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M.Sc. (Cellular and Molecular Medicine)	2003
TITRE DE LA THÈSE / TITLE OF THESIS: IDENTIFICATION & CHARACTERIZATION OF STEM CELL-LIKE SP CELLS IN THE POST-NATAL MYOCARDIUM	

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TITRE DE LA THÈSE - TITLE OF THE THESIS

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Identification and characterization of stem cell-like SP cells in the post-natal myocardium

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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February 23, 2003

Abstract

The field of stem cell biology is continuously expanding, encompassing not only the traditional study of hematopoietic and fetal cells, but also the investigation of adult stem cells from a variety of tissue sources. The definitions and conventions adopted by the field of hematopoiesis have served as a framework for the more recent discoveries in adult stem cell biology. As such, stem cells are defined as immature progenitor cells capable of long term self-renewal and pluripotent differentiation. Using Fluorescence Activated Cell Sorter (FACS) analysis, a sub-population of distinct Hoechst dye-excluding cells, termed the 'side population' (SP), has been isolated from bone marrow (BM) (Goodell et al., 1996). These verapamil-sensitive cells are highly enriched in stem cell activity and have the capacity for lineage conversion. The potential for these cells to play a role in adult tissue maintenance and repair has been established in skeletal muscle, liver and brain. Here we report the existence of endogenous SP cells within the adult myocardium with similar but not identical characteristics to SPs from other adult tissues. These features include the capacity for cardiomyocyte differentiation and the expression of cell surface markers such as Sca-1 and CD34, but not hematopoietic lineage markers Gr-1, Mac-1 and CD4. Conversely, these cardiac-derived SP cells do not express the hematopoietic stem cell (HSC) markers c-kit and CD45. Furthermore, fusion but not transdifferentiation of these myocardial SP cells with skeletal myoblasts was observed. These results offer the first experimental evidence of a resident population of myocardial stem cells and suggest a potential for therapeutic applications in cardiac disease.

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List of Abbreviations

CMV	cytomegalovirus
CT-1	Cardiotrophin-1
D.A.B.	3,3'-Diaminobenzidine
DAPI	Diamidino-2-phenylindole
Dkk	Dickkopf
DMEM	Dubecco's modified Eagle's medium
DMEM/F12	Dubecco's modified Eagle's medium nutrient mixture F12
dn	Dominant negative
DNA	Deoxyribonucleic acid
Dsh	Dishevelled
ES	Embryonic stem
FACS	Fluorescence activated cell sorter
FBS	Fetal bovine serum
FGF	Fibroblast growth factor
FITC	Fluorescein isothiocyanate
GFP	Green fluorescence protein
gp130	Glycoprotein 130
GSK-3	Glycogen synthase kinase-3
HBSS	Hanks balanced salt solution
HSA	Heat shock antigen

HSC	Hematopoietic stem cell
IL	Interleukin
JAK	Janus kinase
JM-MEM	Joklik's modified Eagle's medium
JNK	c-Jun NH2-terminal kinase
LIF	Leukemia inhibitory factor
Lin^-	Lineage negative
LT-HSC	Long term-hematopoietic stem cell
MAPK	Mitogen-activated protein kinase
MEF2	Myocyte enhancer factor
MHC	Myocin heavy chain
NSC	Neural stem cell
PE	Phycoerythrin
PFA	Paraformaldahyde
PKC	Protein kinase C
PNA	Peanut agglutinin
SP	Side population
STAT	Signal transducer and activator of transcription
ST-HSC	Short term-hematopoietic stem cell

Acknowledgments

I would like to take this opportunity to thank all the very important people who, in one way or another, have contributed to the success of this thesis.

First I would like to thank my supervisor Dr. Lynn Megeney, who, throughout the course of my studies, constantly challenged me to reach my potential. It is upon the foundation of his knowledge and support that this thesis came into fruition. In addition to my education in the field of stem cell research, I've had the benefit of many tutorials pertaining to 'the war', international and local political issues, weight lifting, fashion tips and a multitude of other useful topics. But most of all, thank you Lynn, for allowing me to experience the Colorado International Airport!

I also must thank the very special people that I had the pleasure of working with. It is true that you can choose your friends but you can't choose your family, however, as luck would have it, these people are both my family and friends. I especially want to thank each and every one for their undying humour, clever wit, and senseless insanity, which kept me (arguably) sane.
Andrea 'Cobra': who showed me the true meaning of generosity through her actions
Jackie: for her enthusiasm about life; and her 'happy-dance'
Jennifer: my stem cell research and conference drinking partner
Julie: for her motivation and friendship; and for setting high standards
Kim & Beata: for all their technical support and unfailing patience
Pasan: for all the "Lab Games" and other ridiculousness; and his golden knowledge
Simon: for being such a sport and putting up with all the girls!
Steve: for his comic relief and creativity; and for getting all my lame jokes

I also would like to extend my appreciation to Nathalie Tessier, Eric Massicotte and Ruedi Braun for their technical assistance with the FACS machine; to Patrick, for helping me get started and letting me pick his brain throughout this project; and to the Kothary lab for help and know-how.

Lastly, I want to express my gratitude to my family for their love, support and encouragement throughout the course of my studies. During the times of frustration, when motivation wavered, it is their confidence in me that allowed me to continue. Consequently, as I celebrate the end of this road, they can truly appreciate what it took to get here. That means so much to me.

So in closing, always remember... There is safety in numbers!!

Finally, to answer the question that was asked most often over the course of my graduate studies... "Are you done yet?" The answer is *YES*!!!!!

Chapter 1

1.0 Introduction

1.1 Adult Stem Cells

The field of stem cell biology is continuously expanding to encompass not only the traditional study of hematopoietic and fetal cells, but also the investigation of adult stem cells from a variety of tissue sources. The definitions and conventions adopted by the field of hematopoiesis have served as a framework for the more recent discoveries in adult stem cell biology. This amalgamation has led to new definitions for stem cells as well as an increasing burden of proof to validate the stem cell phenotype from non-hematopoietic sources.

1.1.1 Characteristics of adult stem cells

Adult stem cells are currently defined as sharing at least two characteristics: 1) they are capable of self-renewal and, 2) they can give rise to mature cell types that possess appropriate phenotypic and functional characteristic. These rare, unspecialized cells have been found in an increasing number of adult tissue types including skeletal muscle, skin, brain, fat, etc. (Gussoni et al., 1999; Reynolds and Weiss, 1992; Toma et al., 2001; Zuk et al., 2001).

From what little is known about the biological role of adult stem cells, their functions appear to be as diverse as the locales in which they reside. They range from continuous repopulation of the hematopoietic system, to regeneration of damaged tissue, to minor repair and general maintenance of tissue homeostasis (LaBarge and Blau, 2002; Szilvassy et al., 1990). Stem cells are highly sensitive to their microenvironment and thus are able to detect and respond to a variety of signals. In addition to contributing specialized cells

to their host tissue, adult stem cells have displayed a surprising degree of plasticity (Blau et al., 2001; Poulsom et al., 2002).

It is important to consider that, while maintaining several key similarities, stem cells found in diverse tissues or at varied developmental stages are clearly different. However, significant questions remain with regard to their origin, their *in vivo* function, the signals by which they are regulated, and whether they can be manipulated and used for therapeutic purposes. In addition, the biological investigation of adult stem cells is further complicated by the difficulty in identifying, isolating and tracking their fate.

There are several compelling reasons for studying adult stem cells. In the short term, these cells may provide critical information to understand normal biological processes and mechanisms within several tissue types. For example, many questions remain as to the signals and corresponding pathways that govern cellular decisions such as self-renewal versus proliferation or differentiation. Moreover, long-term research on adult stem cells may ultimately reveal clinical applications targeting treatment strategies in a variety of diseases such as cancer, Parkinson's disease, diabetes, spinal cord injury, among others. In particular, the increasing incidence of cardiac disease is a grim reality that our aging population faces. Clearly, the discovery of cardiac stem cells would provide an important first step in designing new therapeutic strategies to fight such a disease.

Two adult stem cell populations that have received a great deal of attention are hematopoietic stem cells and neural stem cells.

1.1.2 Hematopoietic stem cells

Hematopoietic stem cells (HSCs), first identified several decades ago (Till and McCulloch, 1961), are the most widely studied and well-characterized adult stem cells known (Bhatia et al., 1997; Sutherland et al., 1989; Thomas, 1999). The principle function of HSCs is to continually reconstitute the full compliment of blood cells over the life of an individual. Occurring at a very low frequency in adult mouse bone marrow (approximately 1 in 10 000 bone marrow cells) (Szilvassy et al., 1990), these cells have a high proliferative potential and a capacity for self-renewal. HSC are further divided into two classes: long-term (LT-HSCs) capable of reconstituting the blood for the duration of the life of the individual and short-term (ST-HSCs) that survive for less than 2 months. Several markers have been identified that have assisted in the enumeration, isolation and study of various stem cells populations within the bone marrow. Reports of LT-HSC repopulation studies and colony-forming culture experiments have clearly demonstrated that cell surface phenotype can predict stem cell function (Jordan and Lemischka, 1990; Spangrude et al., 1988). The markers that are most commonly used are CD34, Sca-1, c-kit, and lineage negative (Lin^-). CD34, a transmembrane glycoprotein found on many hematopoietic progenitor cells, is involved in cell-cell adhesion and signalling, and is suggested to inhibit hematopoietic differentiation (Krause et al., 1996). Sca-1 (stem cell antigen-1; member of the Ly-6 family) and c-kit (CD117; a tyrosine kinase receptor for stem cell factor) are both cell surface antigens expressed on HSCs and immediate

progenitors. These markers are used in conjunction with CD34 to enrich for an HSC population (Ogawa et al., 1991; Spangrude et al., 1988). Finally, selection for cells that do not express mature hematopoietic cell lineage markers (including CD2, CD3, CD4, CD5, CD8, NK1.1, B220, Ter119, GR-1 and Mac-1), collectively referred to as Lin^-, allows for the enrichment of immature progenitor cells. Together, the combination of positive and negative cell surface marker selection, referred to as KSL (kit-positive, sca-1 positive, lineage negative), have greatly facilitated the investigation and characterization of adult stem cells (Okada et al., 1992).

The use of HSC markers has also facilitated the advancement of therapeutic applications such as bone marrow transplantations for the treatment of cancer and blood disorders (Weissman, 2000). Despite the clinical success of this approach, the use of marker analyses in defining functionally distinct populations has yielded confounding results. CD34, as mentioned above, has been accepted as a definitive marker for primitive stem cells and is used extensively to enrich for and isolate HSCs for the purpose of both research and therapeutic applications (Thomas et al., 1999). Although early studies suggested CD34 positive cells to be an early precursor cell type within the hematopoietic lineage hierarchy, this belief was challenged with evidence that CD34 negative cells may, in fact, be more primitive than their CD34 positive counterparts (Osawa et al., 1996; Zanjani et al., 1998). Reconstitution and *in vitro* culture experiments revealed that approximately 0.58% of CD34 negative HSCs within the bone marrow possessed classical early stem cell characteristics and, in some cases, were capable of generating CD34 positive cells (Goodell et al., 1997; Sato et al., 1999).

In addition to the use of FACS-sensitive markers, other strategies have been devised to enrich stem cell populations. In particular, dye-exclusion protocols have proven to be useful screens to obtain populations of cells enriched for stem cell activity.

Recently, a distinct population of multipotent stem cells was identified within adult bone marrow that stained weakly with Hoechst 33342 dye when analysed using FACS (Goodell et al., 1996). Hoechst 33342 is a fluorescent dye that binds to DNA and is taken up and effluxed by the cells in an active biological process. This differential efflux can be measured and used to separate various cell types by FACS analysis using a UV laser. When signal intensities detected at blue and red wavelengths respectively are plotted against each other, a distinct side population (SP) of Hoechst dye-excluding cells is detected. This SP fraction is highly enriched for stem cell activity (~1000-fold) and contains the majority of cells that label with hematopoietic stem cell markers (Goodell et al., 1996). Moreover, the SP is sensitive to the drug verapamil. This drug prevents the efflux of the Hoechst dye 33342 by blocking multi-drug resistance channel proteins thereby allowing cells to retain Hoechst dye and effectively obscuring the SP population (Goodell et al., 1996; Goodell et al., 1997). Recently, this transporter has been identified as an ATP binding cassette (ABC) transporter. Expression of the Bcrp1/ABCG2 gene, which is responsible for the ABC transporter, is conserved in the SP fraction isolated from a variety of tissues. This gene has been demonstrated to be an important determinant in the dye-effluxing phenotype (Zhou et al., 2001).

This technique has allowed investigators to pursue the identification of stem cells in a variety of adult tissues without preliminary knowledge of specific marker profiles. As a result, adult stem cells have been isolated from a variety of tissue sources on the basis of differential Hoechst dye-exclusion, including bone marrow, skeletal muscle, nervous tissue and pancreas (Gussoni et al., 1999; Jackson et al., 1999; Lechner et al., 2002; Murayama et al., 2002).

1.1.3 Neural Stem Cells

Evidence of adult neural stem cells was first published by Altman and Das in 1965 (Altman and Das, 1965). They and others provided evidence of cell proliferation and *de novo* neuron formation within the post-natal rodent brain (Altman, 1969; Goldman and Nottebohm, 1983). However, these accounts of post-natal neurogenesis were originally dismissed, as they challenged the widely accepted view that the adult brain was a non-regenerative tissue. Compelling data acquired over the last decade, nevertheless, has confirmed the existence of stem cells within the adult brain, and forced the field of neural biology to consider their potential role.

A significant breakthrough in the study of neural stem cells came with the development of techniques for *in vitro* culturing that allowed for growth factor-induced expansion and differentiation of neural precursor cells. The ability to culture multilineage neural stem cells (NSCs) *in vitro*, either as non-adherent neurospheres or adherent cultures (Ray et al., 1993; Reynolds and Weiss, 1992), has played a critical role in the subsequent characterization of adult NSCs. Effective cell surface markers have yet to be identified

for the isolation of living NSCs. As a result, strategies of serial enrichment have been devised on the basis of functional and phenotypic properties including proliferative potential, density differential, and FACS analysis by size, as well as low expression of peanut agglutinin $(PNA)^{low}$ and heatshock antigen $(HSA)^{high}$. These selection procedures are currently used in combination with *in vitro* neurosphere forming assays to study NSCs (Palmer et al., 1999; Reynolds and Weiss, 1992; Rietze et al., 2001). Using these methods, considerable progress has been made in the field of NSCs research.

Adult NSCs have been identified within the ventricular zone (Levison and Goldman, 1993; Morshead et al., 1994), hippocampus (Bayer et al., 1982) and olfactory system (Lois and Alvarez-Buylla, 1994; Schwartz-Levey et al., 1991). However, their specific location and function *in vivo* remains unclear.

Although the physiological role of these cells has not been elucidated, adult NSCs have been shown to give rise to three major classes of neural cells: neurons, astrocytes, and oligodendrocytes (Gage, 2000; Qian et al., 2000; vanderKooy and Weiss, 2000). Furthermore, numerous studies suggest that NSCs are not functionally restricted to regions from which they are derived. For example, hippocampal-derived NSCs, following *in vitro* expansion, will differentiate into hippocampal neurons when implanted back into the hippocampus. Similarly, when explanted into disparate regions such as the subventricular zone, these same cells are able to generate regional-specific neuronal cells typical of the olfactory system (Suhonen et al., 1996).

Taken together, these observations suggest that NSCs behave in an analogous manner to HSCs, as reflected by the propensity to proliferate and differentiate into the tissue of derivation (Gage, 2000). In addition, it is evident from these studies that signals originating from the microenvironment play an important role in determining the fate of these cells.

1.2 Adult stem cell plasticity

The existence of a resident population of stem cells within a particular tissue type, offers exciting possibilities for therapeutic intervention. Even more remarkable is the unanticipated finding that stem cells may not be restricted to a given tissue type. Indeed, plasticity of adult stem cells has been widely reported.

1.2.1 Bone marrow and skeletal muscle

Bone marrow-derived stem cells have been shown to circulate, home to specific tissues and differentiate into various mature cell types in response to damage and disease. Ferrari and colleagues first observed this phenomenon in skeletal muscle following direct injection of genetically marked bone marrow stem cells into chemically induced skeletal muscle damage (Ferrari et al., 1998). They showed donor-derived HSCs participating in the regeneration of skeletal muscle fibers. These results suggest that bone marrow-derived stem cells are not restricted to a hematopoietic fate, but instead, possess multipotent capacity reminiscent of embryonic stem cells. In addition, they performed bone marrow transplantation experiments using genetically marked donor cells followed

by an injury to skeletal muscle. Again, consistent with prior observations, bone marrow-derived stem cells participated in skeletal muscle regeneration, demonstrating a capacity for lineage conversion.

Similarly, Gussoni et al. (1999) observed significant restoration of dystrophin expression in the muscle fibers of mdx mice following hematopoietic reconstitution with bone marrow-derived SP cells. This same group went on to identify a population of SP cells residing within the skeletal muscle that possessed the ability to reconstitute the hematopoietic system (Gussoni et al., 1999). This was the first evidence of HSC potential within skeletal muscle. In addition, the authors demonstrated that skeletal muscle-derived stem cells have a heightened affinity for contributing to muscle fibres compared to hematopoietic stem cells (i.e. an increased number of donor-derived nuclei were detected in recipient muscle fibres when muscle-derived stem cells were used to reconstitute the bone marrow) (Gussoni et al., 1999). These reports illustrate the multipotentiality of both bone marrow- and skeletal muscle-derived stem cells.

Likewise, the hematopoietic potential of skeletal muscle stem cells was reiterated by Jackson et al. (1999). This study demonstrated that skeletal muscle-derived stem cells were capable of long-term repopulation of the hematopoietic system following lethal irradiation (Jackson et al., 1999). Together, these experiments provided the first evidence that skeletal muscle-derived stem cells harbour intrinsic hematopoietic potential.

More recently, Seale et al. (2000) determined that skeletal muscle-derived SP cells represent a population of stem cells, distinct from the satellite cell population. Satellite cells are a self-renewing population of committed muscle precursor cells, located on the surface of the basil lamina of myofibers, which contribute to regenerating muscle tissue. They showed that Pax-$7^{-/-}$ mice do not produce satellite cells, yet they contain similar numbers of SP cells and an increased level of hematopoietic stem cell activity compared to their wildtype counterparts (Seale et al., 2000). Asakura et al. (2002) supported these observations and determined that satellite cells and muscle-derived SP cells have unique developmental potential (Asakura et al., 2002). Skeletal muscle-derived SP cells possess hematopoietic potential, and are capable of myogenic specification, suggesting an inherent multipotentiality.

1.2.2 Bone marrow and brain

Although HSCs arise from the mesodermal germ layer during embryonic development, these cells have been shown to generate ectodermal germ layer tissues such as brain (Brazelton et al., 2000; Eglitis and Mezey, 1997; Mezey and Chandross, 2000). *In vivo* transplantation experiments revealed that retroviral or genetically marked donor-derived bone marrow stem cells are capable of neuronal differentiation as indicated by double labelling of donor cell and neural-specific marker. Conversely, brain-derived stem cells were able to reconstitute the hematopoietic lineages of the bone marrow following lethal irradiation (Bjornson et al., 1999), suggesting that NSC also have HSC potential. Furthermore, NSCs are capable of myogenic differentiation *in vitro* as indicated by skeletal muscle marker expression (Galli et al., 2000; Rietze et al., 2001). In addition,

neural stem cells have the potential to contribute to all three germ layers following injection into chick and mouse embryos (Clarke et al., 2000).

1.2.3 Bone marrow and other non-hematopoietic tissue

The most poignant illustration of somatic stem cell plasticity was demonstrated by Krause et al. (2001), in which a single HSC was engrafted into a lethally irradiated recipient mouse resulting in complete hematopoietic reconstitution as well as differentiation into mature, non-hematopoietic epithelial cells of the liver, lung, gastrointestinal tract and skin (Krause et al., 2001).

The growing list of tissue types, in which similar examples of hematopoietic stem cell plasticity have been described, include liver, skin, kidney, bone and heart (Bittner et al., 1999; Lagasse et al., 2000; Pereira et al., 1995; Petersen et al., 1999; Poulsom et al., 2001; Toma et al., 2001).

1.3 Evidence for stem cell activity within the heart?

Several recent studies have demonstrated that adult stem cells are able to engraft the myocardium. These experiments illustrate that stem cells arising from exogenous bone marrow transplants contribute to repair and *de novo* generation of damaged cardiac tissue *in vivo* (Bittner et al., 1999; Jackson et al., 2001; Kocher et al., 2001; Orlic et al., 2001a). In addition, Anversa and colleagues have presented immunohistological data, which showed donor chimerism in gender-mismatched transplanted heart. They identified

undifferentiated progenitor cells and differentiated myocardial cells, presumably derived from the recipient, contributing to the grafted heart. Furthermore, they reported an increase in the number of stem cells and early progenitor cells in transplanted versus control hearts, suggesting that these cells participate in cardiac repair (Anversa et al., 2002; Quaini et al., 2002).

In the study by Orlic et al. (2001a), fractionated bone marrow-derived stem cells were injected directly into infracted hearts. Donor-derived cells were reported to make up approximately 50% of the of newly formed cardiomyocytes, vascular endothelial cells and smooth muscle cells in the region of cardiac damage, thus providing evidence that stem cells could participate in cardiac repair *in vivo* (Orlic et al., 2001a).

Similar observations were reported by Jackson and colleagues, although compelling differences were evident (Jackson et al., 2001). In this case, researchers reconstituted lethally irradiated mice with genetically marked bone marrow-derived SP cells (previously shown to be enriched for stem cell activity). Ischemic myocardial injury was induced after bone marrow reconstitution. Sections of heart tissue taken two weeks after injury revealed donor-derived cardiomyocyte and endothelial cells surrounding the damaged region, contributing to repair (Jackson et al., 2001). However, in contrast to Orlic et al. (2001a), this group determined the frequency of donor-derived cardiomyocytes to be only 0.02%. Although the dramatic variation in frequency between these reports is disquieting, the results do suggest the possibility that adult-derived stem cells may influence adaptation of the post-natal myocardium.

1.4 Normal post-natal growth of the heart

The principal mechanism of cardiac post-natal growth is believed to be mediated by cardiomyocyte hypertrophy. This mechanism is characterized by cardiomyocyte enlargement without cell division, increased sarcomeric assembly, induction of fetal genes (such as atrial natriuretic factor, beta-myosin heavy chain and alpha-skeletal actin), and upregulation of immediate early gene products (c-fos, c-jun and Egr-1). Key conduits have been implicated in regulating cardiac hypertrophy, including calcium-dependent signalling and mitogen-activated protein kinase (MAPK) pathways (McKinsey and Olson, 1999; Molkentin, 2000). The underlying molecular mechanisms responsible for this process, however, remain elusive. Several factors have been identified that clearly play an important role in post-natal cardiac hypertrophy such as calcineurin, and the Myocyte Enhancer Factor 2 (MEF2) family of transcription factors (Black and Olson, 1998). In addition to being required for early embryonic development, MEF2 has emerged as a central player in hypertrophic signal transduction. Reduced MEF2-dependent gene expression in the post-natal heart restricts cardiac hypertrophy (Kolodziejczyk et al., 1999).

Although cardiomyocyte hypertrophy is well documented, emerging evidence of myocardial regeneration within the heart suggests a possible role for alternate mechanisms of post-natal cardiac maintenance (Anversa et al., 2002). Indeed, the prospect of a resident stem cell population in the post-natal heart cannot be discarded. Condarelli and colleagues, for example, have demonstrated that myocardial vascular

endothelial cells are capable of transdifferentiating into the cardiac muscle lineage when co-cultured with primary cardiomyocytes (Condorelli et al., 2001).

In a second paper by Orlic et al. (2001b), cytokine-mediated mobilization of stem cells led to regeneration of cardiac tissue and improved myocardial function (Orlic et al., 2001b). The authors suggested that the resulting regeneration was the consequence of mobilization and activation of HSCs to the injured area. However, these results are also consistent with possibility that cytokine treatment may in fact simulate a resident population of stem cell-like cells.

1.5 Experimental rationale

The experimental characterization of the existence of stem cells in several adult tissues, together with preliminary observations of stem cell participation in cardiac repair, has raised the possibility that stem cells play an important role in adult cardiac biology. Here we sought to determine whether the post-natal heart maintains an endogenous population of cells with stem cell-like characteristics. The lack of information available regarding sortable markers for a cardiac stem cell population was highly influential in selecting an effective experimental approach.

1.6 Objective:

1) To determine if a resident population of SP cells exists in the post-natal heart, and whether these cells possess stem cell-like characteristics.

2) To investigate the possibility that cardiac-derived SP cells may become activated when the normal mechanism of post-natal cardiac growth is impaired.

1.7 Hypothesis:

Among the recent reports pertaining to adult stem cells and their biological relevance, few have addressed the possibility that functional stem cells may exist within the adult heart. Based on the identification of adult stem cells in other non-regenerative tissue types, such as the brain, as well as evidence of myocardium regeneration following direct injection or recruitment of circulating bone marrow progenitors (Jackson et al., 2001; Orlic et al., 2001b), it is hypothesized that a distinct population of Hoechst dye-excluding, multipotent cells, similar to that found in other tissues, exists in the adult myocardium. Furthermore, this population may contribute to the growth of the heart under circumstances of attenuated hypertrophic growth.

Chapter 2

2.0 Materials and Methods

2.1 Animal models

All transgenic mouse models utilized in the present studies were previously characterized. Models: B6C3F1/J mice were used as the wildtype strain. MEF2Cdn mice, which exhibit a hypoplastic myocardial phenotype (see results), were created and characterized in our laboratory (Kolodziejczyk et al., 1999). Maintenance of the MEF2Cdn colony was carried out by ongoing breeding and genotyping. Tail DNA was used for genotyping, which was performed using PCR under the following conditions: 94°C, 2 min, followed by 33 cycles of 94°C, 30 sec, 53.5°C, 45 sec, and 72°C, 45 sec, and a final elongation of 10 minutes at 72°C; *Primer A- MEF2C - 5'* acg cgt cga cat ggg gag aaa aaa gat tca gat t *Primer B- Human Growth Hormone - 5'* act cca gct tgg ttc ccg aat aga. The resulting 500 bp band was resolved on a 1% agarose/1 x TAE gel. Z/AP mice were developed and published by Lobe, et al. (1999), and contain a β-galactosidase transgene under the control of a combination CMV-enhancer/ chicken β-actin-promoter that allows for its widespread tissue expression pattern (Lobe et al., 1999).

The Z/AP mouse colony was maintained through successive breeding and genotyping, which was performed as follows: 1 cm tail cuttings were fixed in 0.2% gluteraldehyde (Fisher Scientific) in PBS. Tails were then washed in PBS and stained in Lac Z staining buffer: (2mM $MgCl_2$, 5mM ferricyanide, 5mM ferrocyanide, 1mg/ml X-gal (Roche)) for 1-3 hours at 37°C.

The green fluorescence protein (GFP)-expressing mouse strain, STOCK TgN(GFPU)5Nagy, were obtained through Jackson Laboratories. These mice were used

as strain markers, as they harbour the GFP gene regulated by the chick β-actin promoter and CMV intermediate early enhancer. This transgene combination confers extensive tissue expression (Hadjantonakis et al., 1998).

Myf-5/nlacZ mice, created by knocking-in a LacZ transgene into the myf5 loci, were created and described by Tajbakhsh and Buckingham (1994). These mice were used as a reporter for myogenic differentiation (Tajbakhsh and Buckingham, 1994).

2.2 FACS Analyses

2.2.1 Isolation of cardiac cell suspensions for FACS analyses

Single-cell cardiac suspensions were derived from isolated ventricles dissected from mice, aged 1-2 months. Ventricles were digested in collagenase type B (10mg/ml) (Roche)/ Dispase II (2U/ml) (Roche) for 35 minutes at 37^0C with intermittent trituration. Cell suspensions were filtered (74um Costar Netwell inserts) and centrifuged at 1000 rpm for 8 minutes. Supernatant was aspirated and the cell pellet was washed in cold Dubecco's modified Eagle's medium (DMEM) (Invitrogen) containing 2% fetal bovine serum (Invitrogen), 10mM Hepes (Invitrogen), penicillin/streptomycin (100U/ml/100ug/ml (Gibco)) (DMEM+), re-centrifuged as above, and again aspirated. Finally, cells were re-suspended in pre-warmed DMEM+ media at a concentration of 10^6 cells per ml.

2.2.2 FACS: Hoechst dye 33342 staining and immunolabeling

Following preparation of single-cell cardiac suspensions in DMEM+, Hoechst dye 33342 (5ug/ml) (Sigma) was added and incubated for exactly 90 minutes at 37°C. As applicable, verapamil (50μM) (Sigma) was added to an aliquot of cells containing Hoechst dye 33342 as a control. Following staining, cells were placed on ice to limit further biological activity, pelleted and washed once in cold PBS (137 mM NaCl; 2.7 mM KCl; 10 mM Na_2HPO_4; 2 mM KH_2PO_4) containing 2% fetal bovine serum. For antibody staining, cells were resuspended in cold PBS + 2% fetal bovine serum at a concentration of 10^6 cells per ml and appropriate fluorescent-conjugated monoclonal antibodies were added (1:100). Antibodies used were as follows, Gr-1, Mac-1, B220, CD4, CD5, CD45, CD34, Sca-1, c-kit, Flk-2 and Thy1.1 (PharMingen). As a control for background staining, PE-conjugated mouse monoclonal IgG_{2A} isotype control (1:100) (PharMingen) was used. Once staining was complete, cells were washed in cold PBS + 2% fetal bovine serum, pelleted and resuspended in ~500μl of Hanks Balanced Salt Solution supplemented with 2% fetal bovine serum and 10mM Hepes and kept on ice for FACS analyses.

2.2.3 FACS specifications

Cell sorting was performed using a DakoCytomation MoFlo high-speed cell sorter. Forward- and sidescatter was measured at 488 nm (Spectraphysic Argon laser). The Hoechst dye was excited at 350 nm (I90C UV laser from Coherent). Blue emission was

measured at 424nm (424/44 bandpass filter) and red emission above 675nm (675AGLP long pass filter). A 510DLP dichroic mirror was used to split these two wavelengths.

2.3 Primary cardiomyocyte isolation

Primary cardiomyocytes were isolated from ventricles of 3-9 day old B6C3F1 mice as described previously (Argentin et al., 1994). Briefly, hearts were aseptically removed from wildtype neo-natal pups and ventricles were separated and washed in Joklik's modified Eagle's medium (JM-MEM) (Gibco). Following dissection, media was aspirated and ventricles were minced in a petri dish and washed in JM-MEM with trituration to remove red blood cells and debris. Subsequently, the minced tissue was digested in a 50 ml Falcon tube with 1% collagenase B (Roche) 4 x 15 minutes at 37 °C. After each of the incubations, tissue/cell suspensions were triturated and allowed to settle in a 25 ml pipet. Undigested tissue was dispensed back into the 50 ml Falcon tube, while the supernatant and subsequent washes were collected and pooled in a Falcon tube containing 10 ml of cold fetal bovine serum to stop further digestion. In addition, tissue was rinsed with JM-MEM and triturated in between each of the incubations to further dissociate cells, then as above, the supernatant transferred to the same fetal bovine serum-containing collection tube. Following the last incubation, the remaining tissue was washed twice in 10 ml DMEM containing 10% fetal bovine serum and supernatant was added to fetal bovine serum -containing pools. Pooled cell aliquots were centrifuged, aspirated and cell pellets were re-suspended in DMEM containing 10% fetal bovine serum. Suspensions were filtered through a nylon filter (74μm, Costar Netwell inserts) to

remove undigested tissue. Cells were pre-plated 2 x 20 minutes at 37°C on a plastic Petri dish to remove fibroblasts, after which non-adherent cardiomyocytes were counted using a haemocytometer (VWR Canlab) and plated in DMEM containing 10% fetal bovine serum at a density of 10^5 cells per cm^2, on collagen type-1 coated glass chamber well slides (VWR Canlab). After 20 hours, growth media was removed and replaced with serum free Dulbecco's Modified Eagle Medium Nutrient Mixture F-12 (Invitrogen) media containing 1x insulin/transerrin/selenium (VWR Canlab) and penicillin/streptomycin (100U/ml/100ug/ml) (Gibco) in order to preserve enriched differentiated cardiomyocyte cultures. Cardiomyocytes were maintained at 37°C in a CO_2-enriched (5%), humid atmosphere.

2.4 Primary myoblast isolation

Hind limbs from wildtype mice were harvested and skeletal muscle dissected for primary myoblast isolation, as previously described (Megeney et al., 1996; Sabourin et al., 1999). In brief, tissue was digested in collagenase type B (10mg/ml) (Roche)/ Dispase II (2U/m) (Roche) for 35 minutes at 37^0C. Digested tissue was then passed through a nylon filter (74μm pore size; Costar Netwell inserts) to remove tissue debris. Cells were centrifuged at 1000 g and subsequently plated overnight in enrichment Ham's nutrient mixture F-10 (Gibco) supplemented with 20% fetal bovine serum, penicillin/streptomycin (100 U/100 μg/ml) (Gibco), fungizone (400ng/ml) (Gibco), heparin (5ng/ml) (Sigma) and human hepatocyte growth factor (2.5ng/ml) (Gibco) to enrich for myoblasts. Cells were collected and transferred the following day to collagen type-1 coated plates in complete

Ham's nutrient mixture F-10 (Gibco) as described above, with the exception of the substitution of basic fibroblast growth factor (human recombinant, 2.5ng/ml, Pharma Biotechnology) for human HGF. Myoblasts were maintained in culture at 37°C until ~70% confluent and then differentiated in low serum conditions (DMEM/F-12 (Gibco) containing 5% horse serum).

2.5 Co-culture of cardiac-derived SP cells with primary cells and cell lines

2.5.1 Primary cardiomyocyte co-cultures

Primary cardiomyocytes were isolated from wildtype mice as described above, plated on collagen type-1 coated, 2-well chamber slides at a density of 10 000 cells per cm^2, then cultured for one day. Separately, cardiac-derived SP cells were sorted from 1-2 month old EGFP or Z/AP mice (strain details above) and 10^4 cells were added to the wildtype primary cardiomyocyte cultures. Co-cultures were maintained for 10-14 days and then fixed and stained as described below in section 2.6 with the following modifications: For EGFP co-cultures, cells were stained for connexin 43 at room temperature for 1 hour using a specific mouse monoclonal antibody (1:200) (Chemicon), then with a Cy3-conjugated goat-anti-mouse secondary antibody (1:50) (Chemicon). For Z/AP co-cultures, cells were stained overnight at 4°C with a rabbit anti-β-galactosidase antibody (1:250) (Abcam) and subsequently incubated with Cy-3 conjugated goat anti-rabbit secondary antibody (1:50) (Jackson). For double immunolabelling, cells were incubated for 1 hour with the myosin heavy chain-specific monoclonal antibody, MF20 (1:10) (Developmental Studies Hybridoma Bank) diluted in 3.3% BSA, then with the FITC-

conjugated sheep anti-mouse secondary antibody (1:250) (Stressgen). Cells were then visualized and photographed using a Ziess Axioscope microscope equipped with ultraviolet light.

2.5.2 Primary skeletal muscle co-cultures

Primary skeletal muscle myoblasts were isolated from wildtype pups as described above, plated on collagen-coated 2-well chamber slides. Once cells achieved 70% confluence they were differentiated as described. At this time, 10^4 cardiac-derived SP cells isolated from either EGFP mice or Z/AP mice were added to each of the differentiating skeletal muscle cultures. After seven days under differentiation conditions, cells were fixed and stained as described above with the following modifications: For EGFP-containing cultures, cells were fixed and stained using immunocytochemistry methods in section 2.6. Cell cultures were blocked in 2% horse serum in PBS, followed by a 1 hour incubation with anti-myogenin primary mouse hybridoma antibody (1:5) (Developmental Studies Hybridoma Bank) and 30 minute incubation with Cy3-conjugated goat anti-mouse secondary (1:50) (Chemicon). For cell cultures with Z/AP-derived cells, cells were stained for β-galactosidase activity as described in section 2.8 below.

2.5.3 C2C12 and H9C2 co-cultures

The immortalized mouse skeletal muscle cell line C2C12 (ATCC), and the rat cardiomyocyte cell line H9C2 (ATCC) were each grown in DMEM containing 10% fetal

bovine serum, penicillin/streptomycin (100U/ml/100ug/ml) (Gibco) until 75% confluent, then differentiated in DMEM containing 2% horse serum, and penicillin/streptomycin (100U/ml/100ug/ml) (Gibco). Separately, cardiac-derived SP cells were sorted by FACS analysis as described above from one-month-old Z/AP mice, which harbour a lacZ transgene. These SP cells were plated with these cell line cultures at a density of 10^4 cells per plate at the start of the differentiation time-course and maintained for 5 days at 37°C. Cells were then fixed and stained for β-galactosidase activity (see below).

2.6 Immunofluorescence-cytochemistry: General methodology

Cells were fixed in 4% paraformaldehyde for 10 minutes, washed in PBS and subsequently permeablized with 0.3% Triton-X 100 (Fisher) in PBS for 10 minutes. Cells were then blocked in 3% (w/v) BSA (Roche) in PBS for 30 minutes. Specific primary antibodies and secondary antibodies were diluted and applied as described when relevant. Primary antibodies were incubated for 1h at room temperature or overnight at 4°C, washed in PBS, followed by secondary antibody incubation for 45 minutes at room temperature and then washed in PBS. Nuclei were stained by the addition of DAPI (4,6 Diamidino- 2-phenylindole) (Sigma) diluted in PBS (1:10000). A Zeiss Axioscope microscope equipped with an ultraviolet light source was used for visualization.

2.7 Detection of myogenic differentiation: Immunocytochemistry

Differentiated C2C12s and H9C2s were fixed in 90% methanol in PBS for 6 minutes, and washed in PBS. Subsequently, cells were blocked in 5% skim milk powder/PBS for 30 minutes. The anti-myosin heavy chain antibody, MF20 (mouse monoclonal, Developmental Studies Hybridoma Bank, University of Iowa) was diluted 1:40 in blocking solution and added to cells for 1 hour at room temperature then washed in PBS. Secondary antibody horseradish peroxidase-conjugated goat-anti-mouse IgG (BioRad) was diluted 1:2000 in blocking solution and incubated for 30 minutes then washed with PBS. For antibody detection, cells were incubated in the dark in 3,3'-Diaminobenzidine (D.A.B.) solution [0.03% hydrogen peroxide, 1mg/mg D.A.B. (Sigma), in PBS] for 5-25 minutes. Cells were washed in water then visualized under a Zeiss inverted microscope and photographed.

2.8 LacZ staining: Cell culture

Co-cultured cells were stained for β-galactosidase activity using the substrate X-gal. Cells were washed in PBS and fixed for 10 minutes at 37^0C (2% paraformaldehyde, 2mM $MgCl_2$, 0.2% glutaraldehyde, in PBS), washed 3 times in PBS then stained for 2-4 hours at 37^0C in Lac Z staining buffer described above. Cells were then washed in PBS to stop the staining process.

2.9 Hematopoietic activity assay: Methocellulose culture

SP cells were sorted as described above and 10^4 cells were re-suspended in 2.5 ml of MethoCult media GF 3434 (Stem Cell Technologies) using a 5 ml syringe and an 18-gauge needle. Cells were then plated on 3.5 ml plates and incubated in humidity chambers at 37°C and 5% CO_2 with humidity for 10-14 days for colony formation. Colonies were photographed under phase contrast using a Zeiss inverted microscope, and subsequently drawn up using a sterile pipet, washed and re-suspended in PBS then smeared, air-dried and fixed on glass slides using 4% paraformaldahyde. To reduce background staining, 0.3% hydrogen peroxide in PBS was applied and rinsed with water and then PBS. Cells were then blocked with normal goat serum (1:10000) for 30 min to decrease non-specific antibody labelling and subsequently stained for Ly-6G (Gr-1) (Caltag) and Mac-1 (Caltag) (1:200) for 1 hour at room temperature then washed with PBS. Biotin-conjugated anti-rat IgG (1:200) (Pharmingen) was applied for 45 minutes at room temperature as a secondary antibody. Detection was carried out using the VECTASTAIN ABC detection kit (Vector Laboratories Canada) as per manufacturer's instructions. Cells were then counterstained with Hoechst stain (Sigma) diluted in PBS (1:5000) for nuclei visualization.

2.10 Hematopoietic activity assay: MEF2Cdn vs wildtype SP cell activity

Cardiac-derived SP cells were isolated from both wildtype and MEF2Cdn mice as described above and cultured on MethoCult media GF 3434 (Stem Cell Technologies) for 10-14 days also described above. Colony formation was measured using a Zeiss inverted

microscope. Experiments were performed four times and conducted in parallel with total cardiac-derived SP cells.

CHAPTER 3:

3.0 Results

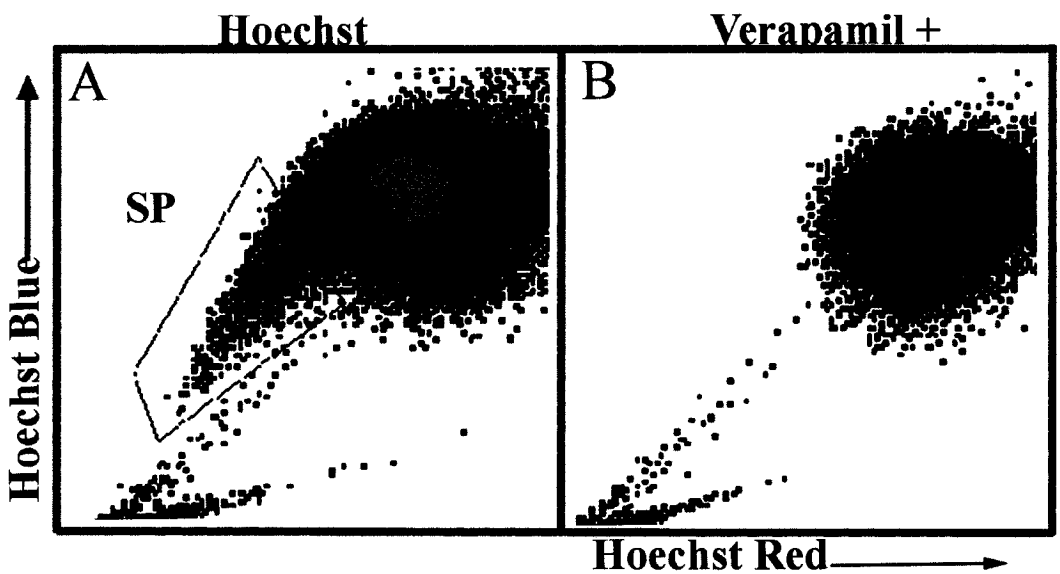
3.1 Identification of a resident SP population in the post-natal myocardium

Stem cells have recently been identified in a variety of unexpected adult tissues, such as brain, skeletal muscle and skin etc. (Gage, 2000; Gussoni et al., 1999; Toma et al., 2001). Based on these findings, we sought to identify a similar population of cells within the post-natal heart that harbour stem cell-like properties. However, lack of suitable stem cell surface markers in non-HSC tissues prompted the use of DNA-specific dyes (Hoechst dye 33342 and Rhodamine 123) with FACS analyses to enrich adult stem cells. First, ventricles from 2-month-old wildtype mice were enzymatically digested with a collagenase/dispase solution to achieve a single cell suspension (Megeney et al., 1996). Subsequently, cells were stained with Hoechst dye or Hoechst dye plus verapamil (an inhibitor of multi-drug resistance-like transporters) then washed (Goodell et al., 1996). FACS analysis was performed using a DakoCytomation MoFlo high-speed flow cytometer equipped with dual lasers. Using this method, a unique population within the heart was identified that stains weakly for Hoechst dye-33342 (figure 1A) and is verapamil-sensitive (figure 1B). Furthermore, this FACS analysis-defined profile shows similarities in appearance to the side populations (SPs) found in other adult tissue sources such as hematopoietic and skeletal muscle tissue (Goodell et al., 1997; Jackson et al., 1999; Seale et al., 2000). Studies on BM report the size of the SP population to range from 0.03% to 0.2% of whole bone marrow (Asakura et al., 2002; Jackson et al., 1999) respectively, while muscle-derived SP cells have been found to range from 1.8% to 2.3% of total muscle cells (Asakura et al., 2002; Seale et al., 2000). Similar to skeletal muscle, myocardial SP cells represented approximately 1.5% of unsorted cardiac cells. This result is the first indication that cells with SP characteristics reside in the adult heart.

These observations beg the question as to what possible role these cells may play in cardiac biology.

Figure 1. Identification of side population in post-natal heart.

FACS analyses of cardiac cell suspensions from 2-month-old wildtype mice stained with Hoechst dye 33342 (A), or Hoechst dye 33342 plus verapamil (B) revealed an ~1.5-2% Hoechst dye-excluding fraction that was sensitive to the drug verapamil.


Hoechst
Verapamil +
A
B
SP
Hoechst Blue
Hoechst Red

3.2 Cardiac-derived SP cells can differentiate into cardiomyocytes *in vitro*

Traditionally, the heart has been classified as a non-regenerative tissue. However, recent evidence has alluded to the possibility that activated stem cells can participate in myocardial repair in situations of injury or disease (Orlic et al., 2001a). Furthermore, reports have shown that bone marrow-derived SP cells can participate in the regeneration of cardiomyocytes within damaged or pathogenic myocardium when introduced into damaged hearts either by direct inter-myocardial injection or via the hematopoietic system following reconstitution of the blood of lethally irradiated mice (Bittner et al., 1999; Jackson et al., 2001; Orlic et al., 2001a). To test if the Hoechst dye-excluding cells residing within the myocardium could contribute to the cardiomyocyte lineage, cardiac-derived SP cells were co-cultured with primary derived cardiomyocytes.

Using 2-month-old EGFP or Z/AP mice as the genetically marked source for cardiac-derived SP cells (isolated as described above), co-culture experiments were set up with primary cardiomyocytes prepared from 6-9 day old wildtype pups. Ventricles from wildtype mice were isolated and enzymatically dissociated in collagenase/JM-DMEM and the supernatants collected and placed in 20% FBS. Upon completion of digestion and subsequent serial pre-plating, purified cardiomyocytes were plated on collagen type-1 coated chamber slides at a density of $1x10^4$ cells per cm^2. Primary cardiomyocyte cultures were maintained for 2 days, at which time 10^4 SP cells derived from either EGFP or Z/AP mouse hearts were added. These co-cultures were maintained for an additional 10 days then fixed with 4% paraformaldehyde, permeablized with 0.3% (w/v) Triton X-100/PBS, and then blocked with 3.3% BSA/ PBS to inhibit non-specific binding of

secondary antibodies. Double-immunofluorescence labelling of the cells was employed to examine the ability of cardiac-derived SP cells (marked by the expression of either GFP or β-galactosidase) to differentiate into cardiomyocytes.

Cardiac-derived SP cells isolated from EGFP mice were identified under UV light using a fluorescein filter. Furthermore, anti-connexin 43 (1:200), a mouse monoclonal antibody that recognizes a cardiac gap junction protein, was used for detection of differentiated cardiomyocytes. Secondary detection was facilitated using Cy3-conjugated goat anti-mouse (1:50). Results from this experiment are shown in figure 2 A-C. Two cardiomyocyte cells identified by connexin 43 labelling are depicted in panel 2A. The arrow indicates a cardiomyocyte cell that originated from the SP fraction as determined by GFP detection (figure 2B). Furthermore a non-GFP-marked wildtype cardiomyocyte was observed (figure 2 A & C). Figure 2C shows these two cells under phase contrast. These observations demonstrate that myocardial SP cells differentiate into cardiomyocytes under *in vitro* culture conditions.

Similarly for SP cells isolated from Z/AP mice, fixed cells were first stained with rabbit anti-β-galactosidase (1:250), then with Cy3-conjugated goat anti-rabbit secondary (1:50) to identify sorted cardiac-derived SP cells. Subsequently, these cells were stained with anti-MF20 (1:10) a second primary mouse antibody targeting muscle-specific myosin heavy chain, and detected with FITC-conjugated sheep anti-mouse secondary (1:250) to mark for cardiomyocyte differentiation. Analyses of these cells revealed cardiomyocytes that were positive for both MF20 and β-galactosidase (arrows) (figure 2 D & E,

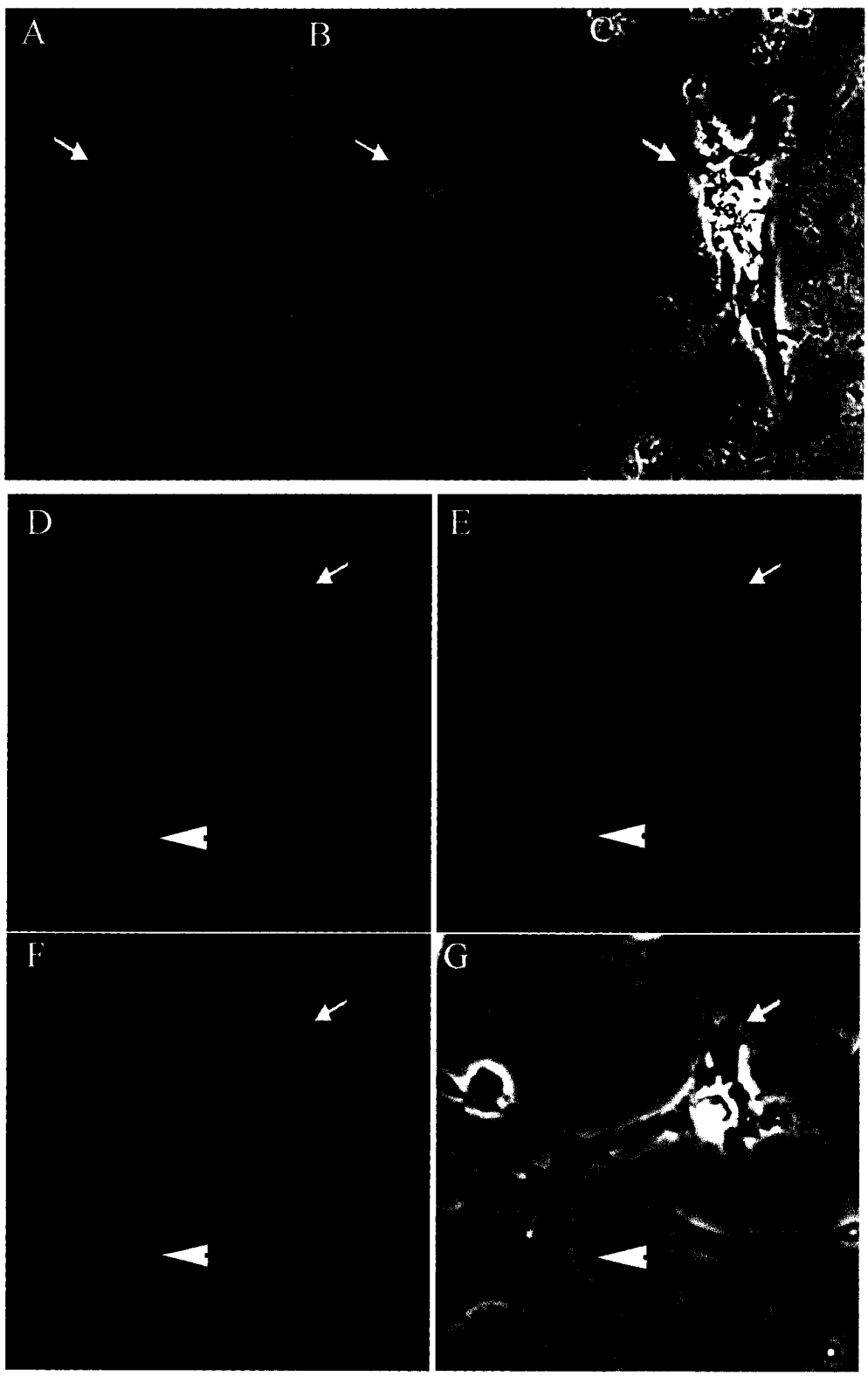
respectively). Also shown are the merged images and the identical field under phase contrast (figure 2F & G, respectively). Arrowheads indicate a non-cardiomyocyte cell type.

Taken together, these results demonstrate the capacity for lineage-specific differentiation by cardiac-derived SP cells, as evidenced by co-localization of genetically marked cardiac-derived SP cells and cardiomyocyte markers. The frequency of differentiation of cardiac-derived SP cells into cardiomyocytes was estimated to be 1 in 1000 cells plated (n=3 as determined by three independent co-culture experiments). However, this number may be a gross underestimation of the actual frequency of SP cell differentiation since it reflects the number of double positive cells per total SP cells plated. The exceptionally low survival rate of these SP cells when cultured alone and their limited ability to adhere to the plate is not taken into consideration. As such, it is likely that the number of surviving cells that attach and converted to the cardiac phenotype would increase with improved culturing methods.

In addition, to verify that the resulting cardiomyocytes are not merely contaminating cell types found within the SP population, freshly sorted cardiac-derived SP cells were dried and fixed onto slides, then labelled with mouse anti-desmin primary antibody (1:200) (Dako), then detected with a Cy3-conjugated goat anti-mouse IgG secondary antibody (1:50) (Chemicon). DAPI staining (1:10000) was used for visualization of nuclei. The frequency of positively identified cardiomyocytes was approximately 1 in $5x10^4$ (data not shown).

Figure 2. Myocardial SP cells differentiate into cardiomyocytes *in vitro.*

Cardiac-derived SP cells isolated from ventricles of either EGFP (A-C) or Z/AP (D-G) mice were co-cultured with wildtype 1° cardiomyocytes for 10 days. Connexin 43 staining designates differentiated cardiomyocytes (A), while GFP positive cells indicate those arising from sorted myocardial SP cells (B). Arrows identify a double-labelled cell. Panel C shows the identical field under phase contrast. In a separate experiment, cardiomyocytes are marked by myosin heavy chain staining (MF20) (D), while β-galactosidase staining (E) indicates cardiac-derived SP cells. The corresponding merged and phase contrast images are shown in panel F & G, respectively. Arrows identify cardiac-derived SP cells expressing the myogenic differentiation marker, myosin heavy chain. Unstained non-cardiomyocyte cell is shown (arrowhead).


A
B
C
D
E
F
G

3.3 Attenuated growth of the heart results in depletion of the SP population

Orlic et al. (2001b) reported that cytokine-mediated mobilization of stem cells resulted in a significant degree of tissue repair in the heart (Orlic et al., 2001b). Although the source of the stem cells was not determined, it was suggested to be bone marrow-derived. However, it is possible that cytokine treatment may have activated a resident population within the myocardium, which ultimately resulted in the observed tissue regeneration and repair. Further investigation into the functional characteristics of these cells was conducted to determine if the cardiac-derived SP cells were able to respond to a growth challenge. Within this context, the MEF2C dominant negative (dn) transgenic mouse was used as a model for restricted cardiac growth. Comparisons between the myocardial SP profiles derived from either the hypotrophic mouse model, MEF2Cdn, or wildtype mice were performed. Our laboratory has previously demonstrated that over-expression of a truncated MEF2C protein (containing the DNA binding and dimerization motifs alone), under the control of the alpha-myosin heavy chain promoter (directing expression in the post-natal heart), competes with and inhibits the transcriptional activity of endogenous members of the MEF2 family. The consequence of over-expression of this transgene is a decrease in cardiomyocyte size leading to a decrease in overall heart size, without additional pathology, such as the infiltration of exogenous cell types or fibrosis. This is an important feature of the MEF2Cdn mouse as its utilization avoids problems of interpretation that may occur with other models of cardiac damage, as seen in instances of ischemia or inflammatory cardiac disease (Kolodziejczyk et al., 1999). Midsaggital sections (6μm) of 4% PFA-fixed hearts from both wildtype and MEF2Cdn mice (2 months old) were counterstained with 0.05% toluidine blue to illustrate the effects of this

transgene (figure 3 A & B, respectively). Comparisons between wildtype and MEF2Cdn heart sections confirm both the failure of cardiomyocytes to increase in size resulting in an increased density of cells per mm^2 (2.3-fold more cardiomyocytes), as well as a lack of overt pathology.

Results of quantitative comparison of FACS profiles between MEF2Cdn and wildtype ventricles showed a significant reduction in the number of SP cells relative to the total number of cells in MEF2Cdn as compared to wildtype hearts (figure 4A vs. 4C). This ~2.5-fold decrease was determined to be significant and reproducible ($P<0.05$) ($n=5$) (figure 4E). Although it was not possible to determine the fate of these cells, one explanation is that in a situation of attenuated growth (i.e. the MEF2Cdn mouse model) the SP cells become activated in response to the myocardial insufficiency and are subsequently depleted.

In addition, examination of the SP profiles of wildtype mice over a 4-month time course revealed a decrease in the size of the SP population with increasing age, thus providing further evidence to support the role for these cardiac-derived SP cells in response to growth requirements and challenges (figure 5). Although the fate of these cells has not been established, it is feasible that they are becoming cadiomyocytes in an attempt to maintain normal cardiac function. Alternatively, technical challenges such as increased difficulty in the preparation of single cell suspensions from older animals due to increasing amount of connective tissues may also account for some of the variation.

Figure 3. MEF2Cdn ventricles show increased density of nuclei.

Comparison between toluidine blue-stained sections from wildtype (A) vs. MEF2Cdn (B) ventricles from 2-month-old mice. Cardiomyocyte nuclei counts are approximately 2.3-fold higher in MEF2Cdn sections, indicative of an increased density of nuclei compared to wildtype. Ten sections of both MEF2Cdn and wildtype were counted per trial (n=3).

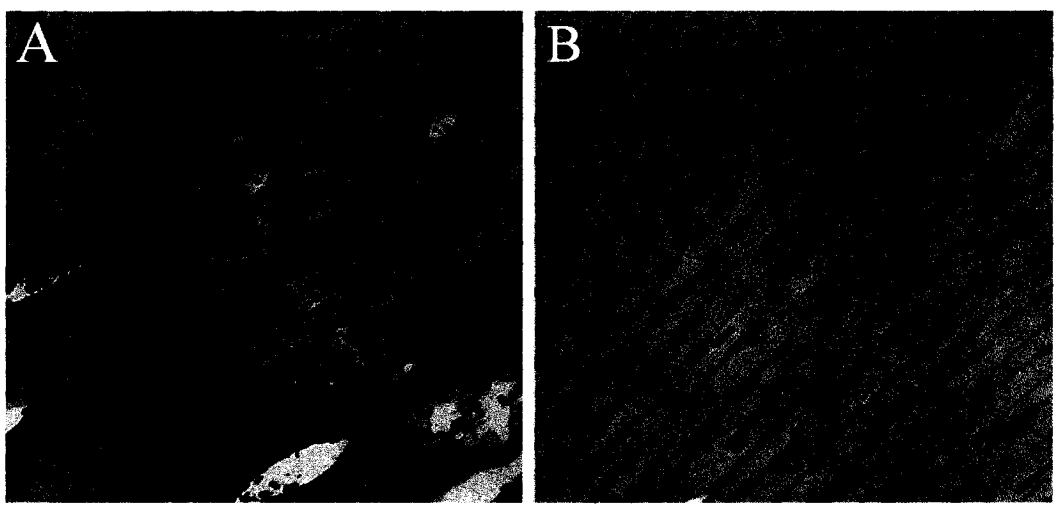

A
B

Figure 4. SP population is depleted in the MEF2Cdn model of cardiac growth attenuation.

FACS analysis of cardiac cells from ventricles of wildtype (A & B) and MEF2Cdn (C & D) mice stained with Hoechst dye 33342 (A & C), or Hoechst dye 33342 plus verapamil (B & D). Comparison of the size of SP fraction in three separate trials shows a >2.5-fold increase in wildtype vs. MEF2Cdn ventricles (E) *($P<0.05$) (n=5).

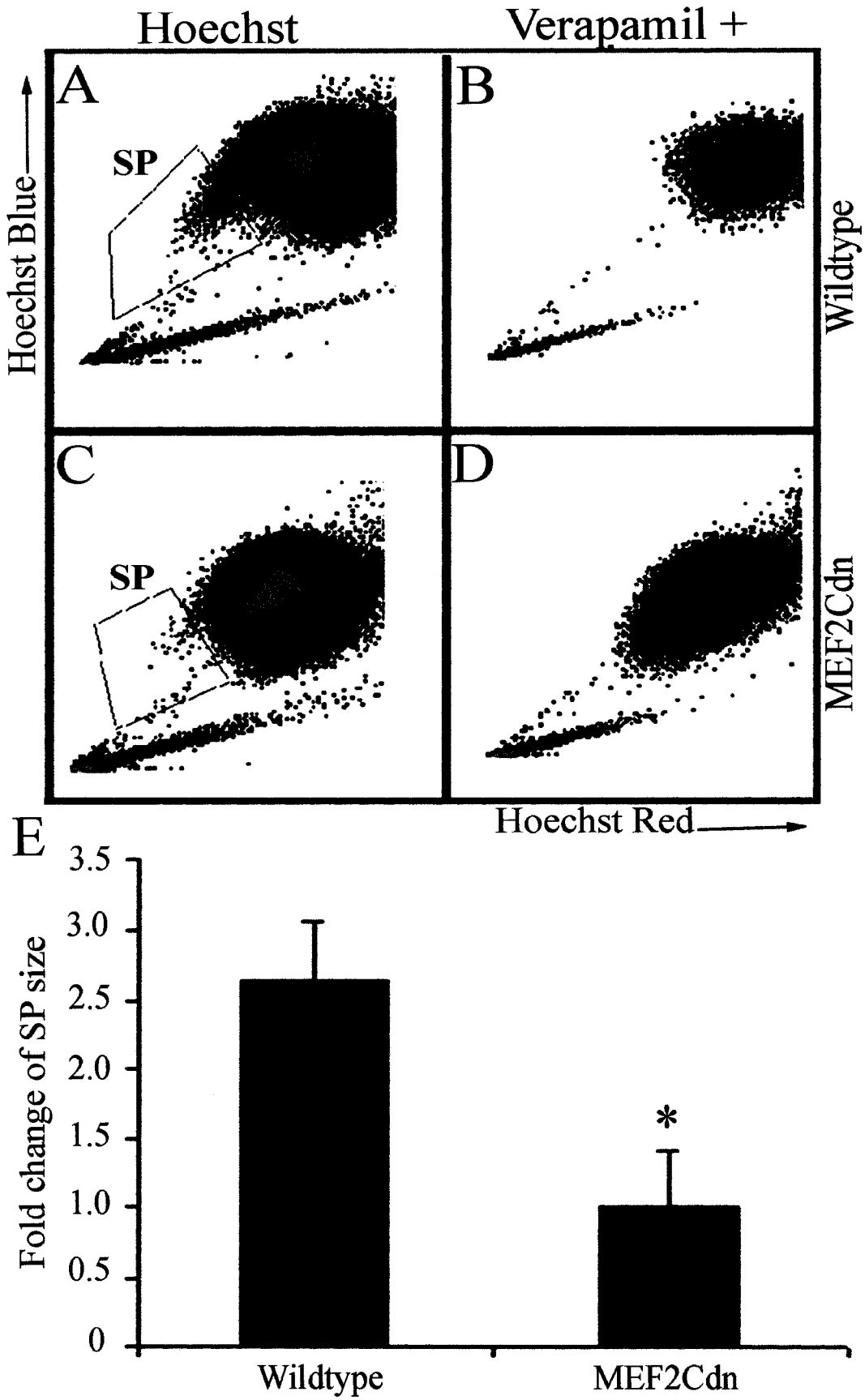
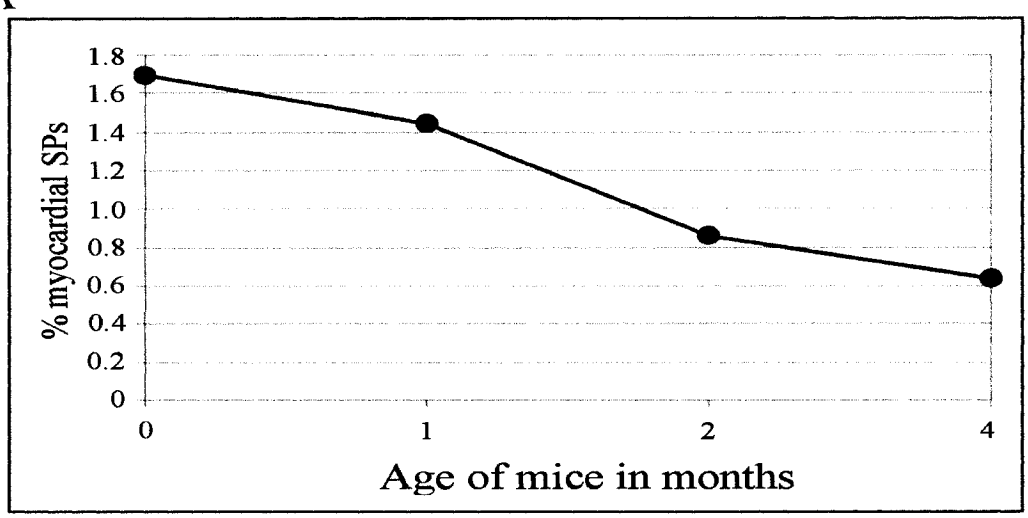

Hoechst
Verapamil +
A
B
C
D
SP
SP
Hoechst Blue
Wildtype
MEF2Cdn
Hoechst Red
E
Fold change of SP size
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0
*
Wildtype
MEF2Cdn

Figure 5. Age related depletion in SP size in wildtype mice

Percentage of SP cells compared to total cells in the normal post-natal heart decreases with progressive aging as determined by FACS analysis (n=2).

A


% myocardial SPs
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0
0
1
2
4
Age of mice in months

3.4 Attenuated growth of the heart leads to activation of SP cells

The reduction in the number of resident SP cells in MEF2Cdn mice inspired further investigation into the stem cell/progenitor-like activity of this population. The hematopoietic colony assay, commonly used to test for stem cell/progenitor-like activity (Seale et al., 2000), was employed to measure differences in activity between cardiac-derived SP cells isolated from either MEF2Cdn or wildtype mice. This semi-solid methocellulose media (MethoCult, Stem Cell Technologies) contains several cytokines and growth factors optimised to promote the proliferation and differentiation of hematopoietic activated stem cells and progenitor cells. (Methylcellulose in Iscove's MDM, Fetal Bovine Serum, Bovine Serum Albumin, Human Recombinant Insulin, Human Transferrin (Iron-saturated), 2-Mercaptoethanol, L-glutamine, rm IL-3, rh IL-6, rm SCF, rh Erythropoietin). The prospective colonies that emerge under these conditions contain mature hematopoietic cell types such as granulocytes, macrophages and natural killer cells.

In this experiment, FACS-sorted SP cells from 2-month-old MEF2Cdn and wildtype mouse ventricles (as described above) were separately cultured in MethoCult media (10^4 SP cells per 3.5 ml petri dish) for 10-14 days. Colony formation was observed as early as 7 days. Examples of typical colony formation are shown in figures 6 A-D. Figures 6 A & C illustrate two separate colonies at 50x magnification, while figures 6 B & D depict both colonies at a 200x magnification.

Analyses of 5 independent culture experiments revealed an average of 3-4 hematopoietic colonies per MEF2Cdn plate (up to a max. of 8 colonies), while the wildtype contained on average less than one colony per plate (figure 7). Although increased colony formation was observed in the MEF2Cdn as compared to wildtype cultures, overall the heart produced drastically fewer colonies in comparison to similar experiments performed with bone marrow or skeletal muscle cell suspensions (with >40 colonies per plate) (Seale et al., 2000).

The colonies that developed consisted of both monocytes and granulocytes as determined by immunolabelling using the cell surface markers, Ly-6G (1:200) and Mac-1 (1:200). Ly-6G, a 21-25 kDa protein also known as the myeloid differentiation antigen Gr-1 (1:200) (figure 6E) and Mac-1 (1:200), a 165 kDa adhesion glycoprotein (Mac-1, integrin αM subunit) (figure 6F), were used to confirm their identity. Gr-1 expression is restricted to granulocyte, and peripheral neutophils as well as being transiently expressed on monocytes lineages. Mac-1, which functions in cell-cell and cell-substrate interactions is expressed by activated lymphocytes, monocytes, granulocytes, and a subset of natural killer cells. Figure 6G shows a negative control in which no primary antibody was added. Nuclei were also stained using DAPI (figure 6H-J).

From this experiment one could interpret that , under circumstances of attenuated growth in the post-natal heart, these resident SP cells become activated as evidence by the increase in colony formation. However, cardiac SP cells derived from the normal adult heart appear to be primarily quiescent.

Figure 6. Hematopoietic colony formation on methocellulose media

Sorted cardiac-derived SP cells were cultured for 14 days on methocellulose media. Examples of colony formation are illustrated in panel A-D. Two distinct colonies are illustrated at 50x magnification (A & C) and similarly at 200x magnification (B & D). Immunohistochemistry was performed on the colonies using hematopoietic markers GR-1 (E), and Mac-1 (F). Negative controls are shown in (G). Nuclei were stained with Hoechst dye (H-J).

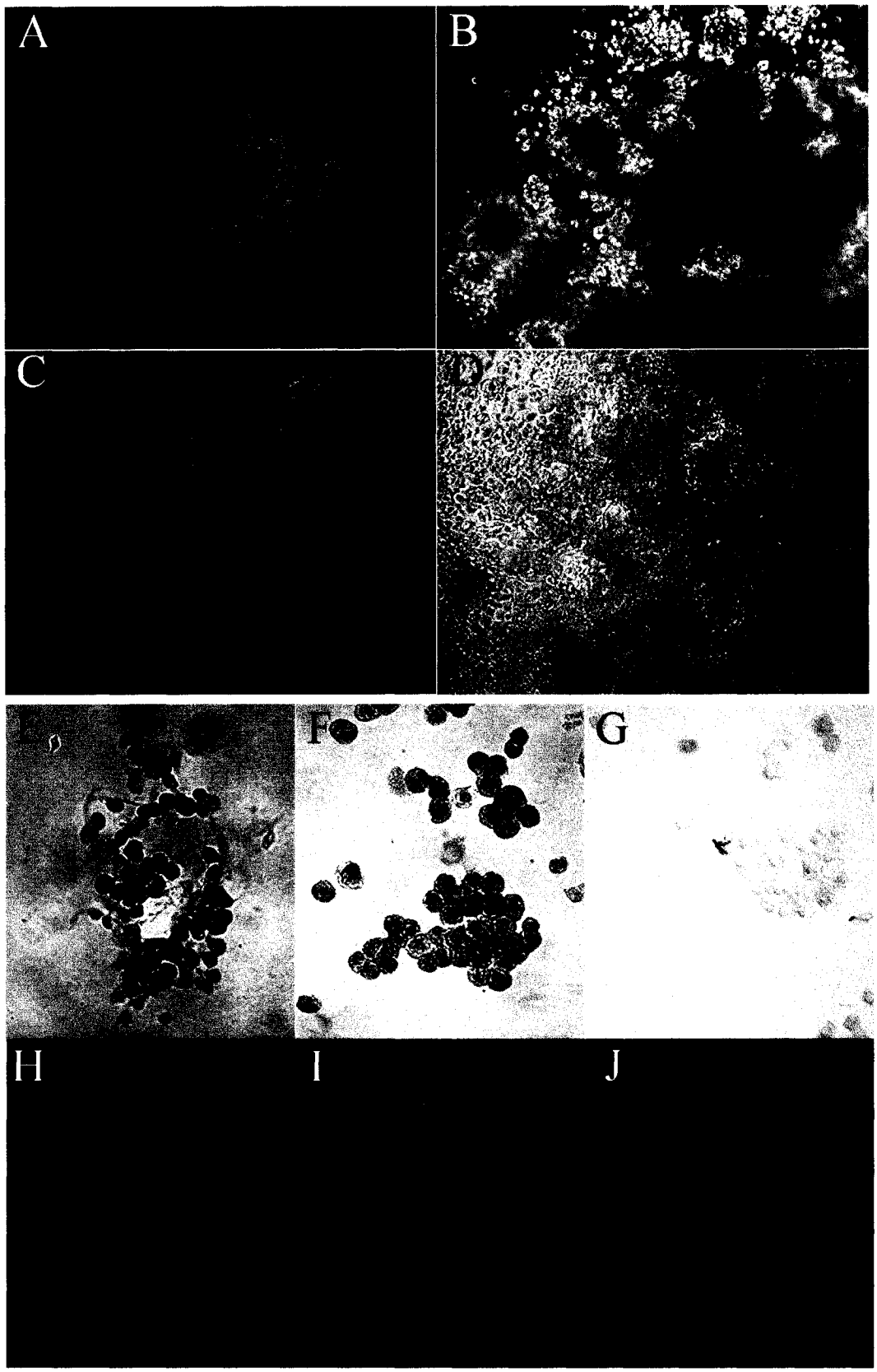
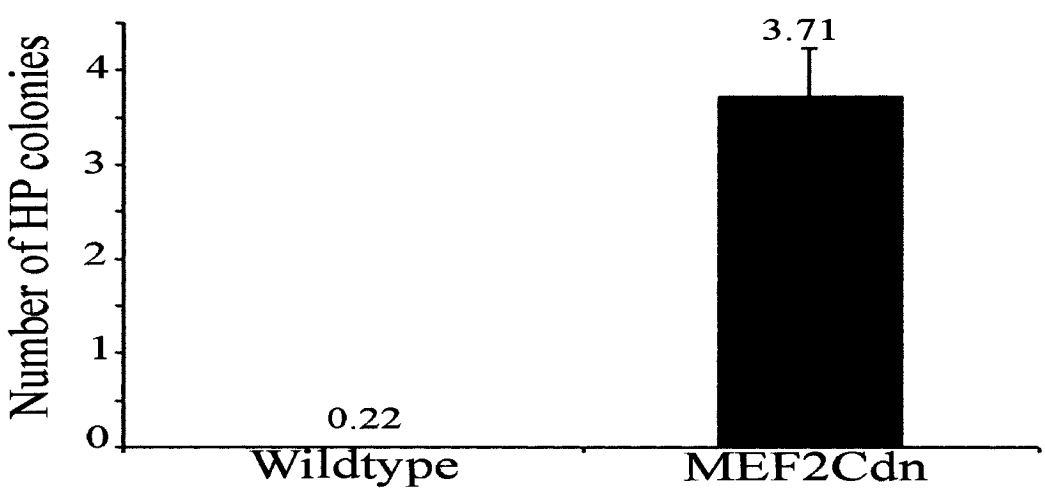

A
B
C
D
E
F
G
H
I
J

Figure 7. Myocardial SP cells activated when heart growth is attenuated.

Comparison of the stem cell-like activity of cardiac derived SP cells between wildtype and MEF2Cdn mice, as determined by the formation of hematopoietic colonies on methylcellulose medium. Myocardial cell suspensions isolated from MEF2Cdn hearts show on average 3.7 colonies per 10,000 cells plated compared to on average less than one colony per plate from wildtype heart cell suspensions.

A



3.5 Examination of hematopoietic cell surface marker expression within the SP population

The potential for cardiac-derived SP cells to form hematopoietic colonies in methocellulose media prompted an examination of the expression profiles for several common hematopoietic cell surface markers using FACS analyses. Extensive research in the hematopoietic field has identified a wide variety of cell surface markers that can be used to define stem cells and their immediate progenitors. These characteristics have facilitated further research and clinical application of hematopoietic stem cells. As mentioned previously, CD34, Sca-1 and c-kit are markers that are readily used to purify HSC from bone marrow (Galli et al., 2000; Krause et al., 1996; Spangrude et al., 1988). In addition to these well-characterized factors, a variety of other markers have been identified that denote functional and phenotypic subpopulations within the bone marrow. However, relevant markers such as those established in the hematopoietic system have yet to be identified within the heart. To this end, using several typical hematopoietic stem cell markers, cardiac-derived SP cells were further characterized for the expression of relevant cell surface markers listed in table 1. Single-cell suspensions of cardiac ventricles isolated from 1-2 month old wildtype mice were stained with Hoechst dye (as previously described) and subsequently labelled with one of several cell surface antibodies conjugated to either phycoerythrin (PE) or fluorescein isothiocyanate (FITC) (table 1). FACS analyses were performed both on the basis of Hoechst dye 33342-exclusion and fluorescence labelling. Both SP and main population (MP) cells were cross-compared. The results in table 1 illustrate the number of positive cells as a percentage of total cells in each population (n=3). As a positive control for antibody

staining, bone marrow-derived SP cells were similarly stained for Hoechst and subjected to HSC antibody labelling (table 2). Standard error between trials is also shown in each table. Representative profiles are shown in appendix A.

Table 1: Characterization of the expression of known HSC markers by cardiac-specific 'side' and 'main' population cells using FACS analyses.

HSC Markers	**SP%**	**Standard Error**	**MP %**	**Standard Error**
CD34	45.08	+/- 14.64	36.7	+/- 12.40
CD45	0.6	+/- 0.07	4.63	+/- 0.37
c-kit	0.39	+/- 0.21	2.19	+/- 0.24
Sca-1	84.1	+/- 1.69	68.46	+/- 1.80
Flk-2	0	+/- 0.00	1.63	+/- 0.23
Thy1.1	0.39	+/- 0.06	0.67	+/- 0.03
Lineage Markers				
Gr-1	0.16	+/- 0.10	1.83	+/- 0.10
Mac-1	0.17	+/- 0.06	2.23	+/- 0.31
CD4	0	+/- 0.00	0.34	+/- 0.08
B220	0.03	+/- 0.02	0.55	+/- 0.13

Table 2: Expression profile of known cell surface markers by bone marrow-derived 'side' and 'main' population cells using FACS analyses.

HSC Markers	**SP%**	**Standard Error**	**MP%**	**Standard Error**
CD34	15.05	+/- 0.75	1.05	+/- 1.05
CD45	42.83	+/- 1.83	78.70	+/- 2.03
c-kit	71.40	+/- 22.90	4.12	+/- 0.62
Flk-2	0.00	+/- 0.00	0.08	+/- 0.01
Thy1.1	0.22	+/- 0.22	0.58	+/- 0.01

Based on these results, an expression profile was devised for cardiac-derived SP cells. Within this population of myocardial SP cells, several HSC markers were expressed. These included CD34 (45%) and Sca-1 (84%), both standard cell surface markers for undifferentiated HSCs and early progenitors. Expressed at a substantially lower level were the stem cell marker, c-kit (0.4%), CD45 (0.6%), a marker for all nucleated hematopoietic cells, and Thy1.1 (0.39%) a proposed regulator of hematopoietic stem cells. In addition, several lineage cell surface markers were tested and found to be expressed at very low or non-detectable levels. For example, Gr-1 (Ly-6) marker for monocytes and granulocytes and Mac-1 marker for monocytes and macrophages showed very low expression at 0.16% and 0.17%, respectively, while CD4 and B220 exhibited undetectable levels within the SP population. The SP contained slightly higher expression levels of the HSC markers Sca-1 and CD34 than the MP fraction. However, for all other typical stem cell markers such as c-kit, CD45, Flk-2 and Thy1.1, the MP had a greater percentage of expressing cells. These observations confirm that standard hematopoietic stem cell markers are individually not suitable for cardiac SP identification. Taken together, these data suggest that cardiac-derived SP cells form a distinct population of heterogeneous cell types. The cardiac-derived SP cells resemble SP populations found within the hematopoietic system and skeletal muscle with respect to certain functional characteristics such as verapamil sensitivity, differentiation capability, and CD34 and Sca-1 expression (Goodell et al., 1997; Seale et al., 2000). Despite these similarities, cardiac-derived SP cells are clearly divergent from other SP populations as suggested by the limited hematopoietic colony formation and lack of expression of markers such as CD45 and c-kit.

3.6 Cardiac-derived SP cells fuse with skeletal muscle primary cells *in vitro*

Evidence supporting the unanticipated plasticity of hematopoietic and non-hematopoietic tissue-derived cells has increased in recent years (Bittner et al., 1999; Ferrari et al., 1998; Orlic et al., 2001a). In particular, SP cells obtained from highly regenerative tissue such as the hematopoietic system and skeletal muscle have been shown to participate in restoration of divergent tissue types following injury or disease. Moreover, similar reports on 'non-regenerative' tissue such as the brain, suggest that these tissues may also contain multipotent capabilities (Galli et al., 2000).

To determine if cardiac-derived SP cells could participate to some degree in the differentiation of non-cardiac cell types, genetically marked cardiac-derived SP cells were co-cultured with primary skeletal muscle cells for 5-7 days under non-growth permissive conditions. Z/AP mice contain a lacZ transgene under the control of a promoter/enhancer combination that allows for widespread tissue expression (Lobe et al., 1999). This transgene provides an easily identifiable marker in the co-culture assays, allowing visual discrimination between wildtype and transgenic cells based on β-galactosidase enzymatic activity. For these co-culture experiments, approximately 10^4 SP cells were FACS isolated from 1-month-old Z/AP ventricles and plated with 70% confluent primary skeletal muscle cell cultures immediately at the start of a low serum induced differentiation time-course. Primary skeletal muscle cells were grown in Ham's F10 media containing 20 % FBS and 2.5 ng/ml bFGF. Differentiation was induced in 70% confluent cultures when media was changed to 5% horse serum in DMEM. Following 7 days of differentiation, cells were fixed (2% paraformaldehyde, 0.2%

glutaraldehyde, 2mM $MgCl_2$ in PBS) and stained for 4 hours at 37^0C (1mg/ml X-Gal, 2mM $MgCl_2$, 5mM ferricyanide, 5mM ferrocyanide, in PBS). Positively stained blue cells were clearly identifiable within the differentiated myotubes of primary skeletal muscle/cardiac-derived SP co-cultures (figure 8A). Results from this experiment indicate that cardiac-derived SP cells are able to effectively fuse with skeletal muscle myoblasts (frequency of blue cells forming myotubes was approximately 10%). In addition, cardiac-derived SP cells from Z/AP mice were co-cultured with a rat cardiomyocyte cell line (H9C2), and differentiated for 5 days in DMEM containing 2% FBS. β-galactosidase positive cells with characteristic cardiomyocyte morphology were clearly observed (figure 8B), suggesting differentiation of SPs into cardiomyocytes. Figure 8C shows control primary skeletal muscle culture grown without the addition of SP cells and stained for β-galactosidase.

Figure 8. Myocardial SP cells are capable of fusion with myoblasts during differentiation.

Co-culture experiments demonstrate cardiac-derived SP cells are capable of fusion with skeletal muscle myoblasts. Cardiac-derived SP cells isolated from Z/AP mice cultured with wildtype 1° skeletal muscle cells (A) and H9C2 cardiomyocytes (B) demonstrate the ability to fuse with muscle cells as determined by β-galactosidase staining. Negative control is shown in panel C, in which no Z/AP cells were added.


A
B
C

3.7 Transdifferentiation of cardiac-SP cells is of limited occurrence *in vitro:*

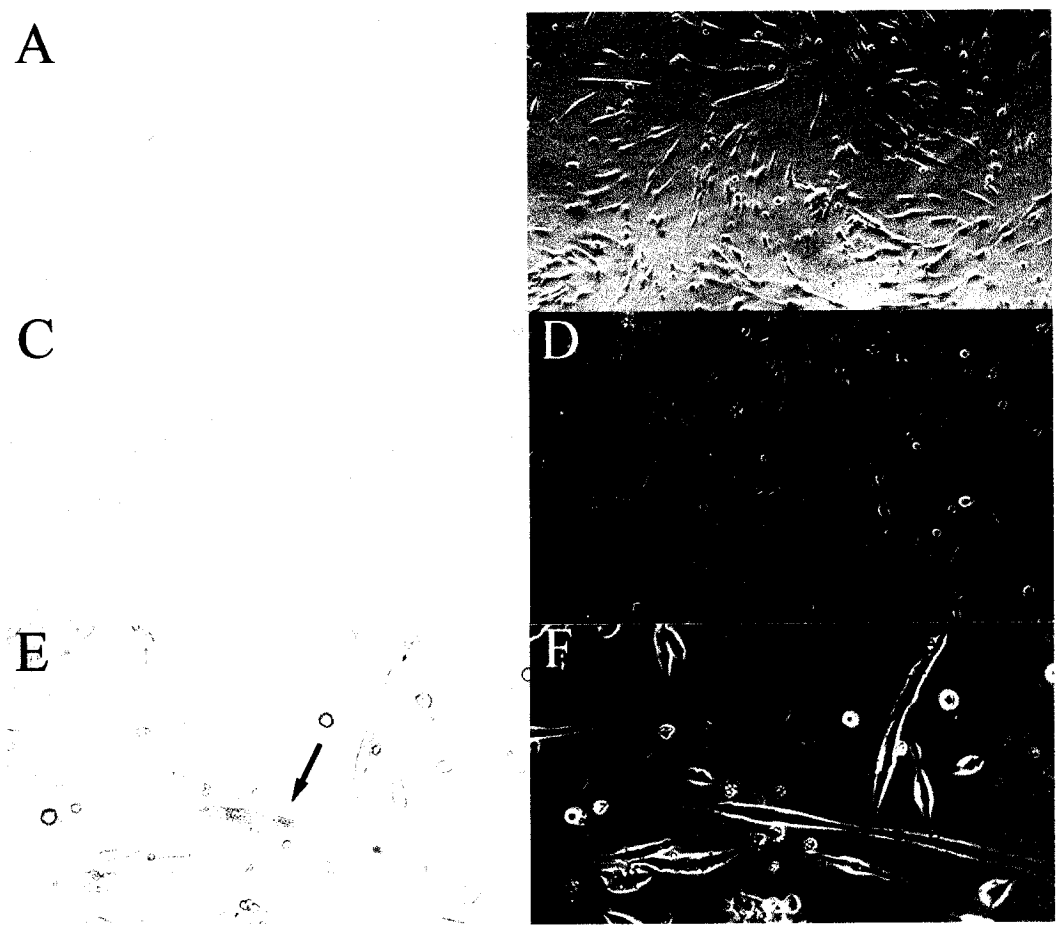
Recent evidence has raised the possibility that cell fusion, and not true genetic reprogramming may account for (some of) the observed transdifferentiation events (Terada et al., 2002; Ying et al., 2002). The term transdifferentiation has been used to denote the process of tissue-specific adult stem cells becoming genetically reprogrammed and differentiating into a specialized cell of another tissue type (Condorelli et al., 2001; Lagasse et al., 2000). This process has also been referred to as plasticity and lineage conversion (Galli et al., 2000; Krause et al., 2001). This new evidence has compelled researchers to differentiate between the discrete processes of fusion and transdifferentiation. The results of the previous co-culture experiments indicated that cardiac-derived SP cells were capable of fusion with skeletal muscle. However, these results were not conclusive evidence for lineage conversion events.

Therefore, to directly address the pluripotency of cardiac-derived SP cells, co-culture assays were performed using skeletal muscle myoblasts and cardiac SP cells derived from a transgenic strain expressing lacZ under the control of a skeletal muscle-specific promoter. Specifically, cardiac-derived SP cells were isolated from myf5/LacZ mice and co-cultured with C2C12 cells and primary skeletal muscle cells as described above. This Myf5/LacZ strain has a lacZ reporter gene targeted to the myf5 locus, thus allowing for β-galactosidase expression when the skeletal muscle specific myf5 gene becomes active during muscle differentiation (Tajbakhsh and Buckingham, 1994). Similar to the cardiomyocyte co-culture experiments, cardiac-derived SP cells were added immediately upon differentiation. Following a 10 day differentiation time course, cells were fixed

with 2% PFA and stained for β-galactosidase. Interestingly, cardiac-derived SP cells were unable to transdifferentiate into skeletal muscle as determined by the absence of β-galactosidase expression under these *in vitro* conditions (bright-field and phase contrast in figure 9A & B, respectively). Similarly, primary skeletal muscle co-cultures were negative for myf5 expressing cells as illustrated in figure 9C & D by the lack of β-galactosidase staining (bright-field and phase contrast, respectively). On very rare occasions (1 out of 10^6 cells), positively stained skeletal muscle cells were observed as shown in figure 9E&F (n=3).

Figure 9. Absence of lineage conversion of myocardial SP cells to skeletal muscle: primary cells

Cardiac-derived SP cells from Myf5/LacZ transgenic mice were co-cultured with C2C12 cells (bright field (A) & phase contrast (B)), and 1° skeletal muscle cells (C-F) (bright field in panels C,E; phase contrast in panels D,F). Results were negative for β-galactosidase expression, with two rare exceptions depicted in panels E & F (arrow shown).

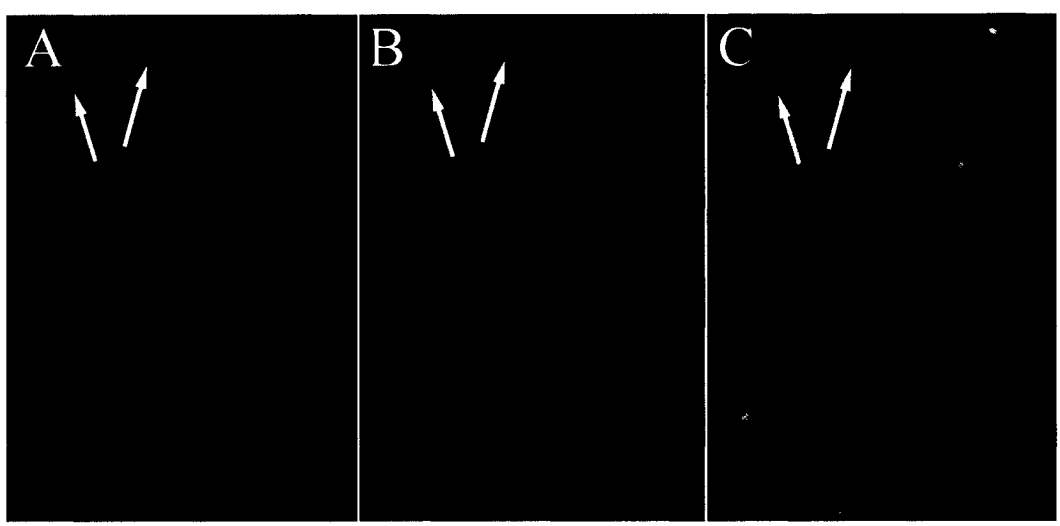

A
C
D
E
F

To verify these results, a similar C2C12 co-culture experiment was conducted with cardiac-derived SP cells isolated from GFP mice (see above). In this experiment, myogenin staining was performed to detect whether or not cardiac-derived SP cells could change fate (myogenin is a skeletal muscle-specific transcription factor). Following a 5 day differentiation time-course, cells were fixed with 4% PFA, stained for myogenin (1:5, Developmental Studies Hybridoma Bank), and subsequently stained with the Cy3-conjugated secondary antibody goat anti-mouse IgG (Chemicon). Results shown in figure 10A illustrate the nuclear-localized myogenin staining within differentiating C2C12 cells. However, GFP positive cardiac-derived SP cells did not stain for myogenin. These observations confirm that cardiac-derived SP cells do not readily transdifferentiate into skeletal muscle cells (figure 10B). DAPI stained nuclei are depicted in figure 10C.

Taken together, the results of these co-culture experiments provide strong evidence that cardiac-derived SP cells are capable of fusion with non-cardiac cell types. However, they are seldom able to transdifferentiate and express skeletal muscle-specific differentiation markers under the *in vitro* conditions used in these experiments.

Figure 10. Absence of lineage conversion of myocardial SP cells to skeletal muscle: C2C12 cells

Verification of skeletal muscle co-cultures were performed with myocardial SP cells from ventricles derived from 2-month-old GFP mice, plated with C2C12s. Panel A shows nuclear-localized myogenin staining, while GFP positive cells are seen in panel B. Nuclei were stained with DAPI (C). No double fluorescent-tagged cells were observed. Arrows show GFP positive cells not seen in panel A.


A
B
C

Chapter 4

4.0 Discussion

4.1 Identification of resident SP cells within the post-natal heart

The results presented here support the hypothesis that a population of stem cell-like SP cells exist within the post-natal myocardium. These cells possess Hoechst dye-excluding properties and verapamil sensitivity, similar to that observed in the hematopoietic system and skeletal muscle tissue (Goodell et al., 1997; Jackson et al., 1999). Further examination of these myocardial SP cells revealed a capacity for cardiomyocyte differentiation. When co-cultured with primary cardiomyocytes *in vitro*, a fraction of the cardiac-derived SP cells showed expression of cardiomyocyte differentiation markers, connexin 43 and myosin heavy chain. In addition, these genetically marked cells were seen to maintain rhythmic contractions, indicative of cardiac function when cultured *ex vivo* (data not shown). Together, these observations demonstrate that myocardial SP cells possess an ability to differentiate into functional cardiomyocytes in permissive environments. However, the identity of the signals that precipitate these events remains unknown.

4.2 Role of signalling during cardiac specification in the adult

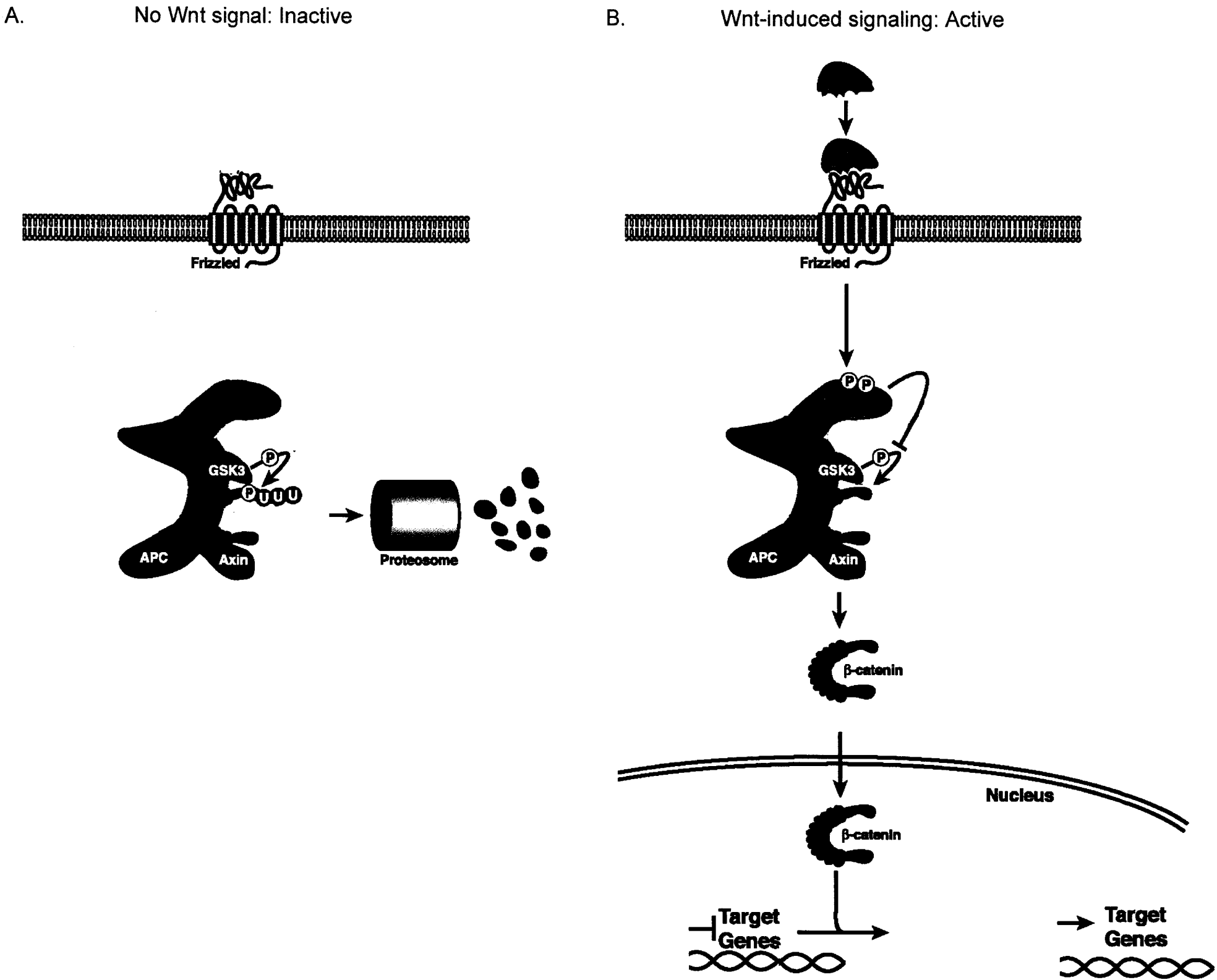
Environmental factors play an essential role in regulating the activities of stem cells. Signals derived from the microenvironment consist of diffusible elements such as growth factors and cytokines, as well direct cell-cell contact and extracellular matrix-mediated communication. Currently, no information is available regarding the cues that regulate cardiac specification within the adult heart. However, insight into this process may be obtained from the current information about cardiac specification during embryogenesis. Consequently, to determine

the signals required for cardiac-derived SP cell specification, it may be necessary to examine signalling pathways that are induced during embryonic cardiogenesis.

Cardiac specification in the developing embryo occurs during gastrulation, through a complex interplay of signals that are now being elucidated. Cardiac progenitor cells, which originate from the anterior lateral plate mesoderm, respond to these signals resulting in the initiation of cardiac differentiation. The Wnt family of secreted growth factors have been identified in embryonic development and implicated during the earliest stages of cardiogenesis. The canonical Wnt/β-catenin pathway has been well established. This pathway functions to regulate β-catenin levels in the cell, ultimately controlling gene expression. In the absence of Wnt signals, a multi-protein "destruction complex" rapidly marks β-catenin for proteosomal degradation (figure 11A). This complex contains cytoplasmic phosphoproteins, including Dishevelled (Dsh), axin, and glycogen synthase kinase-3 (GSK-3). In vertebrates, extracellular Wnt ligands stimulate the transmembrane receptor, Frizzled, resulting in the phosphorylation of Dsh. Activated Dsh inhibits GSK-3-mediated phosphorylation of β-catenin, allowing β-catenin to accumulate in the cell. Free unphosphorylated β-catenin thus enters the nucleus and stimulates the expression of target genes (figure 11B) (Miller et al., 1999; Moon et al., 2002b).

Figure 11. Conical Wnt-signalling pathway.

A simplified representation of the Wnt/β-catenin-mediated signal transduction pathway. In the absence of Wnt, β-catenin is readily ubiquitinated by the deconstruction complex, which marks β-catenin for proteosomal degradation (A). Conversely, extracellular Wnt molecules stimulate the Frizzle receptor, which then activates dishevelled (dsh). Dishevelled, in co-operation with other members of the destruction complex, activates glycogen synthase kinase-3 (GSK-3), releasing β-catenin without marking it for degredation by the proteosome. Subsequently, cellular β-catenin levels increase and β-catenin enters the nucleus, stimulating the expression of target genes.

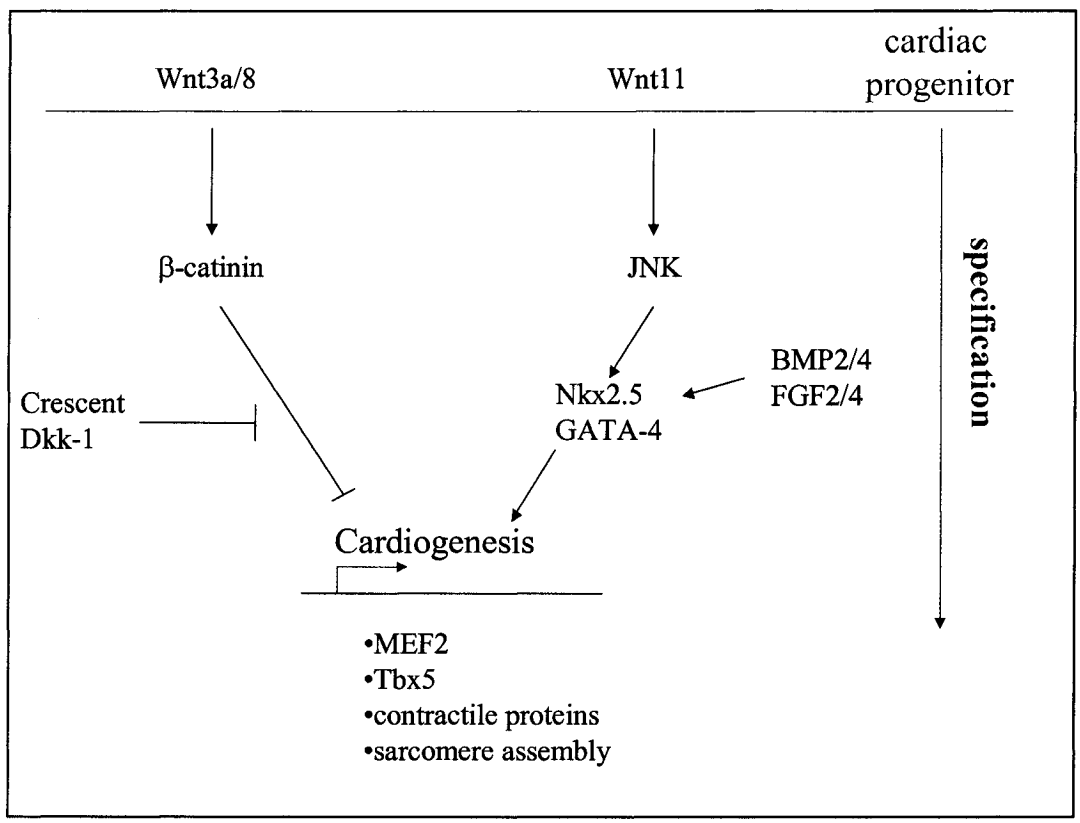

A.
No Wnt signal: Inactive
Frizzled
GSK3
P
P U U U
APC
Axin
Proteosome
B.
Wnt-induced signaling: Active
Frizzled
P P
GSK3
P
APC
Axin
β-catenin
Nucleus
β-catenin
Target Genes
Target Genes

During cardiac development, Wnt-signalling molecules have been demonstrated to act both as inhibitors of cardiac specification through stabilization of β-catenin, (Wnt3a/8), as well as activators of cardiac differentiation through non-canonical pathways involving JNK signalling (Wnt-11) (Marvin et al., 2001; Pandur et al., 2002). The Wnt antagonists, Crescent and Dickkopf-1 (Dkk-1), block Wnt-3a/8 thus inducing expression of cardiac regulatory genes Nkx2.5 and GATA-4, thereby triggering cardiac differentiation (Pandur et al., 2002). Additionally, bone morphogenic proteins (BMPs) 2/4, which are required for the progression of cardiogenesis, have been shown to promote cardiac differentiation by maintaining expression of transcription factors such as Nkx2.5 and GATA-4 (Monzen et al., 1999). A simplified diagram illustrates this Wnt pathway in cardiogenesis (figure 12). It is conceivable that the triggers, which stimulate cardiac specification in the embryo, are again called upon to elicit cardiogenesis in an adult stem cell. Alternatively, the mechanism that induces cardiac differentiation from a precursor cell may be distinct from that seen in the embryo. Condorelli et al. (2001) reported that endothelial cell to cardiomyocyte transdifferentiation was not affected by the administration of either FGF 2/4 or BMP 2/4, suggestive of a divergent pathway for this specialized process (Condorelli et al., 2001).

Interestingly, we demonstrated that co-culturing was necessary for cardiomyocyte differentiation in co-culture experiments (figure 2). Similarly, Condorelli et al. (2001) showed that cell-cell contact is required for cardiac differentiation to occur, although the identity of factors responsible for this fate conversion remains elusive (Condorelli et al., 2001).

Figure 12. Canonical and non-canonical signalling pathways involved in embryonic cardiogenesis.

Wnt-mediated signal transduction pathways have been shown to play a role in early cardiac specification during embryonic development. In vertebrates, Wnt3a and Wnt8 act as inhibitors of cardiogenesis through β-catenin conduits. In contrast, Wnt 11 promotes cardiogenesis through calcium-dependent signalling, thereby activating PKC and JNK pathways. Both these Wnt/β-catenin and Wnt/Ca^{2+} pathways lead to activation of transcription factors that target genes involved in cardiogenesis.


cardiac
progenitor
Wnt3a/8
Wnt11
β-catinin
JNK
specification
BMP2/4
FGF2/4
Crescent
Dkk-1
Nkx2.5
GATA-4
Cardiogenesis
•MEF2
•Tbx5
•contractile proteins
•sarcomere assembly

4.3 Impaired cardiac growth results in activation of the myocardial SP population

Restriction of post-natal growth of the heart (MEF2Cdn mouse model) results in activation of myocardial SP cells, as determined by hematopoietic colony-forming assays. This increase in activation of SP cells is accompanied by a concurrent 2.5-fold depletion of the total size of the population (figure 4). Although the fate of these cells is unknown, it is conceivable that these cells are differentiating into cardiac cells in an attempt to overcome this growth barrier. Additional data in support of this model comes from the observed decline in the number of SP cells occurs with progressive aging, perhaps the result of cumulative demand on the heart over the course of an individual's life (figure 5). Clearly, instructive cues that arise during myocardial adaptation are of primary importance in activation of these SP cells.

4.4 Role of signals in eliciting an adaptive response

Cytokines play a critical role in determining the fate of stem cells. Members of the interleukin (IL)-6 family of cytokines, such as leukemia inhibitory factor (LIF), IL-6, IL-11, and cardiotrophin (CT)-1, have been demonstrated to control complex cellular functions such as mediating intercellular communication as well as regulating survival, proliferation and differentiation of stem cells. These cytokines function through the glycoprotein-130 (gp130)/ Janus kinase (JAK)-signal transducer and activator of transcription (STAT) signal transduction pathway (Heinrich et al., 1998). Briefly, the gp130 family of receptors form homo- or heterodimers and together with the cytokine ligand, create an active signalling complex. This complex then recruits JAK kinases, which mediate phosphorylation and activation of the STAT family transcription factors, resulting in gene activation. Stimulation of this complex pathway has been studied in

several systems including ES cells, HSCs, NSCs and other non-stem cell type cells (Aoyama et al., 2000; Moon et al., 2002a; Raz et al., 1999; Ward et al., 2000). The consequences of this activation are stem cell self-renewal, proliferation and inhibition of differentiation.

Interestingly, CT-1 is a cardiac specific member of the IL-6 family of cytokines. It is tempting to speculate about a potential role for CT-1 in the regulation of SP cells within the heart. For example, CT-1 may act as an inhibitor of cardiac differentiation, analogous to the inhibitory effects of LIF on neural differentiation (Moon et al., 2002a).

4.5 Transdifferentiation: Is this a consistent feature of stem cell populations?

Co-culture experiments between cardiac-derived SP cells and skeletal muscle primary or C2C12 myoblasts revealed that cardiac-derived SP cells can readily fuse with differentiating myotubes, although very rarely did these SP cells transdifferentiate into a skeletal muscle fate (figure 9). As previously mentioned, the transdifferentiation potential of HSCs has been widely reported in recent years (Eglitis and Mezey, 1997; Ferrari et al., 1998; Krause et al., 2001; Theise et al., 2000). Despite these observations, the extent to which transdifferentiation occurs *in vivo* and its physiological relevance remain unclear. The majority of these studies did not provide functional evidence of transdifferentiation, but instead relied solely on immunological and gene expression assays to determine lineage conversion.

Reports of non-hematopoietic tissue types adopting a hematopoietic phenotype, suggest that transdifferentiation is shared by many adult stem cell populations i.e. brain and skeletal muscle (Bjornson et al., 1999; Clarke et al., 2000; Jackson et al., 1999). Our data suggest that these cardiac-derived-SP cells possess limited potential for lineage conversion (figure 9 & 10). For example, the frequency of myocardial SP cells that became genetically reprogrammed when co-cultured with skeletal muscle primary and C2C12 myoblasts was less than 1 in 10^6 cells. The inability of cardiac-derived SP cells to transdifferentiate into skeletal muscle myocytes could be the result of inappropriate or non-permissive conditions. Alternatively, these cells may have lost the inherent plasticity detected in other adult stem cell populations. A more favourable hypothesis comes from new evidence and recent follow-up studies, which challenge the concept of plasticity in non-hematopoietic adult stem cells.

Bjornson et al. (1999) first reported that cultured neurosphere cells, when transplanted back into irradiated mice, were capable of adopting a hematopoietic fate, as determined by *in vitro* clonogenic assays and immunocytochemistry (Bjornson et al., 1999). In an effort to duplicate the experiments, Morshead et al. (2002) were unable to detect NSC-derived hematopoietic cells in any of the tests conducted (Morshead et al., 2002). They did, however, observe anomalous cellular changes under conditions in which cells were cultured for long periods of time. These results suggest that culture-induced abnormalities, acquired by serial passaging, may account for some of the fate-switching phenomenon. Interestingly, Morshead et al. (2002) also hypothesized that these clones may have been atypical. Specifically, they reported a high degree of passage-dependent

transformation, an occurrence not observed in other studies (Bjornson et al., 1999; Shih et al., 2001). In addition, Morshead et al. (2002) reported a cloning efficiency of less than 1%, which is significantly lower than the 7-20% determined by others (Bjornson et al., 1999; Reynolds and Weiss, 1996; Shih et al., 2001). Despite the variability in these studies, the consensus is that hematopoietic transdifferentiation of NSCs is a rare event. One explanation for these observations is that NSCs may represent a heterogeneous population of multipotent stem cells, which contain subpopulations of cells capable of adopting a non-neural fate. Alternatively, a proportion of each distinct subpopulation that exists within a specific neural region may be a genetically distinct cell type.

In a separate study, it was reported that muscle-derived stem cells possessed hematopoietic potential (Jackson et al., 1999). This study revealed that hematopoietic reconstitution of lethally irradiated mice could be achieved with skeletal muscle-derived SP cells. These cells were found to be Sca-1^+/c-kit^+/$CD45^-$ as determined by FACS analysis, suggesting that they were not hematopoietic in origin. However, two recently published manuscripts have provided an alternative model to explain hematopoietic potential in skeletal muscle. Issarachai et al. (2002) examined skeletal muscle-derived stem cells for *in vitro* clonogenic capacity, *in vivo* reconstitution potential and cell surface marker expression. They showed that the hematopoietic potential within muscle tissue was derived solely from the fraction of $CD45^+$ cells (Issarachai et al., 2002). In addition, using cytokine induced stem cell mobilization, they demonstrated an increase in the number of $CD45^+$ genetically marked BM-derived cells in muscle suspensions. The authors concluded that the hematopoietic activity derived from skeletal muscle tissue

originated with BM-derived cells residing within the muscle and was not the product of trandifferentiation events. Furthermore, the authors suggested that skeletal muscle contained a resident population of $CD45^+$ stem cells that originated from the circulation, but were not readily exchanged with those in peripheral blood (Issarachai et al., 2002).

Similar results were observed in a second study from Goodell's lab. The authors reported that hematopoietic potential of skeletal muscle was derived exclusively from a subset of $CD45^+$ cells (McKinney-Freeman et al., 2002). Together, these studies refute the concept that non-hematopoietic $CD45^-$ cells contribute to hematopoietic reconstitution and suggest that the BM is the true source of hematopoietic potential. Furthermore, cells in the $CD45^-$ fraction were found to convert to muscle at a higher frequency than $CD45^+$ cells, suggesting that muscle-derived stem cells have an increased myogenic potential. However, the plasticity of these skeletal muscle stem cells may be reduced. Itinerant BM stem cells may, in effect, provide all hematopoietic potential to the muscle cell population (McKinney-Freeman et al., 2002). Further investigation using more stringent experimental criteria is clearly needed to resolve this debate, such as *in vivo* cell tracking and clonal population analyses.

Confounding the definition of adult stem cell transdifferentiation is the process of spontaneous fusion, in which cells adopt the phenotype of a disparate cell type without genetic reprogramming. Indeed, two separate research groups have provided evidence that spontaneous fusion may be responsible for at least some of the reports of transdifferentiation (Terada et al., 2002; Ying et al., 2002). In one study, Ying et al. (2002) isolated neural stem cells from transgenic mice that possess both GFP and a

puromycin resistance gene under the control of the embryonic stem (ES) cell-specific gene, Oct-4. Using these cells, co-cultures experiments were performed with genetically engineered ES cells, which contained the gene encoding hygromycin resistance. Antibiotic selection for puromycin produced GFP expressing colonies, indicative of neural-derived stem cells converting to an ES cell fate. However, these cell lines contained twice the normal DNA content suggesting that cell fusion had occurred (Ying et al., 2002). Terada et al. (2002) observed similar results with ES cells containing double the normal DNA content, again indicative of a cell fusion event (Terada et al., 2002). These reports, taken together with the results presented in this study, suggest that transdifferentiation may not be a true characteristic of adult stem cell populations.

4.6 Origin of SPs: Common source or distinct population?

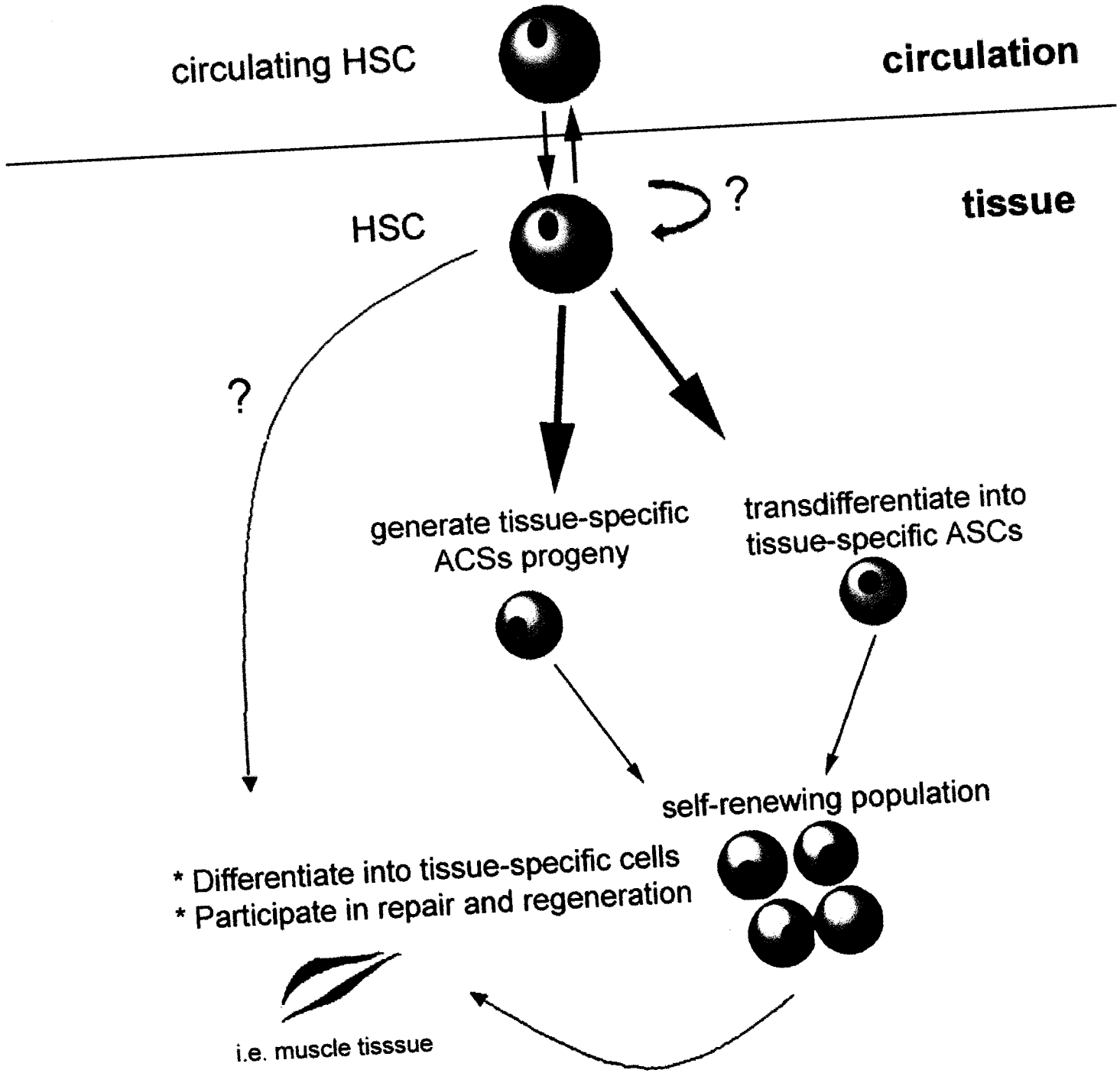
As a means of defining the cardiac SP phenotype, a survey of cell surface hematopoietic antigens was conducted using FACS analyses. Although similarities existed between this SP population and SP cells derived from non-cardiac tissue (i.e. skeletal muscle and the hematopoietic system), notable differences were detected. For example, the hematopoietic stem cell marker, CD45, was not highly expressed in cardiac-derived SP cells. In addition, the HSC markers Sca-1 and CD34 were detected within the cardiac-derived SP fraction, although the proportion of positive cells was only marginally higher than in the MP fraction. Despite the reported similarities of adult stem cell populations, these observations suggest that cardiac SP cells may represent a unique lineage.

The existence of a distinctive subpopulation of stem cells within each tissue/organ system has been proposed. This hypothesis is supported by data illustrating the high degree of variation in marker expression between stem cell populations in disparate tissue types. For example, all HSCs are $CD45^+$. In contrast, skeletal muscle-derived stem cells have been shown to consist of a heterogeneous population of $CD45^+$ and $CD45^-$ cells (McKinney-Freeman et al., 2002). Indeed, it was determined that the hematopoietic reconstitution potential in skeletal muscle is restricted to the $CD45^+$ population alone, whereas $CD45^-$ cells have a significantly higher myogenic potential (Kawada and Ogawa, 2001; McKinney-Freeman et al., 2002). This supposition is also supported by the work presented here, i.e. that the myocardial cell suspensions gave rise to very low numbers of hematopoietic colonies in comparison to colonies generated from bone marrow and skeletal muscle. The lack of $CD45^+$ cells within the cardiac population, in addition to the reduced hematopoietic stem cell activity in colony forming assays, suggests that the adult myocardium harbours a unique stem cell population.

Alternatively, all stem cells may originate from a common source and take on a degree of specialization determined by the host tissue. Indeed, it is feasible that one common stem cell pool exists, from which stem cells are recruited during circumstances of tissue regeneration and repair. Vascular-derived endothelial progenitor cells and circulating HSCs are probable sources for such a common stem cell pool. This is an appealing concept when one considers that all vertebrate tissue systems are inter-woven with a vascular network (Condorelli et al., 2001). Reports on the recruitment of bone marrow-derived cells to both skeletal muscle and cardiac muscle tissue indicates that bone

marrow cells can translocate to vascularized tissues, and imply that HSCs are able to contribute to host tissue repair (Bittner et al., 1999; Gussoni et al., 1999; Orlic et al., 2001a). As described above, critical examination of distinct populations of stem cells within skeletal muscle suggests that a fraction of the heterogeneous population of stem cells within skeletal muscle are HSCs in origin (Kawada and Ogawa, 2001; McKinney-Freeman et al., 2002). It is possible that a hierarchical relationship exists between HSCs and other adult stem cell populations i.e. multipotent HSCs generate non-hematopoietic adult stem cells within the local environment of any tissue. Non-hematopoietic stem cells/early progenitor cells, on the other hand, may be able to self-renew and generate appropriate tissue-specific cell types in response to local signals, however the inherent plasticity of these cells is likely lost. Figure 14 illustrates a proposed hypothetical model. A recent report in skeletal muscle lends support to this idea (LaBarge and Blau, 2002). The authors clearly demonstrate that BM-derived cells are able to change from a stem cells to a skeletal muscle satellite cell and subsequently form muscle fibers in response to injury-induced signals. As suggested in the model presented above, these data illustrate a biological role for BM-derived cells in non-hematopoietic tissues, and suggest a step-wise progression towards a differentiated cellular state.

Figure 13. Hypothetical model of the hierarchical nature of adult stem cells (ASCs). Hematopoietic stem cells or comprised of a heterogeneous population multipotent and self-renewing cells. Circulating HSCs can home to various tissues in response to appropriate signals. These plastic HSCs may be able to either 1) remain quiescent, 2) directly differentiate into host tissue-specific cell types, 3) generate tissue-specific ASCs by asymmetric division, or 4) transdifferentiate into tissue-specific ASCs. ASCs, in turn, are able to maintain themselves within the tissue and differentiate into specialized cell types of the host tissue, however, they no longer possess multipotent potential.


circulating HSC
circulation
tissue
HSC
?
?
generate tissue-specific
ACSs progeny
transdifferentiate into
tissue-specific ASCs
self-renewing population
* Differentiate into tissue-specific cells
* Participate in repair and regeneration
i.e. muscle tisssue

4.7 Role of stem cells in the heart

The potential physiological role of stem cells in the post-natal myocardium remains elusive. Furthermore, elucidating the signals that induce these cells to respond to circumstances of tissue damage may provide novel therapeutic strategies to treat cardiomyopathies.

In defining the physiologic impact of cardiac SP cells, it will be necessary to identify cell surface markers which can be used to specifically isolate or track stem cells within this heterogeneous population. In addition, ongoing characterization of cardiac stem cells would benefit greatly from the development of methods for maintaining and expanding these cells in culture. Although *in vitro* manipulation of these cardiac-derived SP cells will be informative, it is exceedingly difficult to formulate a characteristic profile of stem cells or immediate progenitors when removed from their biological niche. As previously mentioned, the behaviour of stem cells and early progenitor cells is dependent upon the surrounding environment, and thus the mere process of isolation may alter the fate of these cells. Therefore, it follows that *in vivo* techniques are needed to better investigate the nature of these cells.

Despite the uncertainty of the physiological function of cardiac-derived SP cells in post-natal heart, a role for these cells can be postulated. During normal post-natal cardiac maintenance, it is possible that these cells participate in continuous, low-level repair of myocardial damage, which may arise over the lifetime of the organ. Alternatively, the limited regeneration observed in the adult heart (0.0005% of cardiomyocytes are actively

synthesizing DNA during normal murine post-natal cardiac growth) (Soonpaa and Field, 1997) and a lack of effective repair mechanisms, suggests that these cells may represent an ancestral cell type that maintains little potential for physiological contribution. Further investigations of the changes in myocardial SP cells in various models of cardiac damage, such as induced ischemia and in mdx:MyoD$^{-/-}$ mice, may reveal important clues about the injury-induced activation potential of this population. Bone marrow reconstitution experiments using cardiac-derived SP cells may provide valuable insight into the HSC-like characteristics of this population.

This study is the first to report a resident population of SP cells within the post-natal myocardium. These results offer exciting potential for therapeutic application in cardiac disease. Further research on these cardiac stem cells will be necessary to answer outstanding questions regarding mechanisms of action.

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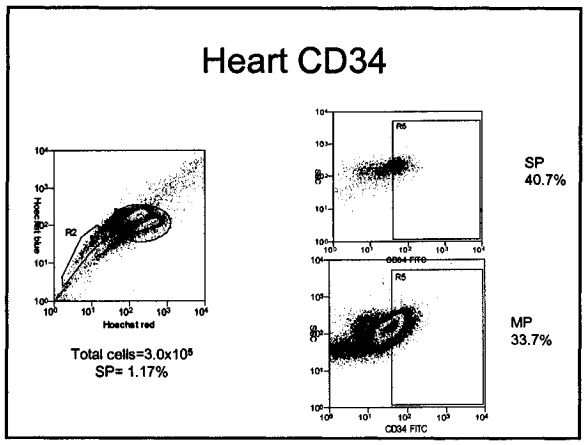
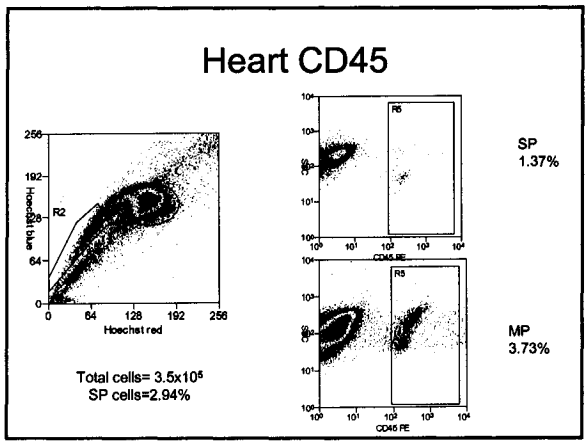
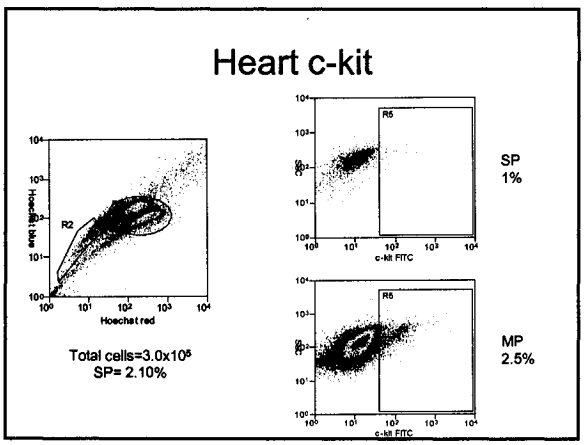
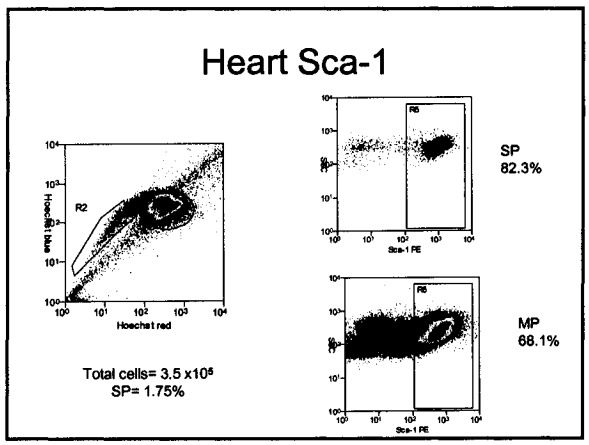
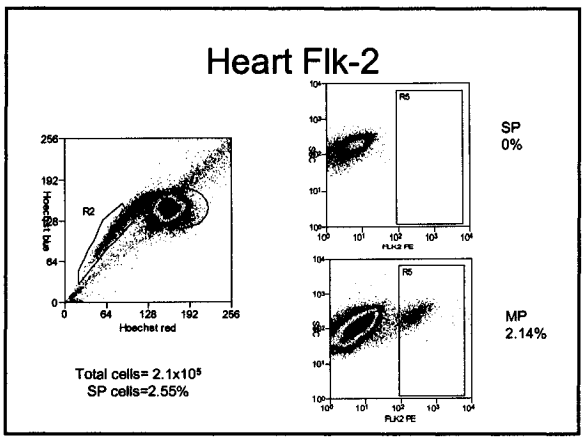
Appendix: Representative FACS analysis profiles from 2-month-old mouse hearts

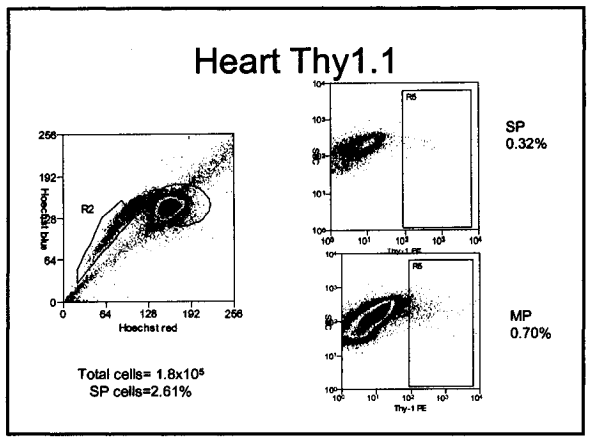




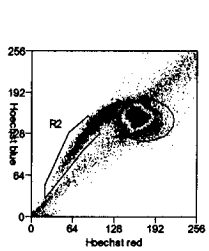


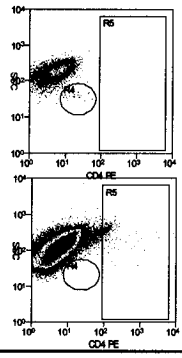






Heart CD4



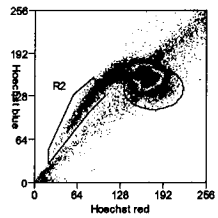


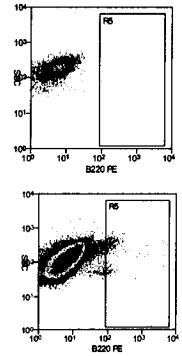
SP
0%

MP
0.49%

Total cells= 2.2×10^5
SP cells=2.01%

Heart B220



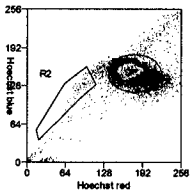


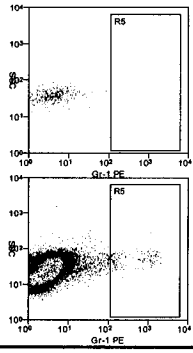
SP
0%

MP
0.78%

Total cells= 2.1×10^5
SP cells=1.78%

Heart Gr-1



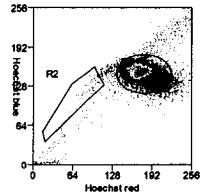


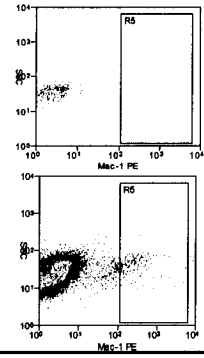
SP
0%

MP
0.65%

Cell count: 2×10^5
SP= 0.2%

Heart Mac-1





SP
0%

MP
1.19%

Cell count: 1.5×10^5
SP= 0.28%