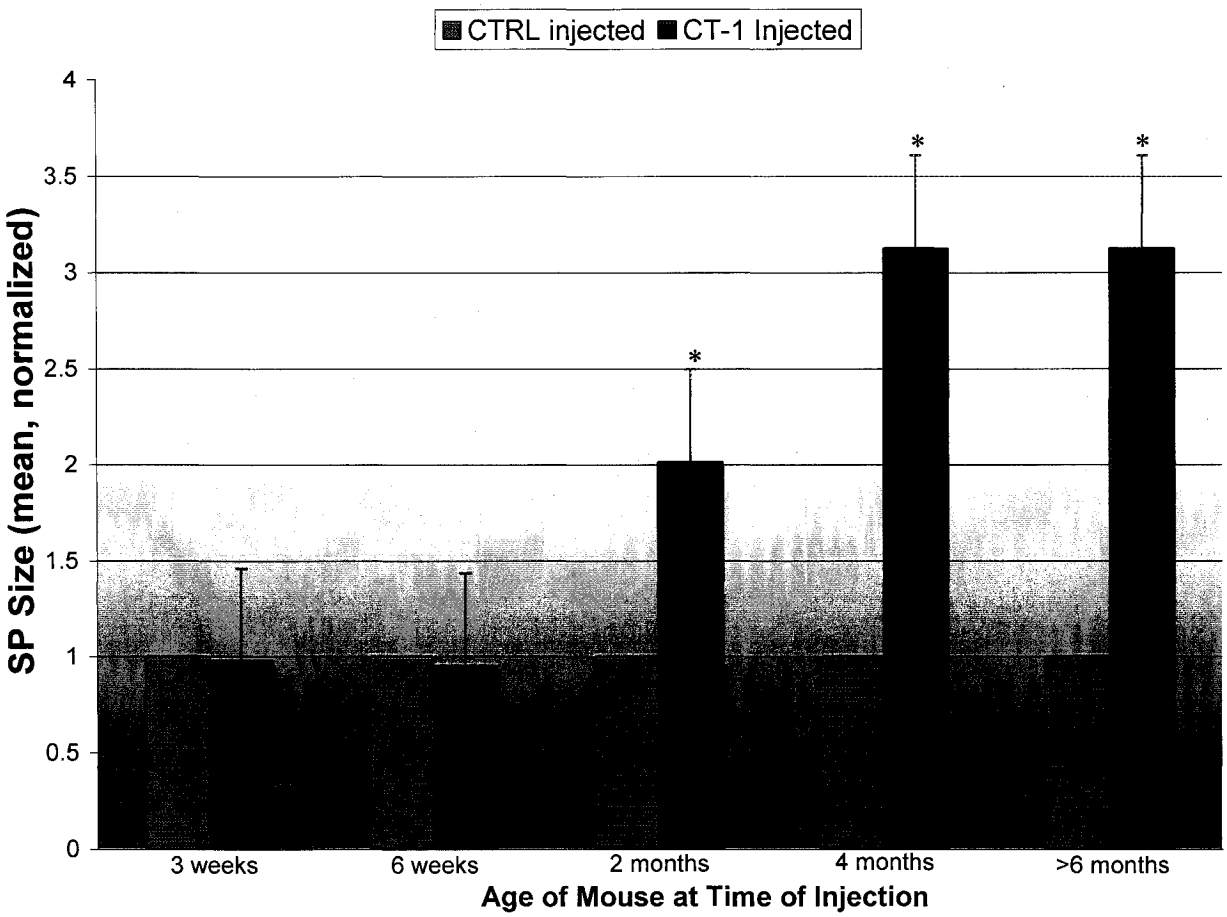
Figure 12: Variation in the cardiac SP population with age.

FACS analyses of cardiac cell populations from wild type mice that had been injected with either Ad-CTRL (A, C, E, G, I) or Ad-CT-1 (B, D, F, H, J). Cell suspensions were stained with Hoechst dye 33342 (A-J). Mice of various ages were analyzed at 48 hours post injection. Mice ranged in age from 3 weeks (A & B), 6 weeks (C & D), 2 months (E & F), 4 months (G & H) to 1 year (I & J). The results revealed an overall decrease in the cardiac SP population with age (A, C, E, G & I). These results also demonstrated that the cardiac SP responsiveness to CT-1 injection was greater in older mice (B, D, F, H & J).


CTRL
CT-1
3 weeks
SP=0.75%
3 weeks
SP=0.68%
6 weeks
SP=0.96%
6 weeks
SP=0.86%
2 months
SP=1.22%
2 months
SP=2.57%
4 months
SP=0.86%
4 months
SP=3.21%
> 6 months
SP=0.65%
> 6 months
SP=3.81%
Hoechst Blue
Hoechst Red

Graph 5: The Effect of Age on the Cardiac SP.

A bar graph illustrating the means obtained by FACS analyses cardiac cells from 3 week, 6 week, 2 month, 4 month or >6 month old wild type mice that had been injected with either Ad-CTRL or Ad-CT-1. Cells were analysed at 48 hours post injection. The results represent the mean SP size at each time point. The means were calculated using normalized data ($P<0.05$ by student's t-test, $n=3$). The results demonstrated that the cardiac SP responsiveness to CT-1 injection was greater in older mice (2 months, 4 months and >6 months).



(* denotes a statistical difference between control and CT-1 injected hearts, where $P<0.05$ by student's t-test, $n = 3$)

3.11 Morphology of Hearts and Skeletal Muscle in CT-1 Adenovirus Injected Mice

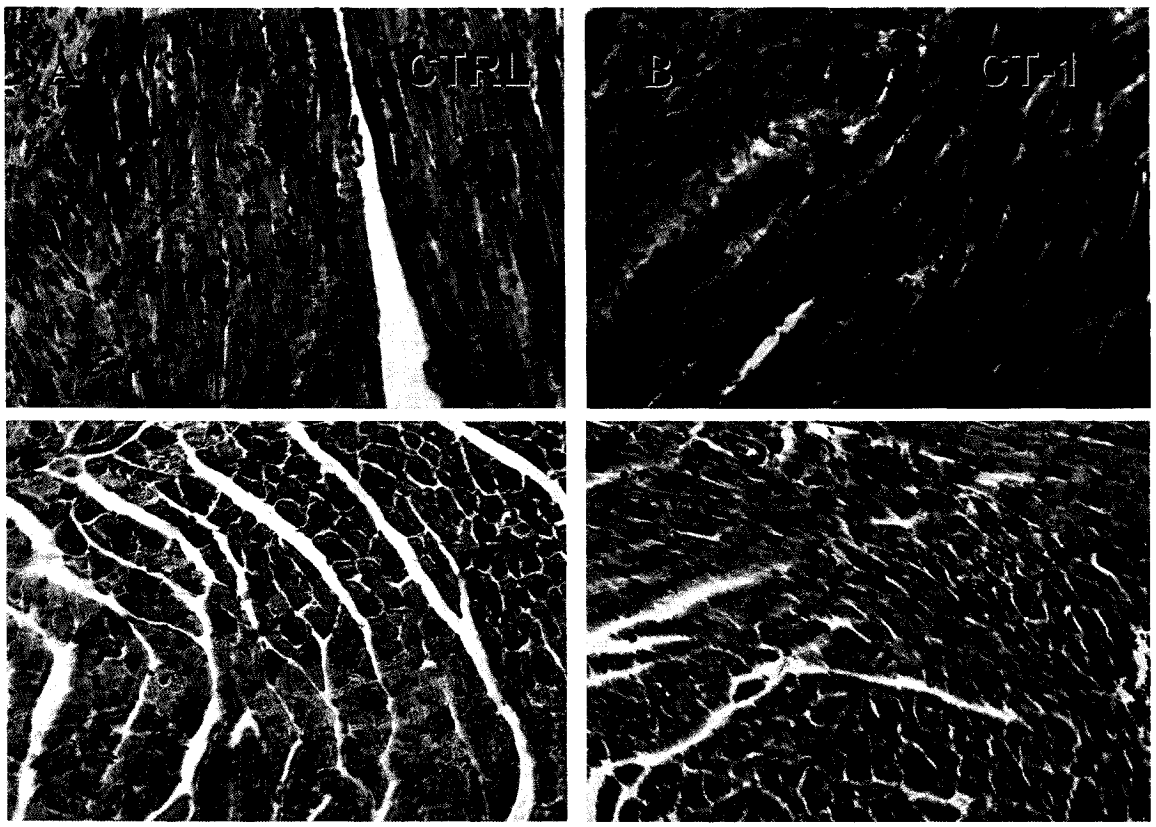
A visual comparison of the hearts and skeletal muscle of CT-1 and CTRL injected mice at 5 days post injection showed no differences in histology between the hearts (Figure 13 A, B) or skeletal muscles (Figure 13 C, D). There were no changes in cell numbers, cell size or the location of the nuclei (not shown). The results were surprising as CT-1 was originally identified as a hypertrophic agent (Pennica, 1995), one would have expected to see enlarged heart cells.

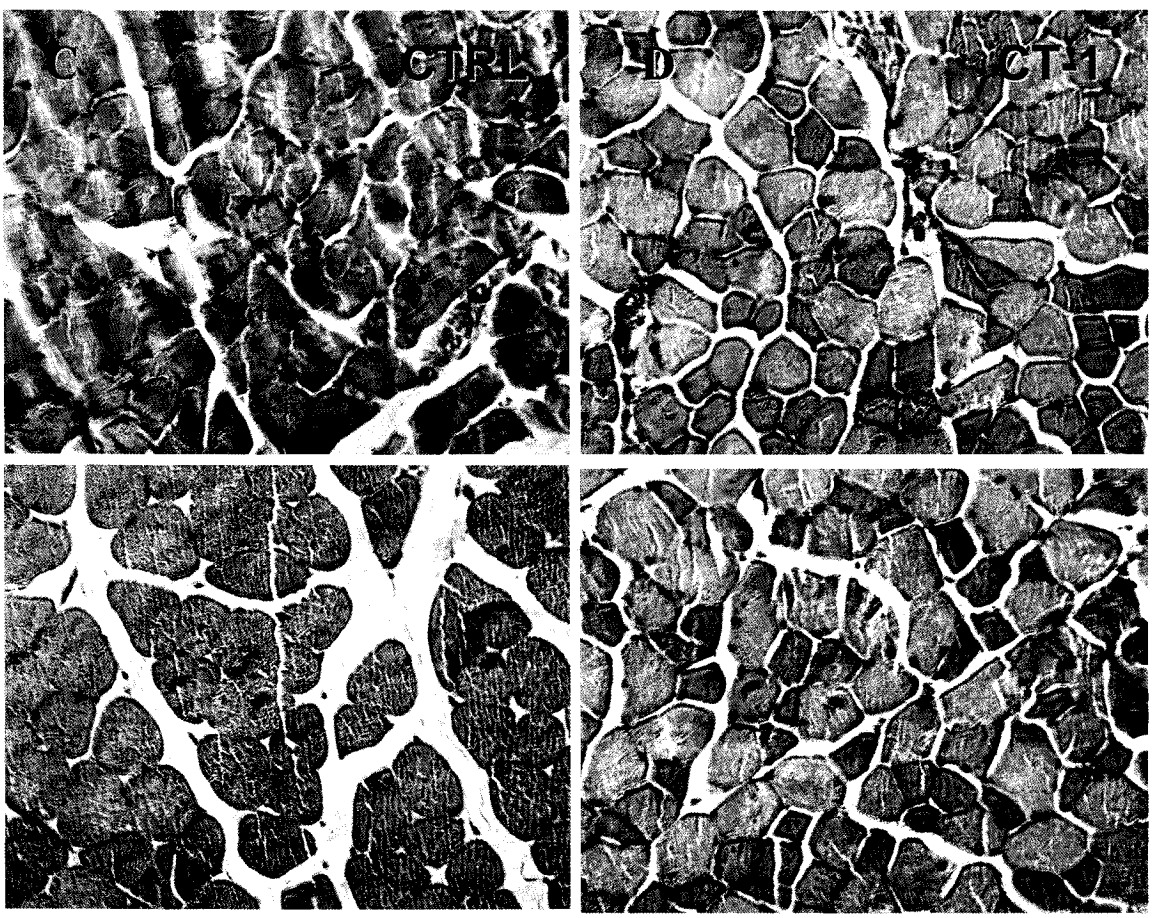
3.12.0 Addressing the Absence of a Hypertrophy Phenotype

As previously mentioned, CT-1 was first identified as a hypertrophic agent in the heart. Cardiac hypertrophy is one of the most important adaptive responses in the heart. Extended periods of hypertension, myocardial injury or other demands for increased cardiac output induce hypertrophy (reviewed by Sugden, 1998). In depth research has shown that CT-1 plays a distinct role in hypertrophy by increasing cardiac cell length (Wollert, 1996), inducing the expression of genes involved with hypertrophy (Wollert, 1996) as well as *in vivo* – it has been shown to increase heart weight (Jin, 1996). Initial sections of hearts from CT-1 injected mice failed to provide evidence of hypertrophy. In order to further investigate these contradictory findings, several approaches were used: ANF northern blot analysis, an *in vitro* assay, and a comparison of heart to body weight ratios.

Figure 13: Histology Analysis of Hearts and Skeletal Muscle Following Ad-CT-1 injection.

Histological analysis of hearts (A & B) and skeletal muscle (C & D) at 5 days post-injection with either Ad-CTRL (A & C) or Ad-CT-1 (B & D). Tissues were sectioned at 10 μm and counterstained with hematoxylin and eosin to visualize the nuclei and cytoplasm. Photographs were taken at 40X magnification. No differences in ultrastructure were observed.


A
CTRL
B
CT-1


C
CTRL
D
CT-1

3.12.1 Northern Blot Analysis: ANF

RNA was collected from the hearts of CT-1 and CTRL adenovirus injected mice. Via northern blot analysis, the RNA was probed using a portion of the atrial natriuretic factor (ANF) cDNA. Increased expression of ANF is an indicator of hypertrophy (Dosch, 2001). The results showed no increase in the level of ANF expressed in the hearts of CT-1 injected mice (Figure 14A). This provided additional evidence that the CT-1 adenovirus was not inducing hypertrophy at the organ level.

3.12.2 *In Vitro* Assay for Hypertrophy in H9C2 Cells

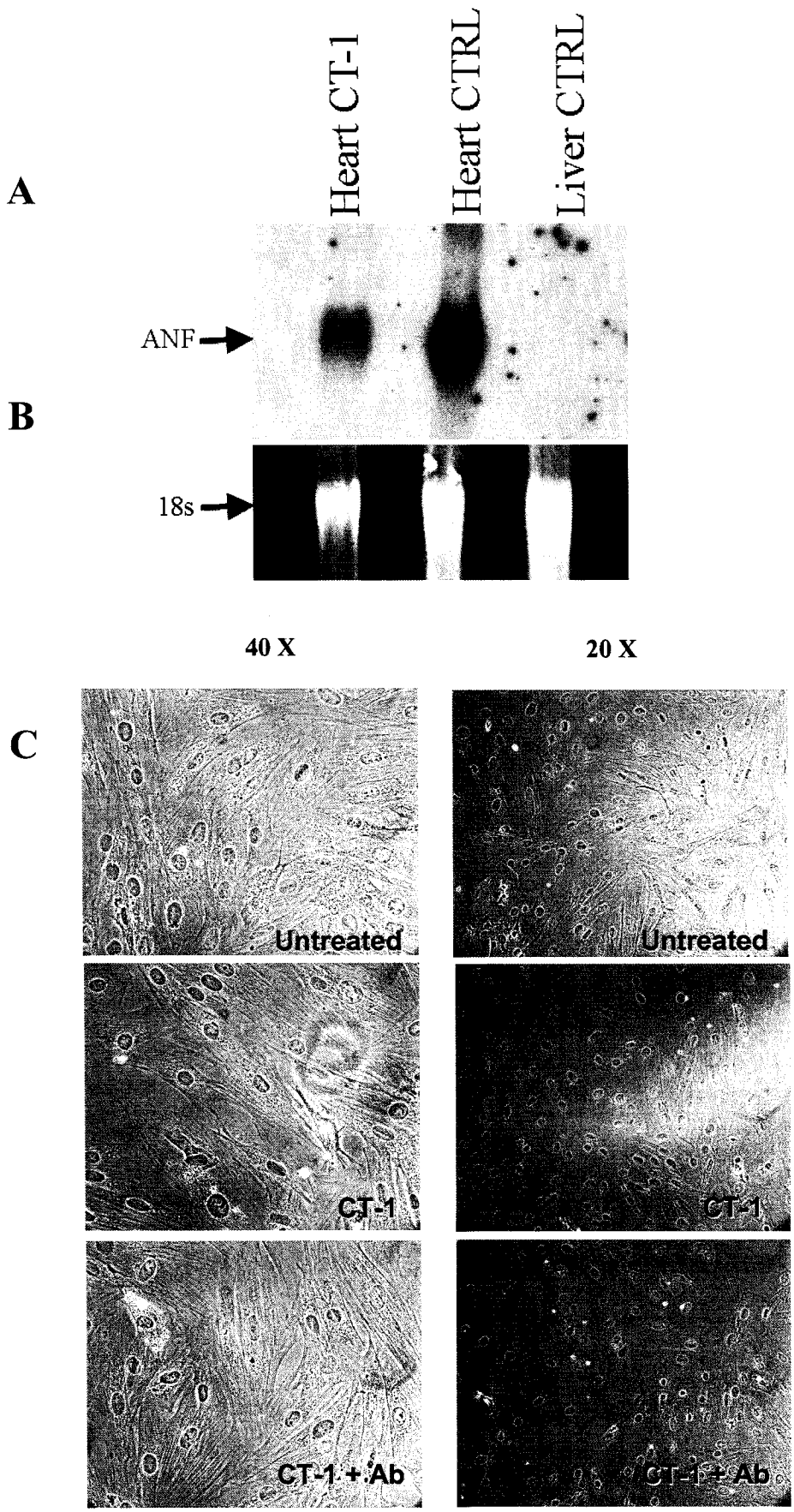
Previously published data reported that when cardiomyocytes were treated with CT-1, the cells became elongated (Kuwahara, 1999). In addition, adding an anti-CT-1 antibody along with the CT-1 could inhibit this increase in size. Several attempts were made to repeat this experiment. Both H9C2 cells (a rat heart cell line) (Figure 14C) and primary cardiomyocytes (not shown) were treated with either CT-1 adenovirus or a combination of adenovirus and anti-CT-1 antibody. However no change in cell size was observed ($P<0.05$). The results provided further evidence against the occurrence of hypertrophy.

3.12.3 Heart to Body Weight Ratio

As a final experiment to address hypertrophy, a comparison of heart weight to body weight was performed. Animals were injected with either CT-1 or CTRL adenovirus. Prior to culling the mice, their body weights were taken. Following excision, the hearts were also weighed. A ratio of heart (g) to body weight (mg) was calculated and

Figure 14: Assessment of CT-1 as an Inductive Cue for Cardiac Hypertrophy.

Northern blot analysis for ANF expression in Ad-CT-1 injected hearts, Ad-LacZ (CTRL) injected hearts and liver (A). The 18s band is used as a loading control (B). There was no increase in ANF expression observed in Ad-CT-1 hearts suggesting that no hypertrophy was occurring. A heart cell line (H9C2) was treated with CT-1 or CT-1 plus a CT-1 antibody or left untreated (C). Cells were fixed and stained with DAPI to visualize the nuclei. Photographs were taken using either 40X or 20X magnifications. A comparison between treated and untreated cells revealed no differences in cell size and suggested that hypertrophy did not occur in CT-1 treated cells.



48 Hours ~ H9C2 cells

Table 3: A Comparison of Heart Weight to Body Weight Following Systemic Delivery of Ad-CT-1.

Wild type mice were injected with either Ad-CTRL or Ad-CT-1. At one-week post injection the body weight and heart weights were taken. As a means to assess hypertrophy, these weights were compared in the form of a ratio, heart weight in mg to body weight in g. The averages were taken from each group, CT-1 adenovirus hearts and CTRL adenovirus hearts and compared. No significant difference was observed between the two ratios ($P<0.05$ by student's t-test, $n=4$) which suggested that hypertrophy was not occurring in CT-1 injected mice.

Mouse Treatment	Body (g)	Heart (mg)	Ratio (heart/body)
CTRL #1	19.65	144.10	7.333
#2	19.96	117.00	5.862
#3	18.06	127.00	7.032
#4	20.94	119.70	5.716
CT-1 #1	29.98	146.10	4.873
#2	21.01	122.90	5.849
#3	23.28	146.20	6.280
#4	22.81	129.20	5.664

compared. The results showed no significant difference between CT-1 and CTRL injected animals ($P<0.05$, $n=4$) (refer to table 3). From these studies it can be concluded that CT-1 administration did not induce cardiac hypertrophy.

Despite previously published data, all three experiments drew a similar conclusion. The CT-1 adenovirus was not inducing hypertrophy in the hearts of injected mice.

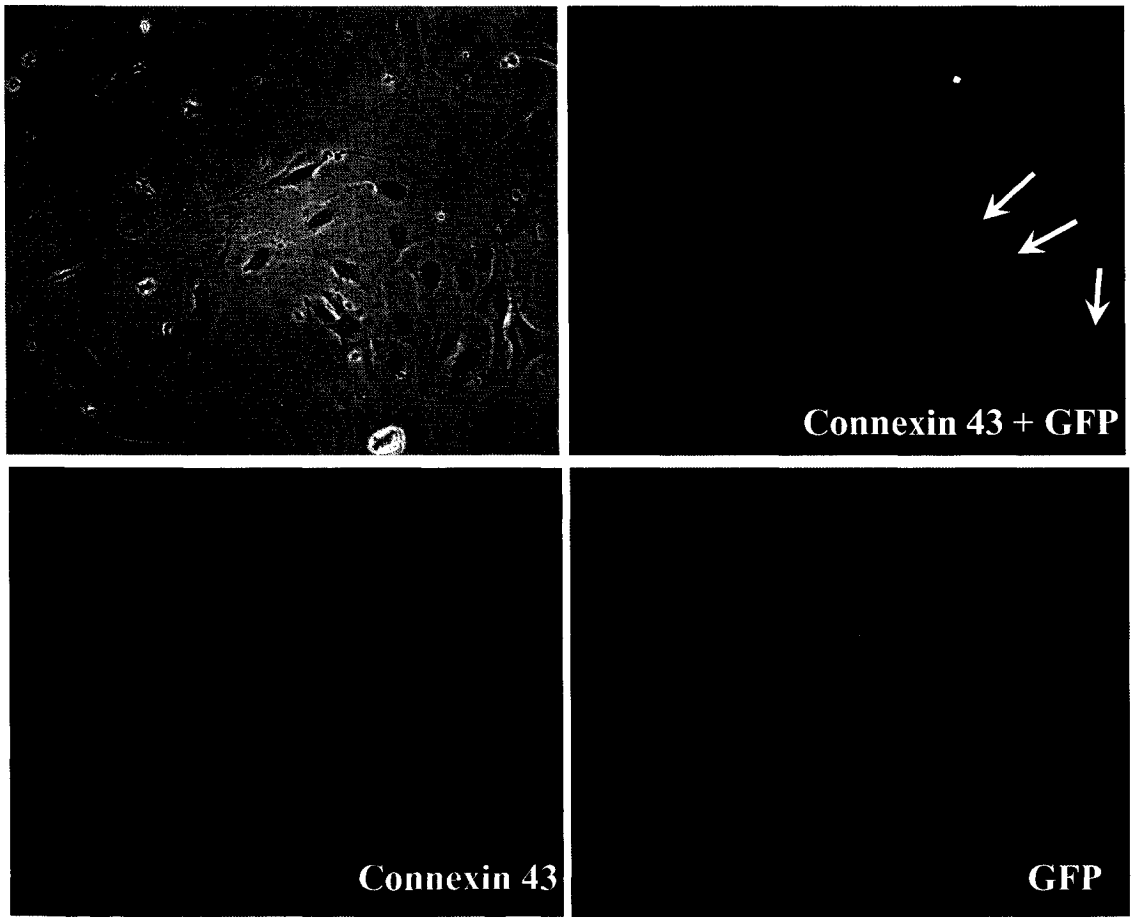
3.13 Co-Culture of Cardiac SP Cells and Primary Cardiomyocytes

Previously published data demonstrated that *in vitro* the murine cardiac SP was capable of differentiating into functional cardiomyocytes when co-cultured with H9C2 cells (a rat heart cell line) (Hierlihy, 2002). To test the effect of CT-1 on the ability of cardiac SP cells to become cardiomyocytes, a co-culture experiment was performed. Cardiac SP cells were collected from Ad-CT-1 or Ad-CTRL injected GFP mice and co-cultured with primary cardiomyocytes (isolated from wild-type mice). GFP mice provided a genetically marked source for cardiac derived SP cells (i.e. all cells originating from the cardiac SP would fluoresce green under UV light). The SP cells and primary cardiomyocytes were co-cultured for 14 days after which they were fixed and stained with DAPI (to visualize the nuclei) and connexin-43 (a cardiac specific marker used to detect differentiated cardiomyocytes) (refer to Figure 15). Co-localization of GFP and connexin-43 indicated cardiac SP cells that had undergone cardiac specific differentiation.

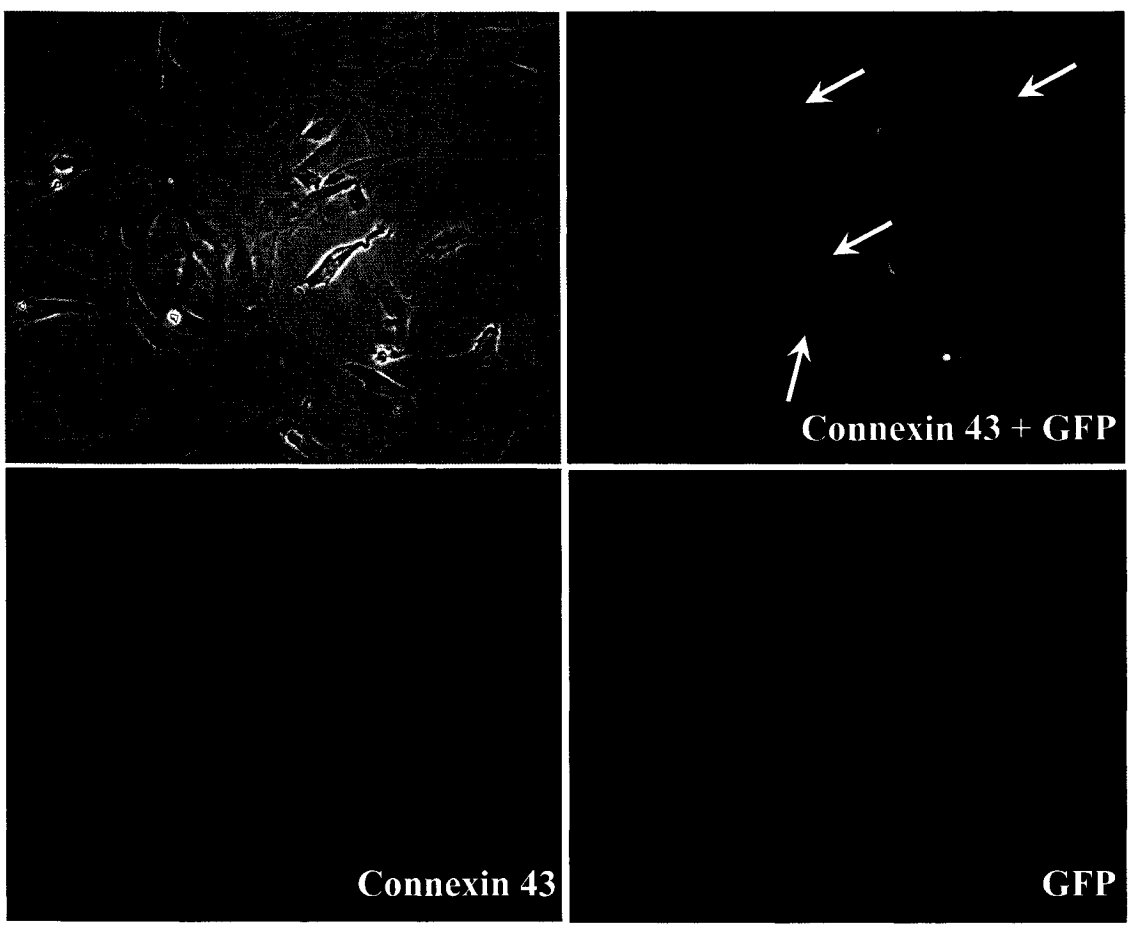
Figure 15: Co-Culture of Cardiac SP Cells and Primary Cardiomyocytes.

SP and MP cells were collected from GFP positive mice using FACS at 24 hours post Ad-CT-1 injection. The GFP mice were injected with either Ad-CTRL (A) or Ad-CT-1 (B). The SP or MP was then co-cultured with primary cardiomyocytes. Cells derived from injected mice fluoresce green under UV light, while the cells derived from the primary culture will not. Cells were co-cultured for one week and then fixed and immuno-stained for connexin-43 and DAPI. Cells originating from the SP fraction should fluoresce green. A double staining of red and green suggests that the SP cell has adopted a cardiomyocyte phenotype. The arrows indicate cells that co-fluoresce and indicate SP cells that may have become cardiomyocytes. Photographs were taken at 40X magnification.

A


Connexin 43 + GFP
Connexin 43
GFP

B


Connexin 43 + GFP
Connexin 43
GFP

A comparison between the SP co-cultures from CTRL injected mice (Figure 15A) and CT-1 injected mice (Figure 15B) suggested that heart SP cells from CT-1 injected mice underwent cardiomyocyte differentiation at a much greater frequency. This suggested that perhaps not only was the cardiac SP size increasing but that a differentiation program was also being activated. These experiments were only performed once and further investigation is required to draw a decisive conclusion.

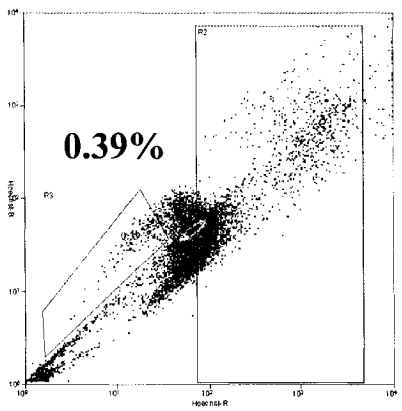
3.14 LIF Receptor expression in the Heart

The CT-1 protein signals via the gp130 pathway through the LIF receptor (Pennica, 1995) (Figure 16). The first event in the CT-1 activation of this pathway involves a ligand-induced hetero-dimerization. The two components involved in this step are gp130 and the LIF receptor beta subunit (reviewed in Heinrich, 1998). As such for SP cells to respond to CT-1, they must express the LIF receptor. Hearts from untreated mice were excised and stained with a LIF receptor antibody (an antibody was available that recognized a portion of the receptor that was expressed on the cell surface and thus FACS could be used to isolate the LIFR responsive cells). The analysis confirmed that there were cells in the cardiac SP fraction that expressed the LIF receptor, suggesting that SP cells have the ability to respond to CT-1 protein (Figure 16). The results also provided insight into a possible mechanism of cardiac SP activation, the gp130 pathway. The pathway is downstream of the LIF receptor.

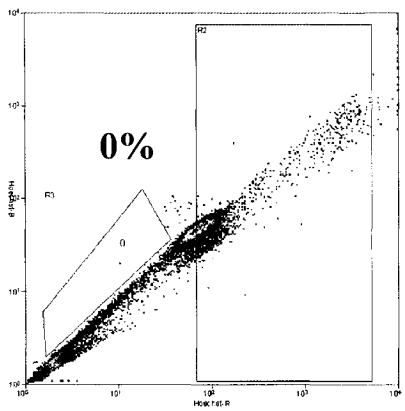
Figure 16: Expression of the LIF Receptor in Cardiac SP cells.

FACS analyses of hearts from 2 month-old wildtype mice. Cells were stained with Hoechst dye 33342 (A) or Hoechst dye 33342 plus verapamil (B) or LIF receptor-Alexa 488 (C-E). The cardiac SP (E) and non-SP/MP (D) were individually analyzed for LIF receptor expression. The results revealed that ~ 20 % of all cardiac cells express the LIF receptor (C) while ~ 22% of all MP cells and ~ 20% of all SP cells expressed the LIF receptor.

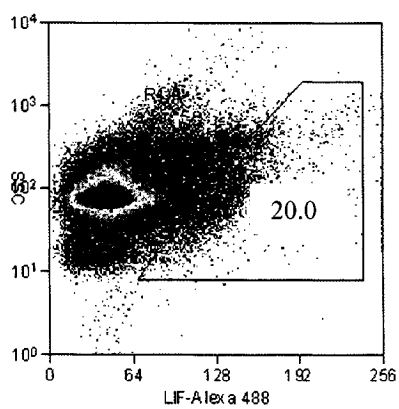
A Hoechst Only



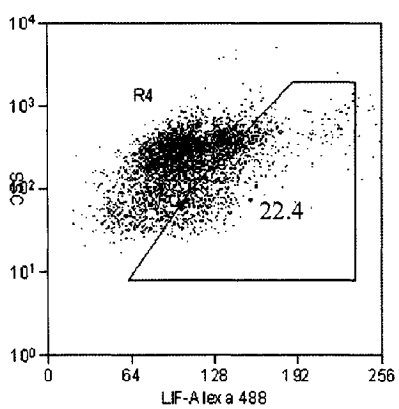
B With Verapamil



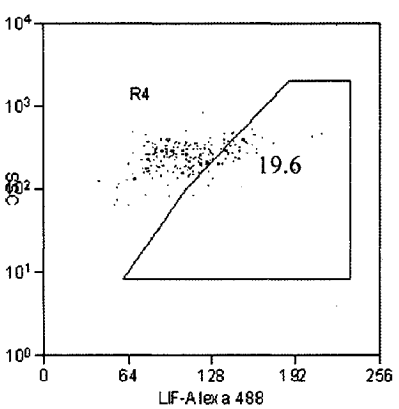
C All Cells



D MP Only



E SP Only



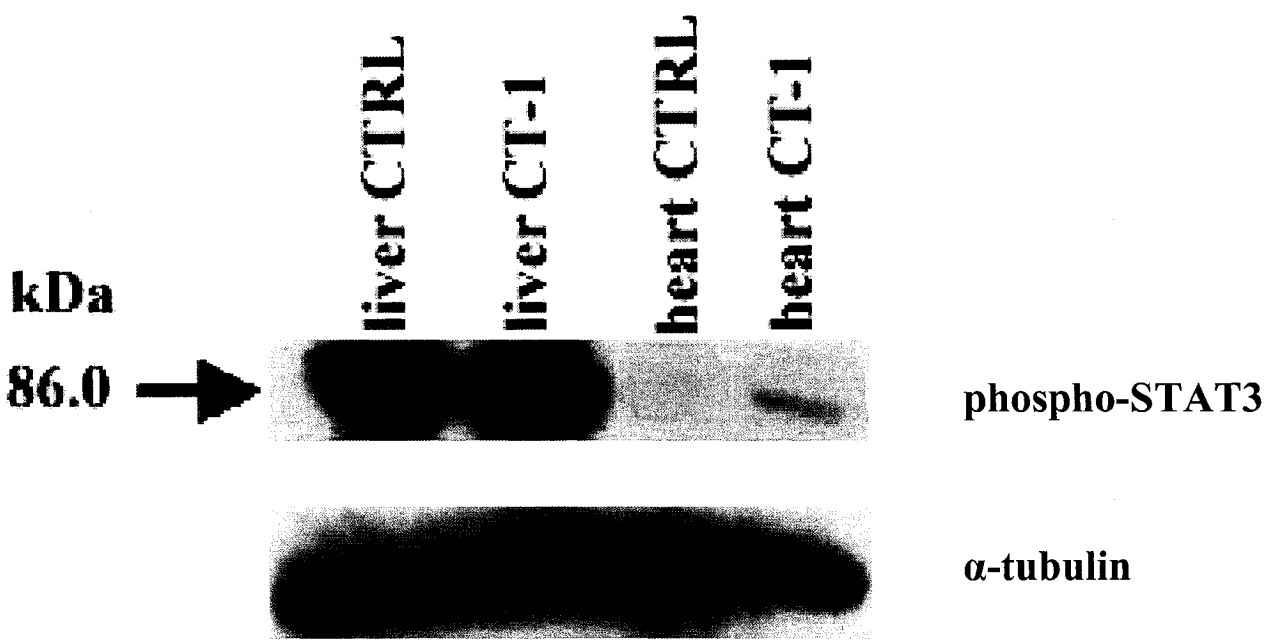
3.15 Activation of the gp130 pathway

Having confirmed that the LIF receptor was present in the cardiac SP fraction, the next step was to examine activation of the LIFR/gp130 signal pathway. The formation of the signaling complex induces the phosphorylation/activation of gp130 associated Janus Kinases (JAKs). Upon activation, the JAKs then phosphorylate tyrosine residues located on the cytoplasmic tails of the gp130 protein. These phosphorylated tyrosine residues are docking sites for the STAT family of transcription factors. The STAT factors, mainly STAT3 and STAT1 bind to matching SH2 domains of the docking sites, and subsequently become phosphorylated. The phosphorylated STATs form dimers and translocate to the nucleus where they function as a bona fide transcription factor, binding to specific enhancer sequences to stimulate or repress transcription of target genes (reviewed in Heinrich, 1998).

When STAT3 is phosphorylated, it becomes activated and moves into the nucleus where it can control gene activity (Ihle, 1995). To determine STAT activation occurred in the hearts of CT-1 adenovirus injected mice, a phospo-STAT3 antibody was used for western blot analysis. Proteins were isolated from CT-1 and CTRL injected heart and liver samples. Protein was then separated by electrophoresis, transferred to a membrane, and labeled with a phospho-STAT3 antibody. The results indicated that in the heart there was an increased amount of phospho-STAT3 protein following Ad-CT-1 was administered (Figure 17). The increase in phospho-STAT3 protein suggested that the gp130/LIFR pathway was being activated by the Ad-CT-1 administration.

Figure 17: Western Blot Analysis for phospho- STAT3 Expression following Ad-CT-1 Injection.

A total of 500 μg of protein from each tissue sample were separated by 10 % SDS-polyacrylamide gel electrophoresis. Protein samples were collected from the hearts, and livers of mice that had received either CT-1 or CTRL adenovirus injections. The membrane was then incubated with phospho-Stat3 (Ser 727) antibody (Cell Signalling) at a dilution of 1:1000 followed by incubation in secondary antibody at a dilution of 1:5000. Enhanced chemiluminescence was performed to visualize the proteins. (phospho-STAT3 size ~ 86 kDa). Western blot analysis using α-tubulin antibody was also performed to show equal protein loading. The results indicated an increase in STAT3 phosphorylation in the hearts of Ad-CT-1 injected mice.


liver CTRL
liver CT-1
heart CTRL
heart CT-1
kDa
86.0
phospho-STAT3
α-tubulin

Chapter 4

Discussion

4.1 Activation of the Cardiac SP Population

The identification of an SP within the adult murine heart infers new methods of cardiac repair, i.e. stem cells compensate for damage by generating new cardiomyocytes. Stem cells have the ability to self renew, that is they can divide and create additional stem cells, as well differentiate into a specific cell type. Identifying the signals that regulate stem cell differentiation is essential. Several studies have emphasized the dominance of the tissue microenvironment on the differentiation & functional properties of stem cells (reviewed in Heinrich, 1998). In order to change stem cell fate, cells must respond to key growth & differentiation factors.

Of particular interest was Cardiotrophin-1 (CT-1), a member of the IL-6 family of cytokines (Pennica, 1995). CT-1 is a multi-functional cytokine. It is responsible for a diverse range of bioactivities in a variety of cell types. CT-1 has the ability to inhibit embryonic stem cell differentiation and aortic endothelial cell proliferation, as well as being capable of modulating local inflammatory responses and promoting the survival of cultured motor neurons (reviewed in Pennica, 1996). In the heart, CT-1 has been identified as a hypertrophic agent (Pennica, 1995) as well as having a cardioprotective effect during times of stress (Stephanou, 1998).

The data presented here demonstrated that adenoviral CT-1 had a short-term effect on the cardiac SP population. CT-1 caused an increase in the percentage SP during the first 72 hours post injection. The increase in the SP population eventually decreased and returned to its initial size by 96 hours following Ad-CT-1 injection. Furthermore, the

increase was cardiac specific and was not observed in skeletal muscle, bone marrow or liver SP cells.

There are several possible explanations for this temporary increase in the cardiac SP. Potentially, after activation and an initial increase in the cardiac SP fraction, these cells begin to rapidly differentiate into cardiomyocytes, thus depleting the cardiac SP. A recent study reported the existence of cardiac progenitor cells in the heart (Oh, 2003). It was found that these cells had the ability to differentiate and contribute to cardiac repair during ischemia. It is possible that the cardiac SP cells reported here are acting in a similar fashion.

Another possibility is that the cardiac SP cells become refractory to continued CT-1 exposure. Self-renewal is the most fundamental property of stem cells and the signals that govern stem cell fate are poorly understood (reviewed in Morrison, 1997). Hypothetically, the tissue microenvironment would intrinsically regulate SP cell self-renewal, thus limiting the number of stem cells under steady state conditions. Presumably the number of cardiac SP cells is limited by the amount of space available in the heart. Cells generated in excess would differentiate and a negative feedback loop would regulate any further renewal. In other words, the sudden increase in cardiac SP cells would activate an additional mechanism, which would inhibit CT-1 signaling from further increasing the SP population.

Preliminary results demonstrated in the co-culture experiment (Figure 15) suggested that this increase was accompanied by SP cell differentiation. In the presence of CT-1, cardiac SP cells co-cultured with primary cardiomyocytes showed an increase in connexin-43 (a cardiac specific marker) expression. This suggested that perhaps not only was the cardiac SP size increasing but that a differentiation program was also being activated. These experiments were only performed once and further investigation is required to draw a decisive conclusion.

4.2 Absence of a Hypertrophic Phenotype following CT-1 Exposure

Initially CT-1 was identified as a hypertrophic agent (Pennica, 1995). In subsequent *in vitro* studies it was observed that the form of hypertrophy was distinct, both in terms of cell morphology and gene expression when compared to α-adrenergic stimulation (Wollert, 1996). CT-1 induced hypertrophy leads to an increase in cardiac cell size, caused by an increase in cell length without a significant change in cell width (Wollert, 1996). These *in vitro* studies were supplemented by an *in vivo* study in which CT-1 was administered to intact mice via intraperitoneal injection (Jin, 1996). In this study, a dose dependent increase in both heart weight and ventricular weight was observed in the mice treated with CT-1. Clearly, these experiments suggested that CT-1 could induce cardiac hypertrophy.

Further investigation provided evidence of an additional role for CT-1 in the heart. Specifically, it was observed that treatment with CT-1 enhanced the survival of neonatal rat cardiomyocytes in serum free media (Sheng, 1996). Moreover, it has been shown

that CT-1 is able to protect cultured neonatal cardiomyocytes against subsequent exposure to heat shock or stimulated ischaemia/hypoxia (Stephanou, 1998). CT-1 exerts the survival promoting effects by minimising the degree of apoptosis (Sheng, 1997; Stephanou, 1998).

The key difference in these two roles is the signalling pathways involved. Like all members of the IL-6 family of cytokines, CT-1 signals via the gp130 pathway (reviewed in Heinrich, 1998). This pathway results in the activation of at least two types of cellular transcription factors that can then activate the expression of specific target genes. Thus following the binding of CT-1 to its receptors, activation of the p42/p44 mitogen activated protein kinase (MAPK) enzymes (Sheng, 1997) results in the phosphorylation of the nuclear factor-IL-6 transcription factor (NF-IL-6), allowing it to activate gene transcription (Nakajima, 1993). Similarly, activation of the Janus kinase tyrosine kinases results in the phosphorylation of the signal transducer and activator of transcription (STAT)-3 transcription factor (Sheng, 1997). The activation of STAT-3 results in its dimerization and transportation to the nucleus where it can activate its target genes (Akira, 1994).

There is evidence that of these two signalling pathways for CT-1, one is used predominantly to induce hypertrophy, while the other is involved in the cardioprotective effect. For example, it was shown that PD98059, an inhibitor of the p42/p4 MAPK pathway, was able to block the protective effect of CT-1 while having no effect on the ability to induce hypertrophy (Sheng, 1997). A similar effect was also observed *ex vivo*

in which PD98059 was able to block the effect of CT-1 added prior to ischemia or after ischemia at the time of reperfusion (Brar, 2001). These experiments indicated that the protective effect is likely to be dependant upon the p42/p44 MAPK pathway.

Phosphorylation of STAT3 occurs in the heart in response to hypertrophic stimuli such as pressure overload and mechanical stretch. In both cases, STAT3 phosphorylation was mediated by the gp130-signalling pathway (Pan, 1998). More direct evidence for the role of STAT3 signalling in CT-1 mediated cardiac hypertrophy was derived from a study in which replication defective adenovirus vectors carrying wild type or mutant STAT3 were used to infect cardiomyocytes (Kunisada, 1998). The hypertrophic effect of CT-1 was augmented by wild type STAT3 and attenuated by mutant STAT3. Furthermore, inhibition of STAT1 or p42/44MAPK signalling did not block the hypertrophic effects of CT-1, whereas inhibition of the STAT3 pathway did block the hypertrophic effect (Railson, 2002). These effects occurred independently from any changes in MAPK activity demonstrating that the STAT3 pathway was specifically required for the hypertrophic response associated with CT-1.

Surprisingly, the data presented here shows no evidence of hypertrophy *in vitro* or *in vivo*. Several hypertrophy assays were performed to evaluate the hypertrophic effects of CT-1 on cardiomyocytes including: ANF northern blot analysis, an *in vitro* CT-1 exposure assay, and a comparison of heart to body weight ratios. All three experiments concluded that hypertrophy was not occurring. This was a surprising result given that STAT3 phosphorylation (Figure 17) was increased *in vivo* in the presence of CT-1,

which as previously mentioned is a part of the pathway involved with CT-1 induced cardiac hypertrophy (Railson, 2002).

While the primary focus of this study was not to assess hypertrophy, it is important to address this discrepancy. Cytokines are pleiotropic in their biological activities and play pivotal roles in a wide variety of responses. Cytokines are responsible for regulating immune response, hematopoiesis, neurogenesis, embryogenesis and oncogenesis (reviewed in Hirano, 2000). It is not surprising that CT-1 would be responsible for numerous activities in the heart. Furthermore it has been demonstrated that STAT3 is involved in the regulation of cell growth, differentiation and survival (reviewed in Heinrich, 1998). For example it has been shown that STAT3 phosphorylation is sufficient to maintain an undifferentiated state in mouse embryonic stem cells (Matsuda, 1999). Other studies have shown that that the self-renewal of pluripotent embryonic stem cells is mediated via the activation of STAT3 (Niwa, 1998). Contrastingly, STAT3 is also responsible for differentiation of M1 leukemia cells into macrophages (Nakajima, 1996), differentiation of cerebral cortical precursor cells into astrocytes (Bonni, 1997) as well as anti-apoptosis in pro-B cell lines (Takeda, 1998). STAT3 has also been implicated in a variety of malignancies, such as prostate, lung, brain, breast, and squamous cell carcinomas (reviewed in Predrazini, 2004). Inhibition of activated STAT3 led to decreased proliferation and apoptosis of many cancer-derived cell lines. It has also been shown that STAT3 participates in liver regeneration. It regulates the primary growth response genes in the early phase of liver regeneration (Cressman, 1995). As illustrated it is possible for STAT3 to exert numerous responses.

While it is possible that the short-term increase in the cardiac SP population is due to STAT3 phosphorylation, direct evidence has not been shown in this study. Further investigation is required to determine the pathway(s) responsible for the CT-1 induced increase of the cardiac SP fraction. Defining these pathway(s) will provide insight into an explanation for the absence of hypertrophy.

Interestingly, a third component of the gp130/LIF receptor complex involved in CT-1 signalling has been identified (Robledo, 1997). It has been suggested that the gp190 receptor protein is also required to initiate a response to CT-1. This observation raises several questions about the CT-1 signalling pathway and indicates that many intricacies of the CT-1 response remain unknown.

4.3 CT-1 Signalling

As previously mentioned, CT-1 signals through the gp130 pathway (reviewed in Heinrich, 1998). The first event in the CT-1 activation of the gp130 pathway involves a ligand-induced hetero-dimerization, i.e. gp130 and the LIF receptor beta subunit. Presumably, if cardiac SP cells were capable of responding to CT-1, these cells would also retain the appropriate receptor components. Using FACS and a LIF receptor antibody it was determined that the cardiac SP population expresses the required LIF receptor (Figure 16). The presence of the receptor provided evidence that the cardiac SP population has the necessary components to be stimulated by CT-1.

The formation of the signalling complex induces the phosphorylation/activation of gp130 associated Janus Kinases (JAKs). Upon activation, the JAKs then phosphorylate tyrosine residues located on the cytoplasmic tails of the gp130 protein. These phosphorylated tyrosine residues are docking sites for the STAT family of transcription factors. The STAT factors, mainly STAT3 and STAT1 bind to matching SH2 domains of the docking sites, and subsequently become phosphorylated. The phosphorylated STATs form dimers and translocate to the nucleus where they function as a bona fide transcription factor, binding to specific enhancer sequences to stimulate or repress transcription of target genes (reviewed in Heinrich, 1998). In particular STAT3 is responsible for CT-1 induced hypertrophy (Railson, 2002). As previously mentioned, STAT3 phosphorylation was examined and the results showed an increase in STAT3 phosphorylation in the presence of CT-1. This lends further support to that CT-1 is acting through the gp130 pathway.

4.4 The Effects of Age and CT-1 Adenovirus on the Heart

Previous findings suggested that with age the cardiac SP population diminishes (unpublished, communication with Hierlihy *et al.*; Capogrossi, 2004). To determine if there was a combinatorial effect of age and CT-1 on the cardiac SP population, mice of various ages (3 weeks, 6 weeks, 2 months, 4 months, and >6 months) were injected with adenoviral CT-1. Two major trends were observed. Firstly, it was confirmed that the cardiac SP population diminished with age. Secondly, the effect of CT-1 on the SP fraction was increased in older mice (2 months and older). It is interesting to note that the mouse is considered an "adult" at two months of age.

Cardiac muscle cells exhibit two distinct modes of growth that are highly regulated during development. Cardiomyocytes rapidly proliferate during fetal life but exit the cell cycle irreversibly soon after birth. At this point the predominant form of growth shifts from hyperplastic to hypertrophic (reviewed in MacLellan, 2000). Traditionally it was believed that adult cardiomyocytes were unable to regenerate. This belief was based on the concept that (1) in the adult heart all cardiomyocytes are terminally differentiated and (2) the myocardium lacks a stem cell population to generate new myocytes (reviewed in Nadal-Gidard, 2003). Theoretically this would suggest that most if not all heart muscle cells in an 80-year old individual would be similarly aged. Recent discoveries in cardiac biology challenge these old ideas. A growing body of evidence suggests that hypertrophy is not the only mechanism responsible for post-natal cardiac growth. Several reports have shown that there are replicating cardiomyocytes in the adult myocardium (Kajustura, 1998; Limana, 2002; Beltrami, 2001).

The diminished SP population in older mice would suggest that the cardiac SP cells have distinct roles at various stages in development. Prior to the "adult" phase when the mouse is still undergoing growth and development there are more SP cells in the heart. However once the mouse reaches the "adult" stage and beyond the SP fraction diminishes. There are several hypothetical explanations for this change. Perhaps the cardiac SP cells are contributing to the heart growth in the younger mouse and are in a proliferative stage. It is also possible that the cardiac SP cells are being used for cardiac repair/maintenance in the older mouse and are undergoing differentiation, thus resulting in a decrease in the cardiac SP fraction. Regardless of the reason, CT-1 appeared to

have a greater effect on the cardiac SP in older mice. This is valuable from a therapeutic perspective, in which cardiac disease occurs more frequently in the aged.

4.5 The Effects of CT-1 on Skeletal Muscle

As previously discussed, the effects of CT-1 on the size of the SP population are cardiac specific however it is important to note that CT-1 does have an effect on the skeletal muscle. The SP phenotype is not the only measure that can be used to evaluate the effects of CT-1 on stem cell like cells. The hematopoietic colony assay has been commonly used to test for stem cell/progenitor-like activity (Seale, 2000), and is based on colony growth in a semi-solid environment (i.e. methocult) (refer to section 3.7). Methocult is a commercial semi solid methylcellulose media that contains various cytokines and growth factors optimized to promote the proliferation and differentiation of hematopoietic activated stem cells and progenitor cells (Stem Cell Technologies). The prospective colonies that emerge under these conditions contain mature hematopoietic cell types such as granulocytes, macrophages and natural killer cells. As such, the assumption in the field is that emergence of these cells types reflects prior activation of a stem cell pool.

The methocult experiments revealed that there was significantly more colony formation in skeletal muscle injected from CT-1 adenovirus injected animals when compared to that from CTRL injected mice, while the number of colonies formed by the heart and bone marrow SP cells showed no variation between the CT-1 and CTRL adenovirus injections.

These results suggested that even though the number of skeletal muscle SP cells was not altered in ad-CT-1 injected mice, there was a dramatic increase associated with stem cell progenitor activity. These findings suggest that while CT-1 does not have a direct effect on the size of the skeletal muscle SP it is having an effect on SP activation.

The notion that CT-1 is having an effect in skeletal muscle is not surprising. Extensive research has been done using CT-1 as a treatment for various skeletal muscle diseases. For example, adenoviral CT-1 gene transfer has been shown to protect progressive motor neuropathy (pmn) mice from disease progression (Bordet, 1999; Lesbordes, 2002). These mice suffer from motor neuron degeneration, muscle paralysis and premature death. When CT-1 was administered to pmn mice they exhibited prolonged survival and improved motor functions. Intra-muscular CT-1 injections in mice suffering from spinal muscle atrophy (SMA) were shown to have improved survival, delayed motor neuron defects and decreased loss of motor neurons (Lesbordes, 2003). The SMA mouse model is characterized by degeneration of the low motor neurons.

Similarly, CT-1 administration has been demonstrated to modify the effects on neuromuscular degeneration in amyotrophic lateral sclerosis (ALS) (Bordet, 2001). ALS mice exhibit a loss of cortical and spinal motor neurons. As with other neurological disease models, ALS mice treated with CT-1 showed delayed onset of motor impairment and slowed axonal degeneration. Taken together there is strong evidence that CT-1 also has a protective role in skeletal muscle as well as a role in promoting motor neuron survival.

4.6 Sca-1 Expression

The discovery of a cardiac stem cell population is relatively new and the characterization of these cells has not been completed. While there are a variety of cell surface markers available to identify the hematopoietic stem cell (HSC) subset within the adult bone marrow (Okada, 1992; Szilvassy, 1990), no such markers have been identified within the heart. Since much of adult stem cell biology is based on what is known about the hematopoietic stem cell population, it was a useful tissue in which to begin the search for an appropriate cell surface marker. Stem cell antigen-1 (Sca-1) is a member of the ly-6 family. Sca-1 is frequently used in combination with negative selection for lineage specific cell surface markers to identify and isolate murine HSCs. Sca-1 positive HSCs can be found in the adult bone marrow, fetal liver and mobilized peripheral blood and spleen within the adult animal (Spangrude, 1993; Morrison, 1997; Kawamoto, 1997; Yamamoto, 1996).

Sca-1 has also been discovered in several non-hematopoietic tissues (Van de Run, 1989), and has been used to enrich progenitor cell populations other than HSCs. Sca-1 has been used to isolate stem cell like populations from the murine mammary gland and skeletal muscle (Welm, 2002; Askura. 2002). In an attempt to find additional markers for the cardiac stem cell population, cells were analyzed for Sca-1 expression. Approximately 25% of all cells within the murine heart express SCA-1. Interestingly, cells within the cardiac SP were enriched for Sca-1 expression (75% compared to 15% in the non-SP). Recently published work confirmed this finding, it was reported that the adult murine heart contains a 93% Sca-1 positive SP fraction (Oh, 2003). Furthermore

they concluded that this population was capable of contributing to cardiac repair. The fact that the cardiac SP is enriched for Sca-1 expression lends further support to the notion that the SP is a stem cell population as well as an additional marker for defining this subpopulation.

These results prompted an investigation of whether CT-1 had an effect on the Sca-1 population within the heart. A comparison of the total cell Sca-1 expression and SP only Sca-1 expression yielded surprising results. The analyses indicated that there was no change in the total number of Sca-1 positive cells in the hearts of CT-1 adenovirus injected mice (Figure 11). Similarly, there was no change in the number of Sca-1 positive cells in the SP fraction (hearts, CT-1 adenovirus injected). The results indicate that CT-1 did not have an effect on the Sca-1 positive fraction of the cardiac SP. A better understanding of the Sca-1 positive SP fraction could give insight into a possible explanation, i.e. at what stage is Sca-1 expression lost? Is the Sca-1 positive fraction closer to the original stem cell and is Sca-1 expression lost by spin off progenitors? The answer is unclear, but perhaps Sca-1 positive SP cells are not responsive to CT-1.

4.7 Origin of Cardiac SP Cells

Recent studies provide compelling evidence that the post-natal heart does contain a resident cell population with a residual stem cell like activity (Hierlihy, 2002; Beltrami, 2003; Oh, 2003). Despite the appearance that the cell population being examined in each of these studies is one in the same, significant differences have been noted between these three reports (See Table 4). While Sca-1 enrichment was commonly

observed, only one of the three studies reported that the putative cardiac stem cell pool was c-Kit positive (Beltrami, 2003). Interestingly, the same study that identified the c-Kit positive stem cell population also demonstrated that these c-Kit positive cells were in turn capable of multiple-lineage conversion events, giving rise to endothelial, smooth muscle and cardiomyocyte cell types upon re-implantation to a damaged myocardium *in vivo* (Beltrami, 2003). However, the presence of c-Kit has been suggested to provide a reliable marker for identifying hematopoietic stem cell populations and not stem cell populations from other cell lineages (McKinney-Freeman, 2002; Nadin, 2003).

Given these disparities, it is likely that the cardiac c-Kit stem cell population (as described and tested in Beltrami et al) is a qualitatively different cell type than the SP/Sca-1 cell population described in the other studies (Hierlihy, 2002; Oh, 2003). What is not clear at the present time is the relationship (if any) between these cell populations. It is possible that the cardiac c-Kit stem cell pool represents a more primitive cell population within the same continuum as the SP/Sca-1 cell population. This model would predict that c-Kit expression defines a primordial pluripotent cell type and the SP/Sca-1 cell is the derivative/daughter cell that possesses a restricted fate choice owing to the influence of exogenous influences/factors (See Figure 18). This is an appealing model, and evidence to support such a supposition has been reported in skeletal muscle tissue. For example, CD45+ Sca-1+ cells derived from skeletal muscle have been shown to readily form either skeletal muscle cell types when instructive

Table 4: A comparison of the Post-Natal Cardiac Stem Cell Populations.

Paper	Organism	Side Population	Sca-1 Expression/ Enrichment	c-Kit Expression/ Enrichment	Lineage Conversion	Fusigenic Properties
Hierlihy, 2002	Mouse	Yes	Yes	No	No	Yes
Oh, 2003	Mouse	Yes	Yes	No	No	Yes
Beltrami, 2003	Rat	ND (not determined)	Yes	Yes	Yes	ND

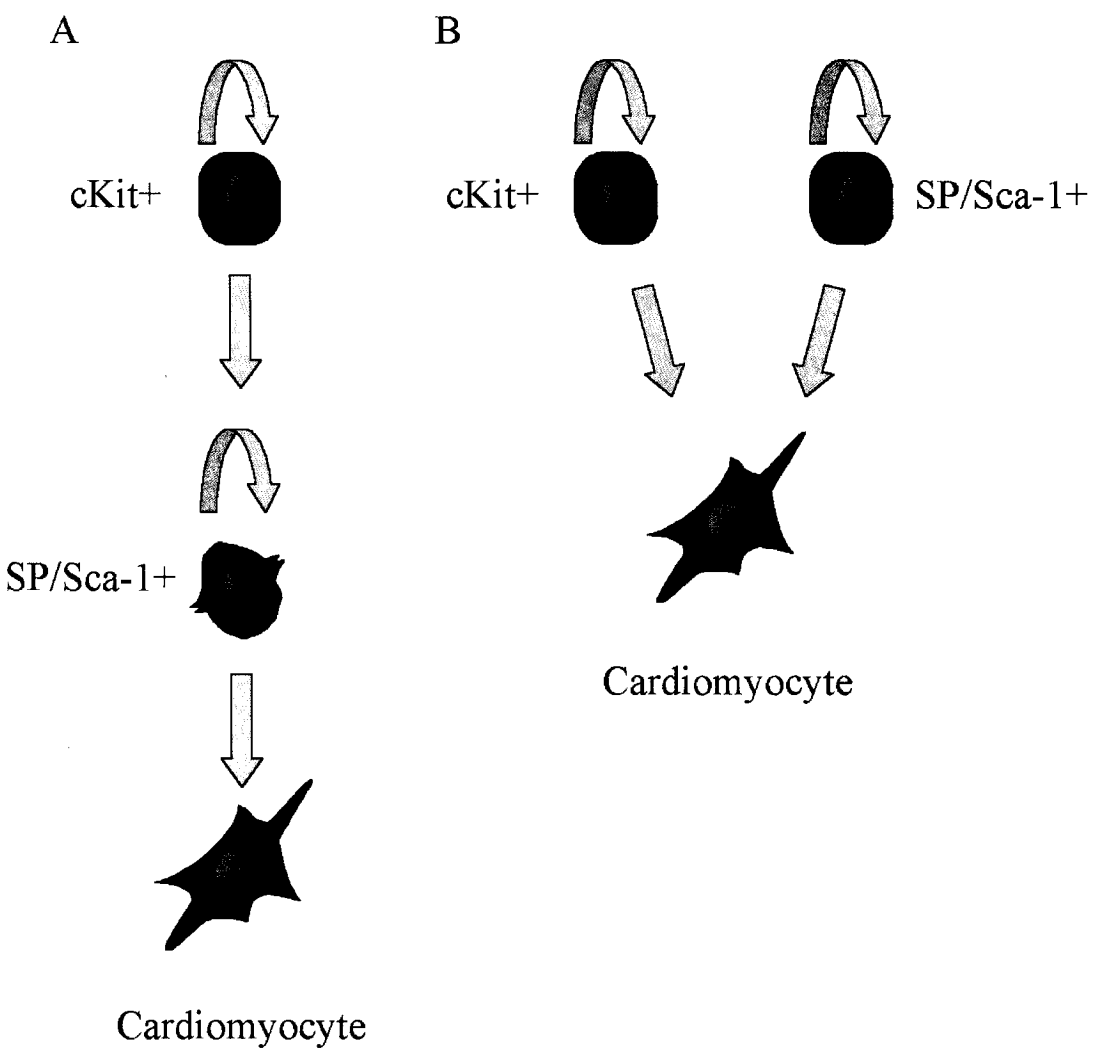
cues/diffusible ligands are present or hematopoietic cell types when these cues are not provided (Polesskaya, 2003). Alternatively, the cardiac c-Kit and SP/Sca-1 populations may originate from divergent sources and occupy separate niches within the heart (See Figure 18). Indeed, examples of divergent stem cell pools within one tissue reservoir have been documented, with skeletal muscle having been suggested to contain stem cell populations that specify myogenic or hematopoietic cell types exclusively (McKinney-Freeman, 2002). Clearly, more research is required to determine the biologic origin of these cardiac stem cell populations.

4.8 Application of CT-1 in Cardiac Disease

The majority of studies characterizing CT-1 have utilized the mouse protein. However the human CT-1 protein has also been studied (Pennica, 1996). The human CT-1 is encoded by 201 amino acids (compared to 203 in mouse) and is 80% identical to the mouse sequence. Similar to mouse CT-1, human CT-1 also signals through the LIF/gp130 receptor complex. Interestingly it has been shown that human CT-1 binds to the LIF receptor on both human and mouse cell lines. This indicated that CT-1 has a lack of species specificity (Pennica, 1996). Human CT-1 is expressed in a variety of tissues, including heart, skeletal muscle, ovary, colon, prostate, testis as well as fetal kidney and lung (Erdmann, 1998). As such, it appears that the human and murine CT-1 proteins may be functionally interchangeable, i.e. an ability to cross-react and bind to each respective receptor complex. This is important when considering the application of CT-1 in humans. A strong similarity between human and murine CT-1 is suggestive that CT-1 will have a similar function in the human heart.

Figure 18: Post-Natal Cardiac Progenitor/Stem Cells – One or Multiple Origins?

A number of studies have confirmed the existence of a resident cardiac stem cell population in the postnatal heart. Despite the similarities in the cell types, considerable differences have been noted. One interpretation may be that the c-kit+ stem cell population represents a more primitive cell population, with the SP/Sca-1+ cell being the derivative/daughter cell. The SP/Sca-1+ cell possesses a more restricted fate choice than the c-kit+ cell (A). An alternative model envisions that the c-kit+ and SP/Sca-1+ populations may originate from different niches within the heart (B). Each population is capable of giving rise to cardiomyocytes.


A
cKit+
SP/Sca-1+
Cardiomyocyte
B
cKit+
SP/Sca-1+
Cardiomyocyte

The data presented here suggests that CT-1 could be a possible mechanism in which to activate the resident population of stem cells in the heart. While more research is required, there are several lines of preliminary evidence to consider such a possibility. The effects observed are cardiac specific, with no changes seen in the bone marrow, skeletal muscle or liver. The effect in the heart is immediate and short term. Cardiac hypertrophy is the heart's natural adaptive response to a variety of pathological stresses (reviewed in Steinberg, 2000). However prolonged hypertrophy has damaging effects on the heart. The adenoviral CT-1 used in this study appeared to have no hypertrophic effects, eliminating hypertrophy as a possible negative side effect.

Previous experiments suggest that the cardiac SP possesses stem cell like activity (Hierlihy, 2002; Oh, 2003). If the SP cell fraction does indeed possess stem cell like activity, an increase in the cardiac SP would mean an increase in the number of cells capable/available to induce cardiac repair. Although of a preliminary nature, the co-culture experiment (reported in section 3.16) suggested that CT-1 induced cardiac SP cells to adopt a cardiomyocyte cell fate.

Taken together the results in this study offer a potential therapeutic treatment for cardiac disease. However there are still many questions that must be answered before this therapy can reach its full clinical potential. In particular some basic biological questions about the signalling pathways that govern the fate of cardiac stem cells. For example, what causes these cells to differentiate into cardiomyocytes? What causes cardiac stem cells to home to the injured tissue? What factors stimulate the expansion of

this population? Many of these questions may be answered by studying the cardiac biology associated with cardiotrophin-1 exposure.

The existence of a potential biologic stimulant (cardiotrophin-1) for cardiac stem cells is an exciting prospect. What is not known at this stage is whether CT-1 application will provide a protective or regenerative effect to damaged myocardium. If cardiotrophin-1 can be shown to mitigate cardiac damage, then cardiac repair may become a viable therapeutic option in the not too distant future.

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Appendix

The appendix contains tables of data used to generate statistics and graphs for the time course trials. These numbers have been normalized (as described in section 2.19). Each table contains the data for trials #1-4 {A) heart, B) skeletal muscle and C) bone marrow} or #1-3 {D) liver} for 24, 48, 72, 96 hours post-injection with either Ad-CTRL (CTRL) or Ad-CT-1 (CT-1). The last table (E) contains the data used to assess the effects of age on the cardiac SP. It contains the data for trials #1-3 generated from the hearts of mice aged 2 weeks, 6 weeks, 2 months, 4 months and >6 months of age at 48 hours post-injection with either Ad-CTRL (CTRL) or Ad-CT-1 (CT-1).

A) HEART	24 hours	48 hours	72 hours	96 hours
CTRL # 1	1.0	1.0	1.0	1.0
2	1.0	1.0	1.0	1.0
3	1.0	1.0	1.0	1.0
4	1.0	1.0	1.0	1.0
CT-1 # 1	1.143	1.659	1.757	1.016
2	2.981	2.313	2.609	1.064
3	1.767	5.860	2.941	0.902
4	2.062	2.257	2.236	1.163

B) Skeletal muscle	24 hours	48 hours	72 hours	96 hours
CTRL # 1	1.0	1.0	1.0	1.0
2	1.0	1.0	1.0	1.0
3	1.0	1.0	1.0	1.0
4	1.0	1.0	1.0	1.0
CT-1 # 1	1.154	0.915	0.841	1.187
2	1.083	0.968	0.959	1.102
3	0.691	0.895	0.816	1.207
4	1.353	1.071	1.728	0.912

C) Bone Marrow	24 hours	48 hours	72 hours	96 hours
CTRL # 1	1.0	1.0	1.0	1.0
2	1.0	1.0	1.0	1.0
3	1.0	1.0	1.0	1.0
4	1.0	1.0	1.0	1.0
CT-1 # 1	0.981	1.113	1.061	1.208
2	0.913	0.688	0.923	1.087
3	0.965	0.951	1.582	0.948
4	1.278	1.854	0.948	1.005

D) Liver	24 hours	48 hours	72 hours	96 hours
CTRL # 1	1.0	1.0	1.0	1.0
2	1.0	1.0	1.0	1.0
3	1.0	1.0	1.0	1.0
CT-1 # 1	1.052	0.877	1.026	1.191
2	0.793	0.819	0.961	1.039
3	0.970	1.000	1.455	1.207

E) Age Course	2 weeks	6 weeks	2 months	4 months	>6 months
CTRL # 1	1.0	1.0	1.0	1.0	1.0
2	1.0	1.0	1.0	1.0	1.0
3	1.0	1.0	1.0	1.0	1.0
CT-1 # 1	0.800	0.860	2.107	3.733	4.862
2	1.124	0.974	1.979	2.754	2.789
3	1.006	1.025	1.955	2.887	3.894