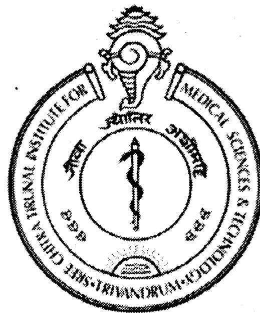
REGULATORY SIGNALS FOR EXPANSION OF HUMAN ADULT CARDIAC STEM CELLS

KG. Aghila Rani

Ph.D. Thesis March 2009



SREE CHITRA TIRUNAL INSTITUTE
FOR
MEDICAL SCIENCES AND TECHNOLOGY
THIRUVANANTHAPURAM - 695 011

REGULATORY SIGNALS FOR EXPANSION OF HUMAN ADULT CARDIAC STEM CELLS

A thesis presented

by

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Thiruvananthapuram 695 011, India

in partial fulfillment of the requirements for the degree of

Doctor of Philosophy

of

SREE CHITRA TIRUNAL INSTITUTE
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MEDICAL SCIENCES AND TECHNOLOGY
THIRUVANANTHAPURAM, INDIA

March 2009

CERTIFICATE

I, KG. **Aghila Rani**, hereby certify that I had personally carried out the work depicted in the thesis entitled ***"Regulatory signals for expansion of human adult cardiac stem cells"***, except where external help sought and acknowledged.

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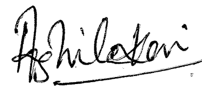

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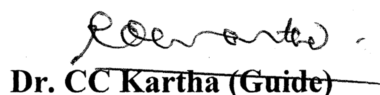

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The thesis entitled

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for

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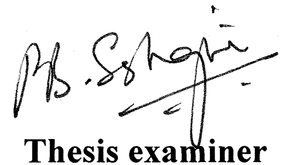

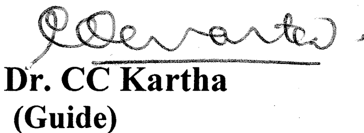

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ABBREVIATIONS

ASCs	Adult stem cells
bFGF	basic fibroblast growth factor
BMDSCs	Bone marrow derived stem cells
BSA	Bovine serum albumin
CABG	Coronary artery bypass grafting
CAD	Coronary artery disease
CCM	Cellular cardiomyoplasty
CDCs	Cardiosphere derived cells
CFUs	Colony forming units
CNS	Central nervous system
CSs	Cardiospheres
CSCs	Cardiac stem cells
CTT	Cell transplantation therapy
CVRF	Cardiovascular risk factor
DAB	3, 3' - diamino benzidine
DEPC	Diethyl pyrocarbonate
DMEM-Ham F12 mix	Dulbecco's Modified Eagle's medium-Ham F12
DMSO	Dimethyl sulphoxide
EDTA	Ethylene diamine tetra acetic acid
EGF	Epidermal growth factor
EPCs	Endothelial progenitor cells
ERK ½	Extracellular signal regulated kinase ½
EGCs	Embryonic germ cells
ESCs	Embryonic stem cells
FBS	Fetal bovine serum
FITC	Fluorescein isothiocyanate
FACS	Fluorescence activated cell sorting analysis

G-CSF	Granulocyte colony stimulating factor
GIST	Gastrointestinal stromal tumor tissue
HGF	Hepatocyte growth factor
HRP	Horse radish peroxidase
HSCs	Hematopoietic stem cells
ICM	Inner cell mass
IGF-1	Insulin-like growth fator-1
IL	Interleukin
IMDM	Iscove's modified dulbecco's medium
MACS	Magnetic activated cell sorting
MAPCs	Multipotent adult progenitor cells
MAPK	Mitogen activated protein kinase
MI	Myocardial infarction
MR	Mitral regurgitation
MSCs	Mesenchymal stem cells
NSCs	Neural stem cells
NYHA	New york heart association
PBS	Phosphate buffered saline
PCI	Percutaneous coronary intervention
PDGF	Platelet derived growth factor
PE	Phycoerythrin
RWMA	Right wall motion abnormality
SCs	Stem cells
TCA	Trichloroacetic acid
TCSCs	Tissue committed stem cells
TGF-β	Transforming growth factor-β
TVRS	Total vascular risk factor score
VEGF	Vascular endothelial growth factor

SYNOPSIS

INTRODUCTION

Regenerative therapy for heart diseases is a rapidly growing domain and involves application of multiple enabling technologies. Several strategies are currently being evaluated for regeneration of the damaged myocardium in heart failure (Hughes 2002). A major breakthrough in the field of cardiac regeneration is the discovery by Piero Anversa and colleagues, of a resident pool of stem cells in post-natal hearts (Anversa *et al.* 2004). This pool of cardiac stem cells can divide, grow in size and acquire structural and functional characteristics similar to myocardial cells (Urbanek *et al.* 2005a). Transplantation studies using cells isolated from cardiac tissues in animals have been successful, raising hopes of isolating resident cardiac stem cells from human heart tissue and using them for therapy in patients with heart disease (Messina *et al.* 2004; Smith *et al.* 2007). The availability of adult cardiac stem cells for autologous therapies in patients offers distinct advantage over embryonic and bone marrow derived stem cells. When compared to other cell types, adult cardiac stem cells are believed to be faster in achieving structural and functional characteristics favorable for regenerating the damaged myocardium (Sussman and Anversa 2004). Stem cells from adult heart thus provide an excellent source that can be used for tissue regeneration and neovascularisation in the infarcted heart. Considering the clinical plausibility in treating patients with end-stage cardiac disorders, the present *in vitro* study was aimed at identifying an easy and cost effective method for the isolation and expansion of human adult resident cardiac stem cells from right atrial biopsy samples as well as analyzing the role of disease severity and coronary artery disease risk factors in determining the yield of stem cells isolated from

myocardial tissue was subjected to mild enzymatic digestion after which the tissue fragments were cultured (Messina *et al.* 2004). Whereas in the second case, myocardial tissue after mincing were directly seeded onto culture dishes (Urbanek *et al.* 2005b). Phase-bright cells migrated from adherent explants were then expanded in a growth factor supplemented medium to form cardiospheres. The cells were verified as stem cells by their positive staining for ckit, MDR1 and CD34 markers by MACS and FACS analysis.

The clinical parameters of the patients were analyzed for their role in determining the yield of ckit positive cells migrated from the explants. Prevalence of cardiovascular risk factors such as age, hypertension, smoking, diabetes and dyslipidemia among the patients were noted. The total vascular risk score was calculated for each patient by considering the risk factors- hypertension, diabetes, smoking, and dyslipidemia. Patients were grouped depending on the presence or absence of the individual risk factors as well as according to severity of coronary artery occlusion, NYHA functional class, mitral regurgitation grades, wall motion abnormalities, total vessel blockage, involvement of right coronary artery and intake of drugs.

The cardiosphere formation was analyzed under different growth conditions in order to select an appropriate growth factor that favors maximum sphere formation. The growth factors employed include epidermal growth factor (EGF), basic fibroblast growth factor (bFGF), vascular endothelial growth factor (VEGF) and insulin-like growth factor (IGF).

Cardiospheres were plated on fibronectin-coated culture dishes in 10% FCS containing medium and expanded as monolayers to form cardiosphere derived cells (CDCs). The effect of different growth conditions in CDC proliferation was also analyzed. Various growth factors employed for the assay include EGF, bFGF, VEGF, IGF-1, TGFβ, HGF and PDGF. Non radioactive cell proliferation assay was performed using WST cell proliferation kit.

The effect of EGF on the migratory activity of cardiosphere derived cells was analyzed by trans-well migration and wound healing assays. 2% FCS containing media without any additives was used as control and the test medium was supplemented with 10ng/ml EGF.

Direct effect of pathway specific pharmacological inhibitors –such as those specific for p42/44 MAPK [PD098059, 10μM], p38 MAPK [SB203580, 10μM], and ERK1/2 [Wortmannin, 100nM] pathways on EGF induced CDC proliferation was studied. CDCs were pre-treated with inhibitors for one hour and then supplemented with growth medium containing 10ng/mL EGF. Cell proliferation in the presence of inhibitors was assayed by WST rapid kit.

STATISTICAL ANALYSIS

The values are expressed as mean ± SD. Sample means were compared using Student's t-test and group means by one-way ANOVA where necessary. A level of $p<0.05$ was considered statistically significant.

RESULTS

Isolation, characterization and expansion of adult resident cardiac stem cells

Phase-bright cell migration was obtained with both the enzymatic and non-enzymatic isolation methods. The isolated cells were characterized as stem cells by their morphology and expression of markers - ckit, MDR1 and CD34. The ckit-positive cells isolated by both methods were able to form clusters termed cardiospheres when grown in a growth factor supplemented medium. The cardisphere cells stained positive for stem cell (ckit, MDR1 & CD34), endothelial (vWF) and cardiac differentiation markers (cTNI & MHC).

Comparison between the enzymatic and non enzymatic isolation methods

The non-enzymatic method was found easy and cost-effective for the isolation and expansion of adult cardiac stem cells. The cell yield and cardiosphere count obtained with both the methods were analogous.

Effect of disease severity and CAD risk factors on the number of migrating cardiac stem cells

The cardiovascular risk factors and the other clinical parameters did not seem to affect the number of ckit positive cells migrating from explanted atrial tissue samples. The number of migrating cells was also not affected by any of the drugs taken by the patients. Among the various clinical parameters analyzed, only age of the patient had a statistically significant relationship with the stem cell number ($p \leq 0.05$). A significant negative correlation between age of the patients and the number of migrated ckit^{+} cells ($r= -0.419$) was observed.

Effect of growth factors on cardiosphere formation

Among the growth factors analyzed, EGF was found to cause maximum enhancement of cardiosphere formation regardless the presence/absence of serum in the medium ($p<0.05$). EGF accelerated cardiosphere formation was confirmed by the sphere count and proliferation assays.

Cardiosphere culture and characterization

Cardiospheres were expanded into monolayers termed cardiosphere-derived cells (CDCs). CDCs exhibited antigenic and cytochemical similarities to cardiospheres. CDCs were positive for the markers c-Kit, MDR-1, CD34, CD31, cTN1 and MHC. These cells were largely $CD133^-$ and $CD45^-$, as well as negative for a cocktail of blood lineage markers (B220, Mac-1 & Gr1). CDCs also consisted of subpopulations that phenotypically resemble endothelial cells ($CD34^+$ $CD31^+$). However, in the presence of EGF, percentage of cells expressing myocyte markers (cTN1 & MHC) was high when compared with the untreated controls.

Effect of EGF on the mitogenic, migratory and wound healing activities of CDCs

Among the seven different growth factors analyzed, maximum enhancement of cardiosphere-derived cell proliferation was obtained in medium supplemented with EGF ($p< 0.005$). A potent effect of EGF on CDC migration was documented (0 versus 10 ng/ml EGF = 0.857 ± 1.2 versus 8 ± 3.2 cell/well, $p <0.0005$). EGF significantly increased the rate of wound closure ($p<0.005$) as well.

Signaling pathway involved in epidermal growth factor induced proliferation of cardiosphere derived cells

Incubation of cardiosphere-derived cells with EGF supplemented medium, after pretreatment with SB 203580 decreased the cell proliferation rate by 42% compared to control conditions ($p<0.005$). The results suggest that p38MAPK pathway might be involved in EGF induced CDC proliferation.

CONCLUSION

Phase-bright cells isolated from right atrial biopsy samples manifest features characteristic to stem cells, confirmed by marker expression. The study demonstrated an easy and cost-effective method for the isolation and expansion of human adult cardiac stem cells. The yield of stem cells from cardiac biopsies was not influenced by either disease severity or risk factors for coronary artery disease. However, a significant inverse correlation was observed between the age of the patients and the cardiac stem cell counts. This observation suggests that in older patients stem cell isolation from cardiac biopsies may not succeed and in them, such cells may not be available for therapy.

Cardiac stem cells respond to epidermal growth factor more efficiently when compared to the other widely used growth factors. Maximum cardiosphere formation and cardiosphere-derived cell proliferation were obtained in medium supplemented with EGF. EGF significantly favored CDC migration, proliferation and healing process as assessed by *in vitro* tests. EGF treatment seemed to also enhance cardiomyocyte differentiation in CDC populations. Preliminary results suggests that p38MAPK signal transduction pathway might be involved in EGF mediated CDC proliferation.

SIGNIFICANCE OF THE STUDY

The findings confirm the existence of $ckit^{pos}$ cardiac stem cells in post natal human hearts. Except in older patients, isolation of stem cells from adult heart tissue could provide an avenue for developing regenerative cell therapy in patients with coronary artery disease. The study provided evidences suggesting role for EGF in encouraging migration of transplanted CDCs directed towards sites of wound and stimulating proliferation of thus attracted cells.

I. INTRODUCTION

I.1. THE ROLE OF STEM CELLS FOR TREATMENT OF CARDIOVASCULAR DISEASE – CELLULAR CARDIOMYOPLASTY

Current therapeutic strategies for cardiovascular diseases fail to address the underlying scarring and cell loss, which are the causes of ischemic heart failure. The heart is one of the few organs of the body that does not appear to contain a resident stem cell population on call to repair basis, although there are more recent evidences to the contrary (Beltrami *et al.* 2003). Perhaps this is why cardiovascular diseases, resulting in chronic ischemia and myocyte degeneration, are among the leading cause of death worldwide. Cellular transplantation can overcome these problems and new impetus has been introduced into this field following isolation of human embryonic and adult stem cells (Lovell and Mathur 2004). The transplanted cells have shown remarkable ability to regenerate cardiomyocytes and vascular cells *in vitro* and *in vivo,* the procedure termed cardiomyoplasty. However, facts regarding signals for homing, differentiation, and engraftment of transplanted cells remain to be unearthed. The role of cell fusion and the mechanisms by which transplanted cells improve cardiac function are the other matters of concern. Adult stem cells provide an excellent source of multipotent stem cells that can be used for tissue regeneration and vascularization. Their accessibility for autologous therapies in adult patients offers them a distinct advantage over embryonic stem cells as well. The low number of stem cells in adult tissues however necessitates identification of strategies to facilitate expansion and mobilization of the resident adult stem cells. The keys to harnessing the potential of these progenitors will be reproducible isolation, characterization, and

direction toward specific fates *in vitro* and *in vivo*. In addition, the engraftment levels currently observed will have to be enhanced for maximum utility.

I.2. THE CARDIAC STEM CELL

The dogmatic view of heart as a post mitotic organ has been challenged by two important discoveries. One is the discovery of small dividing cells in the heart expressing cardiac contractile proteins and with stem cell properties (Beltrami *et al.* 2001, Kajstura *et al.* 1998, Urbanek *et al.* 2003). Second is the development of cellular therapy for cardiovascular diseases. Recent studies suggest that several subpopulations of cardiac stem or progenitor cells (CPCs) reside within the adult heart and that under appropriate stimulus they can differentiate into cardiac myocytes, vascular smooth muscle cells and endothelial cells (Quaini *et al.* 2002, Beltrami *et al.* 2003, Messina *et al.* 2004). CPCs or their progeny have been found in the hearts of multiple animal species including mouse, rat, dogs, pigs and human (Urbanek *et al.* 2005a, Urbanek *et al.* 2005b, Linke *et al.* 2005). These cells are distinguished by expression of different marker proteins which include ckit (Beltrami *et al.* 2003, Messina *et al.* 2004), stem cell antigen-1 (Sca-1) in mice (Oh *et al.* 2003), Sca-1 like antigens in other species, multiple drug resistance-like protein-1 (MDR1) (Linke *et al.* 2005), side population (SP) antigen (Martin *et al.* 2004), islet-1 antigen *etc.* None of the surface antigens that have been utilized to characterize the cells are however specific for CPCs. The expressed surface markers may differ between species and could also change in the course of differentiation from progenitor to mature cells. In addition, it is difficult to determine whether CPCs as studied by different investigators represent the same cellular lineage at various differentiation stages or entirely

different cell lineages. In animal studies, *in vitro* expanded CPCs have been observed to improve cardiac performance in pathological conditions as well. Regenerative potential of CPCs remains to be confirmed. Although there are promising results, several issues need to be addressed before administering CPCs to patients. The issues to be resolved include establishing standard methods for isolation, culture and expansion of the cells, identifying appropriate methods of delivery of the cells to the heart and finding out how to control the growth and maturation of the cells delivered.

I.3. CCM EMPLOYING RESIDENT CARDIAC STEM CELLS

Adult cardiac stem cells hold great potential for use in new strategies aimed at the regeneration and repair of damaged or diseased myocardium and may have potential value for improving treatment of myocardial ischemia and cardiac failure. When transplanted into animals with myocardial infarction, these cells appear to recover heart function (Urbanek *et al.* 2005). CPCs are able to differentiate into functional myocytes and vessels which integrate into the diseased myocardium (Beltrami *et al.* 2003, Dawn *et al.* 2005, Linke *et al.* 2005). Besides directly replenishing cardiac tissue, heart derived stem cells could also stimulate angiogenesis and improve survival of existing cells through paracrine effects. Isolation of a truly resident pluripotent cardiac stem cell thus could change our understanding of the native homeostatic processes in the heart and may also provide with the ultimate cell type for therapeutic cardiac regeneration.

I.4. CHALLENGES IN CARDIAC STEM CELL THERAPY FOR MYOCARDIAL REGENERATION

Cardiac stem cells isolated from atrial biopsy specimens obtained from patients with coronary artery disease, when transplanted into diseased heart have improved cardiac function in such animals, as observed in experimental studies (Smith *et al.* 2007). For many patients with severe degree of cardiac failure, a small sample of tissue as obtained by biopsy technique may not yield sufficient cells for expansion and transplantation. Lack of sufficient cell numbers hinders the clinical utility of cells isolated from biopsies. Recent studies reveal that cardiosphere-derived cells, expanded from biopsy specimens could supply reasonable cell numbers for transplantation. Human cardiosphere-derived cells (CDCs) when injected into the border zone of myocardial infarcts engraft and migrate into the infarct zone. Injected CDCs resulted in increase in the percentage of viable myocardium and improved left ventricular ejection fraction as well (Smith *et al.* 2007). Hence, strategies favoring augmentation of the yield of transplantable stem cells are currently being rigorously investigated.

I.5. OBJECTIVES OF THE PRESENT STUDY

The broad aim of the study was to explore the possibility of isolating and culturing $ckit^{pos}$ cardiac stem cells from biopsy samples obtained from human heart and to identify factors for expansion of cardiosphere-derived cells.

The specific objectives were

1) To isolate and characterize human adult resident cardiac stem cells.

2) To identify an easy and cost-effective method for the isolation and expansion of adult cardiac stem cells.

3) To determine whether the cells grow in normal culture conditions to form cardiospheres and to characterize the cardiosphere cells.

4) To determine whether any clinically relevant factors influence the yield of the isolated stem cells.

5) To select an appropriate growth factor that favors maximum cardiosphere formation and cardiosphere-derived cell proliferation.

6) To characterize the cardiosphere derived cells (CDCs) and to determine the effect of epidermal growth factor (EGF) in the mitogenic, migratory and wound healing activities of CDCs.

7) To elucidate the signaling mechanism involved in EGF induced cardiosphere derived cell expansion.

I.6. RESULTS AT A GLANCE

I.6.1. Selection of an easy and cost effective method for the isolation and expansion of cardiac stem cells and characterization

An easy and cost-effective technique, the non-enzymatic method, has been identified for the isolation adult cardiac stem cells. The isolated cells from right atrial biopsy samples were identified as stem cells by their morphology and positive staining for ckit, MDR1, CD34 stem cell markers. The isolated cells were expanded in a growth factor supplemented medium to form dividing clusters termed cardiospheres. The

cardiosphere cells retained the markers of stemness confirmed by positive staining for the ckit, MDR1 and CD34 stem cell markers. The cardiosphere cells stained positive for endothelial and cardiac differentiation markers.

I.6.2. Clinical determinants of the yield of migrating cardiac stem cells from atrial biopsy samples

Cardiovascular risk factors and disease severity do not seem to affect the number of $ckit^{pos}$ cells migrated from explanted atrial tissue samples. The number of migrating cells was also not affected by any of the drugs taken by the patients. However, age related depletion in stem cell counts was observed.

I.6.3. Epidermal growth factor promotes cardiosphere formation, mitosis, migration and wound healing in cardiosphere-derived cells

Epidermal growth factor (EGF) enhanced cardiosphere formation. EGF also accelerated cardiosphere derived cell (CDC) proliferation and migration. EGF promoted wound healing in cultured CDCs as well. Together, the data suggest an effective role for EGF in the mitogenic, migratory and healing activities of cardiac stem cells.

Data from further experiments suggest that the signaling cascade induced by EGF, leading to the proliferation of cardiosphere-derived cells might be p38 MAPK dependent. Increased proportion of cells among EGF treated CDCs expressing cardiac differentiation markers is a noteworthy finding given its significance in developing therapeutic strategies employing these cells.

II. REVIEW OF LITERATURE

II.1. STEM CELLS – A BRIEF OVERVIEW

The isolation of stem cells from human pre-implantation embryos is considered as one of the biggest breakthrough in the 21st century. These mysterious cells hold great promise in a new era of reparative medicine by providing source for an unlimited supply of different tissue types suitable for transplantation therapy. Stem cells are defined functionally as cells that have the capacity to self-renew and generate differentiated cells (Smith 2001; Weissman *et al.* 2001). They can generate daughter cells identical to their mother (self-renewal) as well as produce progeny with more restricted potential (differentiated cells). The best example of a stem cell is the bone marrow stem cell that is unspecialized and is able to specialize into blood cells, such as white blood cells and red blood cells with special functions such as ability to produce antibodies, act as scavengers to combat infections and transport gases. Stem cells have the remarkable property of developing into a variety of cell types in the human body depending on the external signals. When a stem cell divides, each new cell has the potential to remain as a stem cell or become another cell type with new special functions, such as blood cells, brain cells *etc.* They also serve as a repair system by being able to divide without limit to replenish worn out cells of the body. In mammals, most tissue repair events are brought about by the activation of pre-existing stem cells or progenitor cells. During the last decade, several important discoveries have been made and these have shed light on the biology of stem cells.

There are different types of stem cells. They are:

1. the most primitive totipotent stem cells – the zygote that is able to produce the embryo and placenta (The only totipotent cells are the fertilized egg and the first four cells produced by its cleavage)

2. the pluripotent stem cells – cells that have the potential to become any differentiated cells in the body but cannot contribute to making extraembryonic membranes (eg:- embryonic stem cells, embryonic germ cells and embryonic carcinoma cells) and

3. the mulipotent stem cells – stem cells that give rise to the three germ layers of the body, but can differentiate only into a limited number of cell types and

4. the tissue committed stem cells (TCSC) which give rise to cells, which build specific tissues (Lemoli *et al.* 2005).

II.2. CLASSIFICATION AND SOURCES OF STEM CELLS

Stem cells can be classified into four broad types based on their origin. They are: - stem cells from embryos, stem cells from the fetus, stem cells from the umbilical cord, and stem cells from the adult. Each of these can be grouped into subtypes (Figure 1).

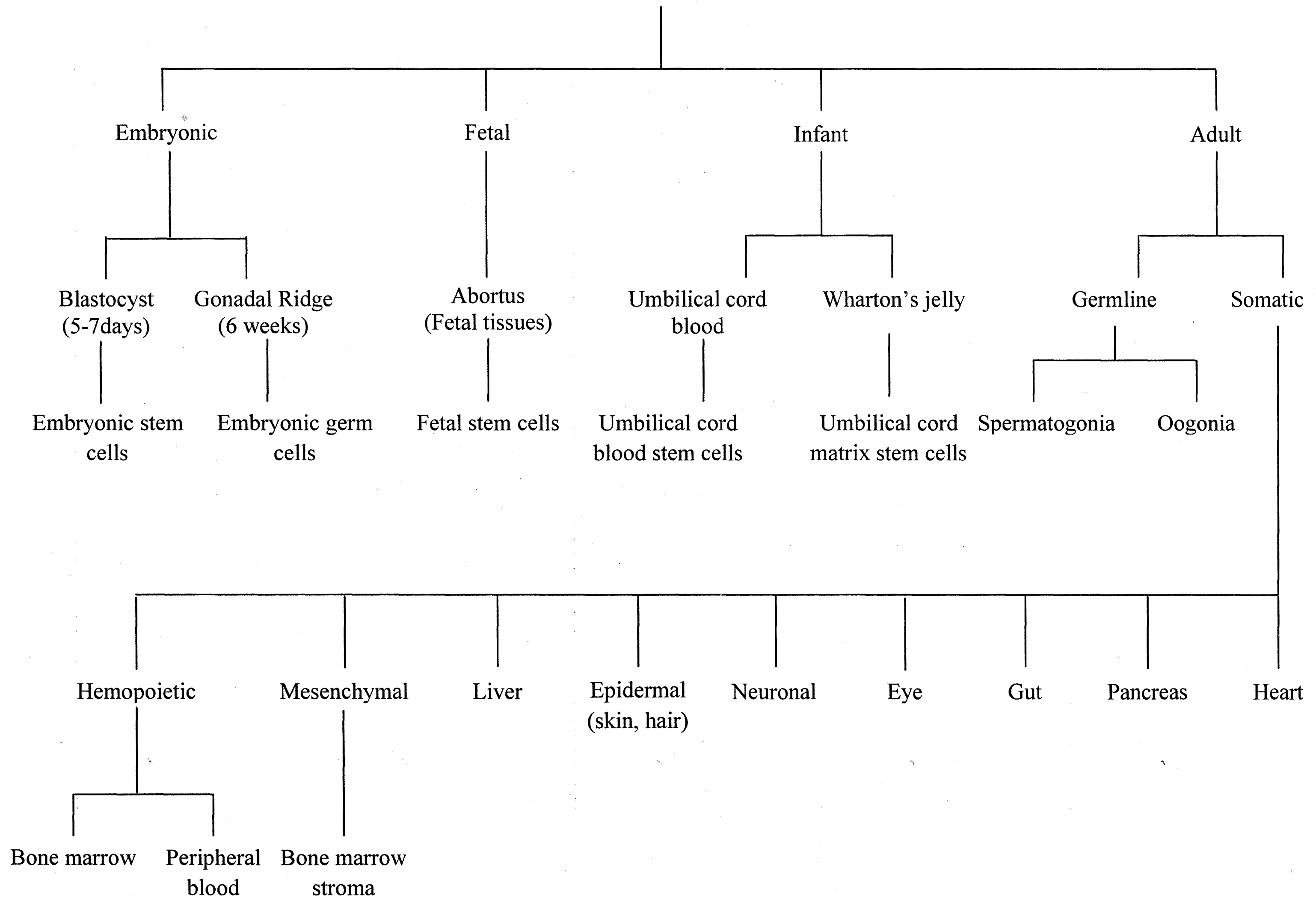

Figure 1. Classification of human stem cells

Source: Bongso A and Lee EH 2004 (modified)

II.2.1. EMBRYONIC STEM CELLS

The inner cell mass (ICM) of the 5- to 6-day old human blastocyst is the source of pluripotent embryonic stem cells (hESCs). During embryonic development, the ICM develops into two distinct cell layers, the epiblast and the hypoblast. The hypoblast forms the yolk sac and the epiblast differentiates into three primordial germ layers (ectoderm, mesoderm, and endoderm). Knowing what drives these developmental pathways is crucial to understanding the factors and events that lead to differentiation of embryonic stem cells to desirable tissue types. Pluripotent embryonic stem cells can give rise to many cell types *in vitro* including cells specific to endodermal tissues. Advances in understanding the ESC biology will provide answers for re-programming stem cells of adult tissue origin.

The basic characteristics of an ESC include properties such as self-renewal, multi-lineage differentiation *in vitro* and *in vivo*, clonogenicity, a normal karyotype, high telomerase activity, extensive proliferation *in vitro* under well defined culture conditions and that they can be frozen and thawed. These cells form embryoid bodies and teratomas containing differentiated cells of all three germ layers. Human ESCs have been shown to be positive for the expression of well-known molecular markers of pluripotent cells (Brivanlou *et al.* 2003).

II.2.2. EMBRYONIC GERM CELLS

Primordial germ cells or diploid germ cell precursors transiently exist in the embryo before they closely associate with somatic cells of the gonads and then become committed as germ cells. Human embryonic germ cells (hEGCs) originate from the

primordial germ cells of the gonadal ridge of 5- to 9-week old fetuses. hEGCs have been successfully isolated and characterized (Shamblott *et al.* 1998). These stem cells are pluripotent and are able to produce cells of all three germ layers. But the cell types derived from embryonic germ cells are limited.

II.2.3. FETAL STEM CELLS

These are primitive cell types found in the organs of fetuses. Neural crest stem cells, fetal hematopoietic stem cells and pancreatic islet progenitors have been isolated from abortuses (Beattie *et al.* 1997). Fetal neural stem cells found in the fetal brain differentiate into both neurons and glial cells (Brustle *et al.* 1998; Villa *et al.* 2000). Fetal blood, placenta and umbilical cord are rich sources of fetal hematopoietic stem cells.

III.2.4. UMBILICAL CORD STEM CELLS

Umbilical cord blood contains circulating stem cells. The cellular contents of umbilical cord blood appear to be distinct from those of bone marrow and adult peripheral blood (Rogers and Casper 2004). Umbilical cord hematopoietic stem cells produce large colonies *in vitro*. They have different growth factor requirements, long telomeres and can be expanded in long term culture. Compared with bone marrow stem cells, cord blood cells induce decreased graft versus host reaction. This is possibly because of high interleukin-10 (IL-10) levels produced by the cells and/or decreased expression of beta-2-microglobulin. Cord blood stem cells are multipotent cells and are able to differentiate into neurons and liver cells (Rogers and Casper 2004).

Matrix cells from the umbilical cord also contain potentially useful stem cells (Mitchell *et al.* 2003). This matrix termed Wharton's jelly is a source of mesenchymal stem cells as well. These cells express typical stem cell markers and have high telomerase activity. They have been propagated for long population doubling times and can be induced to differentiate into neurons *in vitro*.

II.2.5. ADULT STEM CELLS

II.2.5.1.Hematopoietic stem cells (Bone marrow and peripheral blood)

Bone marrow contains stem cells that are hematopoietic and mesenchymal in origin. The process of production and maintenance of blood stem cells and their proliferation and differentiation into the cells of peripheral blood is termed hematopoiesis. The hemaopoietic stem cells are derived early in embryogenesis from the mesoderm and later colonize in very specific hematopoietic sites within the embryo. The sites include bone marrow, liver and yolk sac. Bone marrow stem cells are multipotent and hence more plastic and versatile. They can differentiate into many cell types both *in vitro* and *in vivo*.

II.2.5.2. Mesenchymal stem cells (Bone marrow stroma)

Mesenchymal stem cells (MSCs) are found in the non-hematopoietic bone marrow stroma. The stroma is made up of a heterogenous population of cells, which include reticular cells, adipocytes, osteogenic cells, smooth muscle cells, endothelial cells and macrophages (Bianco and Riminucci 1998). The turnover of stromal tissue in response to injury and the subsequent repair process occur through the participation of a population of stem cells found in the stromal tissue (Owen 1988). MSCs can also be

derived from periosteum, fat and skin apart from the bone marrow stroma. They are multipotent cells capable of differentiating into connective tissue such as cartilage, bone, muscle, tendon, ligament and fat (Caplan 1994).

II.2.5.3. Gut stem cells

The gastrointestinal epithelial lining undergoes continuous and rapid renewal throughout life. The cell renewal process is maintained by a group of multipotent stem cells located in the crypts of Lieberhahn (Wright 2000). Dilineating properties of these stem cells and the molecular mechanisms underlining their fate decisions are important to understand the developmental pathways. In the small intestine, epithelial cells of enterocytic, goblet and enteroendocrine origin differentiate as they migrate from the crypt up an adjacent villus and leave the intestine once they reach the villus tip. But the developmental process is different in the colon region. Epithelial cells migrate from the crypt to a flat surface cuff that surrounds its opening. The stem cell hierarchy and their well defined anatomic location in the gut make the gut stem cells an ideal *in vivo* model for stem cell research (Alison *et al.* 2002).

II.2.5.4. Liver stem cells

In mammals, 75% of the liver can regenerate after liver injury. The lost tissue can be restored within 2-3 weeks. This is in contrast to most other organs such as the kidney or pancreas in which regenerative potential is substantially less. Recent evidence strongly suggests that different cell types and mechanisms are responsible for organ reconstitution and these depend on the type of liver injury.

II.2.5.5. Bone and cartilage stem cells

Mesenchymal stem cells in bone marrow can differentiate into bone and cartilage under appropriate conditions. But recent evidences indicate that bone itself contain both uncommitted stem cells as well as committed osteoprogenitor cells (Nuttall *et al.*1998; Gronthos *et al.* 1999). When bone is fractured, marrow is exposed and abundant bleeding occurs with hematoma formation in the marrow space, which results in good repair potential. When cartilage is injured, stem cells participate in the repair process. The numbers however are small and the regulatory factors are limited (Metsaranta *et al.* 1996; Nakajima *et al.* 1998).

II.2.5.6. Epidermal stem cells (Skin and Hair)

Epidermal stem cells are housed at the base of the hair follicle and they allow re-growth of hair and skin cells continuously. New keratinocytes are formed continuously during adult life to replace worn out outer skin layers and hairs. Stem cells differentiate into an intermediate cell called the 'transient amplifying cell' which gives rise to the more differentiated cell types inclusive of the keratinocytes and sebocytes (Blanpain *et al.* 2004).

II.2.5.7. Neuronal stem cells

Neurogenic turnover occurs continuously in some limited areas of the central nervous system (CNS). The neurogenic regions involved in this process are: the subventriclar zone (SVZ) of the forebrain (Reynolds and Weiss 1992; Luskin 1993; Lois and Alvarez-Buylla 1993) and the dental gyrus of the hippocampus (Palmer *et al.* 1995; Seaberg and Van der Kooy 2002). These regions are considered to be the reservoirs of

new neural cells. Thus neural stem cells (NSCs) reside in these two areas and they consistently generate new neurons (Mckay 1997; Gage 2000; Temple 2001). *In vivo* studies have shown that endogenous NSCs are able to produce neurons exclusively while under *in vitro* conditions, NSCs are competent to generate neurons, astrocytes and oligodendrocytes (Bottai *et al.* 2003). NSCs are multipotent progenitor cells that have self-renewal capacity. But the final fate of a NSC is under tight environmental control.

II.2.5.7.8. Pancreatic stem cells

Whether true stem cells are present in the pancreas is controversial. It has been however reported that the endocrine cells of the rat pancreatic islets of Langerhans, including insulin-producing beta cells turn over every 40-50 days by apoptosis and that there is proliferation and differentiation of new islet cells (neogenesis) from progenitor epithelial cells located in the pancreatic ducts. The pancreatic islets contain an unrecognized population of cells that express the neural stem cell-specific marker nestin. These nestin-positive cells which are distinct from the ductal epithelium, have an unusually extended proliferative capacity *in vitro,* can be cloned repeatedly and appear to be multipotential. They are able to differentiate *in vitro* into cells that express liver and exocrine pancreas markers. These nestin-positive cells participate in the neogenesis of islet endocrine cells as well (Zulewski *et al.* 2001).

II.2.5.9. Eye stem cells

Stem cells have been identified in the adult mouse eye (Tropepe *et al.* 2000). Single pigmented ciliary margin cells can clonally proliferate *in vitro* to form sphere colonies of cells, which can differentiate into retinal-specific cell types, including rod photoreceptors, bipolar neurons and Muller glia. The adult retinal stem cells were localized to the pigmentary ciliary margin and not to the central and peripheral retinal pigmented epithelium.

II.2.5.10. Cardiac stem cells

A major breakthrough in the field of cardiac regeneration is the discovery by Piero Anversa and colleagues, of a resident pool of stem cells in post-natal hearts. Their key observations, made in rodents, based on simple mathematical calculations on cell number and cell death showed that a rat left ventricle contains 23×10^6 myocytes at 4 months and 19×10^6 myocytes at 29 months. Since the average death rate is 1.34×10^6 myocytes/month, all myocardial cells should die in 17 months. Clearly, this is not the case and thus their observations challenged the long-standing dogma about heart regeneration (Beltrami *et al.* 2001). The group claims that in the heart there exists a pool of resident stem cells, which can divide, grow in size and acquire structural and functional characteristics similar to myocardial cells. Moreover, when compared to other cell types, for example bone marrow derived stem cells (BMDSCs), these cells in the heart are believed to be faster in achieving structural and functional characteristics favorable for regenerating the damaged myocardium. Transplantation studies in animals using such cells isolated from the heart have been successful,

raising hopes of isolating resident cardiac stem cells from patient's heart tissue and using them for therapy.

II.3. THE PARADIGM SHIFT IN MYOCARDIAL CELLULAR REPLICATION

Myocyte death and regeneration are proposed as central events determining adult cardiac cellular homeostasis. But the existence of myocyte death in the normal myocardium has been quite controversial (Schaper *et al.*1999; Anversa 2000; Kang and Izumo 2000). Recent reports suggest that myocyte death occurs in the normal and pathological heart of both human and experimental animals throughout the lifespan of the organism, independently from cardiac diseases. Conversely, in the absence of new myocyte formation, the normal heart would lose most of its mass in a few decades and the senile and failing heart would disappear in a matter of several months or, at most, a few years (Anversa and Nadal-Ginard 2002; Nadal-Ginard *et al.* 2003a; Nadal-Ginard *et al.* 2003b). This indicates that there exists cycling myocytes in the normal and pathological adult mammalian heart of several species, including humans (Anversa and Nadal-Ginard 2002; Nadal-Ginard *et al.* 2003a). Thus myocyte death, hypertrophy and new myocyte formation characterize normal cardiac homeostasis and its evolution through time explain why coronary artery disease and its complications are major risk factors in the elderly and why myocardial infarction is associated with increased morbidity and mortality in this population ((Nadal-Ginard *et al.* 2003a; Nadal-Ginard *et al.* 2003b).

The number of cycling myocytes and the rate of new myocyte formation increases dramatically in old age (Chimenti *et al.*2003) and in response to acute (Beltrami *et al.*

2001) and chronic overload (Urbanek *et al.* 2003), suggesting that their generation responds to physiological demands. The observations raised questions about the nature of the cycling myocytes, their origin, as well as their physiological relevance in the adult myocardium. The assumption that adult cardiomyocytes are non-replicating cells is supported by the observation that positive cell cycle regulators, cyclins and CDK, are highly expressed in the embryonic heart and down-regulated after birth, whereas negative cell cycle regulators and CDK inhibitors are increased in the adult heart. It was soon noted that some myocytes divide and express early and late growth-related genes immediately after infarction. The levels of cell cycle related proteins - cyclins - E, A and B are increased and their associated kinase activities are elevated significantly (Anversa and Kajstura 1998). High levels of DNA replication, karyokinesis and cytokinesis have also been identified (Anversa and Kajstura 1998; Kajstura *et al.* 1998; Beltrami *et al.* 2001) and many expressed functional telomerase activity, an enzyme that so far has only been detected in cells that are either in or capable of entering the cell cycle (Leri *et al.* 2000). There is also an inverse correlation between DNA replication and expression of cell cycle inhibitors, such as p16, expressed in terminally differentiated myocytes. This phenotype raised the possibility that the cycling myocytes represented a subpoplation of newly born, immature cells, which had not yet reversibily withdrawn from the cell cycle. This finding was further reinforced by the finding that in the acute phase of human myocardial infarction, the border zone and the myocardium remote from the damage exhibited very high levels of myocyte replication with newly formed myocytes of small size (Beltrami *et al.* 2001). Taken together, these findings shed light on the

origin of cycling myocytes in adult hearts which in turn paved the way for the development of an exciting therapeutic approach for treating ailing human hearts.

II.4. CHALLENGING THE DOGMATIC VIEW ON THE HEART AS A POSTMITOTIC ORGAN AND THE IDENTIFICATION OF CARDIAC PRIMITIVE CELLS

Although stem cells have been identified in several organs including the blood, skin, central nervous system, liver, gastrointestinal tract, and skeletal muscle (Blau *et al.* 2001), the search for a stem cell in the heart was perceived as a futile effort given the accepted lack of regenerative potential of the myocardium. However, data challenging this belief began to accumulate (Bolli 2002; Muller at al. 2002; Leri *et al.* 2005). Questions concerning two apparent contradictory lines of evidence: the irreversible withdrawal of cardiac myocytes from the cell cycle and the presence of cycling myocytes in the infarct area raised question regarding the origin of dividing myocytes, pointing to the presence of a residing stem cell pool in the heart, termed cardiac stem cell (CSC). The pool size of these adult stem cells are most likely set during prenatal life or early after birth, and their migration, proliferation, maturation, and death regulate the homeostasis of the adult heart (Leri *et al.* 2005).

In the sex-mismatched cardiac transplantation experiment, primitive cells of donor and recipient origin that expressed the stem cell surface antigens c-kit, Sca-1, and MDR1 were identified. Identical cells were found in human control hearts as well (Leri *et al.* 2005). During fetal life, c-kit-positive cells are reported to colonize the yolk sack, liver, and probably other organs, and the colonized organs express SCF, the ligand for c-kit receptor (Kunisad *et al.* 1998; Teyssier-Le Discorde *et al.* 1999).

Hence it was reasonable to assume that stem-like cells are present in the heart from fetal life itself. The rapid induction of SCF during myocardial ischemia (Frangogiannis *et al.* 1998) strengthened the notion that SCF was involved in the activation of resident primitive c-kit-positive cells and, thereby, in the increased formation of myocytes in the acutely infarcted heart (Beltrami *et al.* 2001). The presence of these undifferentiated cells together with early committed progenies was suggestive of a true CSC as the critical modulator of the homeostasis of the normal and stressed myocardium. These observations were the foundation for the work that ultimately led to the identification and characterization of a resident CSC pool in the adult heart (Beltrami *et al.* 2003). The replenishment of the parenchymal and non-parenchymal cells in the heart is regulated by the ability of these primitive cells to self renew and differentiate.

II.4.1. MYOCARDIAL INFARCTION – A BRIEF OVERVIEW

Myocardial infarction (MI) is the leading cause of death in most industrialized nations throughout the world. Approximately 800,000 people in the US are affected and 250,000 die prior to presentation to a hospital. But it has been estimated that the survival rate for MI patients hospitalized immediately after the occurrence of the event is approximately 90% to 95%. This significant improvement in survival is related to improvements in emergency medical response and treatment strategies adopted. At present, developing countries contribute a greater share to the global burden of CVD than the developed countries. In India, around 8% of the population is probably suffering from heart diseases. It has been reported that Indians are 3.8 times more prone to cardiovascular diseases than the residents of Singapore, three times

more than Fijians, 2.4 times more than people of Trinidad, 1.4 times higher than South Africans and 1.5 times higher than British (Touze *et al.* 2007). India is considered on the threshold of an epidemic of cardiovascular diseases.

Why does heart remain an ill-fated organ in spite of the tremendous pharmacological advances? The issue remains unanswered. The breakdown in function, seen particularly in the elderly, stems from the irreversible loss of a specific group of heart cells, called cardiomyocytes, which are terminally differentiated cells, eventually resulting in organ failure. Cardiovascular diseases, including hypertensive diseases and myocardial infarction lead to loss of cardiac tissue through death of the cells by apoptosis and necrosis. The remaining myocytes are unable to reconstitute the lost tissue, and the diseased heart deteriorates functionally with time. Cardiac myocytes have been often compared to neurons for their inability to regenerate and replace damaged myocardium. Even though evidence now exists for adult neurogenesis and neural stem cells (Horner and Gage 2000), the concept of myocyte regeneration has not been embraced by the medical community and remains highly disputed (Soonpaa and Field 1998) and mostly neglected. Therapeutic modalities currently available for treating diseased hearts provide relief, but do not lead to a definitive therapy for patients who have had heart attack.

Until recently, the goals of therapy in AMI were the restoration of normal coronary blood flow and the salvage of functional myocardium and these were met to an extent by a number of medical interventions and adjunctive therapies. These include administering thrombolytic agents, use of left ventricular assist devices, heart transplantation therapy *etc.* Even though these interventions benefit patients,

retrieving a complete functioning heart still remains a long-standing goal. Current therapeutic approaches suffer limitations and are primarily focussed at limiting disease progression rather than repairing and restoring healthy tissue and function. The limited efficacy and co-morbidity of current treatments have spurred the interest to investigate alternate options and additional long-term therapeutic approaches. In this perspective, cell transplantation therapy (CTT) seems to have potential as a new therapeutic strategy to achieve cardiac repair.

II.4.2. RISK FACTORS FOR MI

The risk of coronary artery disease (CAD) is traditionally high in patients who had an ischemic stroke or a transient ischemic attack (TIA). High-risk patients may be identified using the traditional cardiac scoring systems (Touze *et al.* 2007). The main causal and treatable risk factors for MI include hypertension, hypercholesterolemia or dyslipidemia, diabetes mellitus, and smoking. In addition to these risk factors, recent studies have shown the importance of age, male gender, poorly controlled hypertension, Type A personality, family history, sedentary lifestyle, genetic factors and interactions between multiple genes and environmental factors. Recent studies reported an association between traditional coronary artery disease (CAD) risk factors and progenitor cell counts (Kunz *et al.* 2006). Clinical studies demonstrated that classical risk factors for atherosclerosis or coronary artery disease (CAD) associate with impaired number of endothelial progenitor cells (EPCs) (Vasa *et al.* 2001a; Vasa *et al.* 2001b; Hill *et al.* 2003; Fadini *et al.* 2005). The number of $CD34^{+}vegfr2^{+}$ or circulating EPCs correlate inversely with the severity of CAD as well as cardiovascular events. (Wang *et al.* 2005; Schmidt-Lucke *et al.* 2005; Kunz *et al.*

2006). Another study reported the activity of EPCs to form CFUs in relation to cardiovascular risk factors and endothelial function in a group of healthy volunteers (Hill *et al.* 2003). The formation of clusters by culture-enriched EPCs is a representative of their fundamental functional features such as proliferation, migration, adhesion, survival and differentiation. It has been reported that CAD risk factors may not only lead to reduced EPC pool in the bone marrow but may also modulate/impair their proliferation, senescence and differentiation, thus affecting endothelial repair mechanism *in vivo*, subsequently leading to increased vascular disease severity (Kaur *et al.* 2007). These observations are relevant to the efforts to develop a strategy for myocardial regeneration in patients with coronary artery disease employing autologous stem cells.

II.4.3. CELLULAR THERAPY FOR MYOCARDIAL INFARCTION

Historically, tissue regeneration techniques based on cell transplantation technology have been used for the treatment of hemopathies (chronic lymphocytic leukemia, aplastic anemia, immunodeficiencies, myeloma), in ophthalmology (transplantation of limbal stem cells for corneal regeneration), and in orthopedics (implantation of chondrocytes for articular defects) (Chachques *et al.* 2005). Current clinical investigations concern the following specialties: endocrinology (transplantation of Langerhans islets in diabetes mellitus), neurology (Huntington's chorea, Alzheimer and Parkinson's diseases, spinal cord regeneration), hepatology (implantation of hepatocytes as a bridge to liver transplantation), myology (transplantation of myoblasts in Duchenne's dystrophy) (Law 1992), dermatology (implantation of cultured keratinocytes and fibroblasts in burn patients), and vascular surgery

(implantation of angiogenic stem cells in critical limb ischemia). Given the limited efficacy of contemporary treatment for end stage heart disease, there is increasing interest in developing cell transplantation technology (cellular cardiomyoplasty, CCM) and gene therapy to regenerate functional muscle and blood vessels in previously infarcted, scarred and dysfunctional myocardium. Despite its very early stage, almost every major cardiovascular company is involved in at least one cell therapy effort. Companies are viewing at multi-billion dollar markets in cell therapy in the future.

Over the years, a number of pre clinical and small clinical trials have shown that heart tissue damaged during myocardial infarction can sometimes regenerate, when cells of various types—embryonic stem cells, stem cells from cord blood and bone marrow, and skeletal myoblasts—are injected into heart scar tissue. Cardiac regeneration is expected to have an important impact on the treatment of heart failure, a major cause of disability and death. Several strategies are currently being evaluated for regeneration of the damaged myocardium in heart failure. These are aimed at 'reviving' existing malfunctioning cells, repopulating the myocardium by new cells from exogenous or endogenous sources, altering the extracellular matrix, or increasing blood supply by enhancing vasculogenesis. Several small clinical trials have reported varying degrees of functional improvement. Currently, there is evidence both in animals and humans, for myocyte regeneration through migration, differentiation and proliferation of stem cells. A variety of cell populations have been examined to generate adult cardiac myocytes and have yielded promising results though certain deficiencies remain to be redressed. These cell types include fetal and neonatal cardiac myocytes, autologous skeletal myoblasts, immortalized myoblasts,

bone marrow derived adult hematopoietic stem cells and cardiomyocytes differentiated from ESCs (Soonpaa *et al.* 1994; Koh *et al.* 1995; Reinecke *et al.* 2000; Zhang *et al.* 2001). Endothelial cells derived from embryonic stem cells from dorsal aorta and from differentiated cells of the human umbilical vein have been shown to trans-differentiate into beating cardiomyocytes when co-cultured with neonatal cardiomyocytes or when injected into post-ischemic adult mouse heart. However, while most of studies indeed reported better contractile properties, almost all failed to produce cardiac myocytes that are electrically coupled to the pre-existing cardiac myofibers (Reinecke *et al.* 2000). Alternatively, ES cells are an abundant source of tissue specific stem cells. However, given the bio-ethical issues involved in the use of human ES cells and tissues, other sources of pluripotent stem cells are now considered timely for use in tissue regeneration and gene therapy. Adult stem cells thus provide an alternative source of multipotent stem cells that can be used for tissue regeneration and vascularization. The low number of stem cells in adult tissues necessitates strategies to facilitate expansion and mobilization of stem cells to be deeply probed to significantly enhance our ability to isolate an appreciable population of stem cells that may ultimately be used for transplantation.

Adult stem cells are, by definition, capable of becoming a variety of differentiated cell types *in vivo*. Although stem cells derived from adult tissues have not been definitively shown to exhibit pluripotent capabilities like their embryonic counterparts, their accessibility for autologous therapies in adult patients offers them a distinct advantage over embryonic stem cells. The keys to harnessing the potential of these progenitors will be reproducible isolation, characterization, and direction toward specific fates *in vitro* and *in vivo*. In addition, the engraftment levels currently

observed will have to be enhanced for maximum utility. Since bone marrow is reported to be a rich source of stem cells, attempts to mobilize adult marrow stem cells by administering cytokines for bringing out cardiac regeneration were attempted. The major objective was to enable cell-based myogenic and angiogenic regeneration for the damaged hearts of patients suffering with chronic ischemic heart diseases. The ultimate goal is to retard the progression of ventricular remodeling and heart failure in patients with ischemic and non-ischemic cardiomyopathies. But the use of allogenic bone-marrow stem cells holds several clinical complications. Patient derived autologous cells are preferred in order to avoid host rejection over implanted cells (Ye *et al.* 2006).

Safety and efficacy of cellular cardiomyoplasty, although appears promising in pre-clinical studies, have not been adequately evaluated (Ellis *et al.* 2000). Its future will depend on conducting carefully controlled, randomized clinical trials with appropriate selection of end points. Controversies exist over the specific cells to be used, the dosages needed for tissue repair, route of administration and how the transplanted cells would affect the electrical activity of the myocardium. Whether the cells can improve myocardial function after transplantation over long term is also not yet clear.

The challenge in regenerative therapy in cardiac diseases is not simply to arrest cardiac dysfunction but is to achieve cell engraftment with electromechanical integration into the heart, arrest adverse myocardial remodeling and improve contractility of the diseased heart (Figure 2). To understand underlying mechanisms and to answer the many unknown questions related to regenerative therapy requires

the knowledge and expertise of many disciplines. Be that as it may, therapy for cardiac regeneration has undoubtedly arrived.

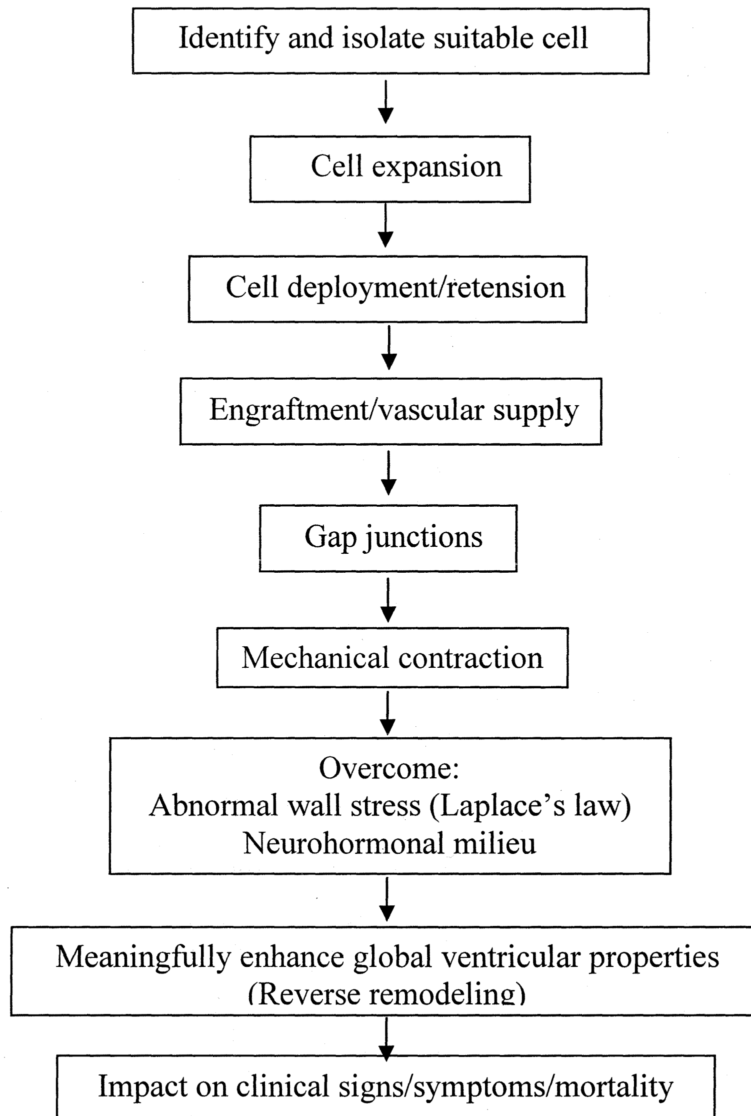

Figure 2: Summary of the milestones that need to be achieved in order to arrive at a successful cell therapy for heart failure (Source: Klotz and Burkhoff 2004)

II.4.4. STEM CELL THERAPIES FOR HUMAN DISEASES

The ultimate goal in the field of stem cell biology is the successful restoration of damaged tissue, healing of degenerative joints or organ compartments and compensating for metabolic or genetic deficiencies. But the scientific community continues to debate concerning the most appropriate stem cell source to be used in order to acheive optimal cellular function and related biological integrity, and minimal patient risk. In order to fulfill clinical and therapeutic requirements, future customized cellular products envisaged to treat human diseases should: 1) not harm the patient 2) satisfy the biological needs they are given for 3) avoid host-mediated rejection in order to circumvent immunosuppressive regimens 4) be at hand in adequate quantity and 5) be able to be administered within reasonable period in case of acute organ failures.

The most widely studied source of stem cells for therapy are the mesenchymal stem cells (MSCs) and multipotent adult progenitor cells (MAPCs). MSCs are capable of differentiating into osteoblasts and fat cells and more recently, they have been shown to generate cell types consistent with differentiation into multiple tissues. MAPCs arise in cultures of MSCs and seem to have an even broader potential for transdifferentiation than the MSCs. MAPCs are however less ideal for therapy. The reasons include: 1) many population doublings in MSC cultures are required for the generation of MAPCs 2) it is not known whether they exist *in vivo* or what their *in vivo* phenotype may be 3) MAPCs cannot be prospectively isolated from a tissue such as bone marrow and 4) there is no quantitative assay for MAPC so that it is difficult to

predict how much tissue would be required to supply sufficient cells for a specific application.

Preclinical studies have been performed in a variety of animal models of tissue damage and degeneration with promising results and clinical studies have demonstrated the safety and potential efficacy of stem cell therapy. Examples include the studies conducted by Horwitz *et al*, Perin *et al* and Stamm *et al* (Hoevitz *et al.* 2002; Perin *et al.* 2003; Stamm *et al.* 2003). In the study conducted by Horwitz *et al*, patients with osteogenesis imperfecta was treated with allogenic bone-marrow derived MSCs which resulted in acceleration of growth velocity without clinically significant toxicity. Perin *et al* investigated the effect of transendocardial injections of autologous mononuclear bone marrow cells in patients with end-stage ischemic heart failure and observed improved blood flow and ventricular function. Stamm *et al* injected autologous $AC133^{+}$ hematopoietic stem/progenitor cells into the infarct border and found improved perfusion of the infarct tissue implying that the injected cells facilitated angiogenesis. In an animal model of tyrosinemia type I, transplantation of as few as 50 purified hematopoietic stem cells restored both the hematopoietic and biochemical liver functions in the recipient by correcting the genetic aberration (Lagasse *et al.* 2000). Likewise, hepatocytes that express human albumin were identified in immunodeficient mice in which purified human hematopoietic stem cells had been transplanted (Petersen *et al.* 1999). In other studies, purified hematopoietic stem cell delivery have been shown to generate functioning cardiomyocytes and vascular structures (Ferrari *et al.* 1998) as well as neo-intimal smooth muscle cells and endothelial cells that contribute to arterial remodeling in various models of vascular lesions. Krause and colleagues (Krause *et al.* 2001) isolated single cells using

the limiting dilution technique and differentiated these cells into mature hematopoietic cells and epithelial cells of the skin, lungs and gastrointestinal tract. Transplantation of these cultured cells resulted in hematopoietic engraftment, retinal neovascularization and the generation of functioning glomerular mesangial cells.

Several investigators have reported the potential of circulating human stem cells, which are mobilized into the peripheral blood by cytokine administration and contribute to the generation of nonhematopoietic tissue. Endothelial progenitor cells mobilized by the administration of human recombinant granulocyte colony-stimulating factor and granulocyte-macrophage colony-stimulating factor contribute to ocular neovascularization in mice and neovascularization of ischemic myocardium in rats. Orlic *et al* provided evidence for the generation of cardiomyocytes in a murine model of myocardial infarction after the induction of circulating stem cells in peripheral blood. In their model, the strategy further improved ventricular function and survival (Orlic *et al.* 2001).

The success in pre-clinical experiments provided hope for a stem cell mediated therapy for treating patients with devastating disorders. Eventhough animal experiments have favorable outcomes, extrapolating knowledge in clinical settings should be done with caution. There are essentially two strategies for using adult stem cells derived from hematopoietic tissue for tissue repair. One approach is based on identifying and expanding *in vitro* multipotent adult progenitor cells for therapeutic studies. The other approach is based on the *in vivo* availability of a pool of systemic and circulating adult stem cells that can be manipulated to generate or repair solid-organ tissue. A potential clinical concept for tissue repair would require three

conditions. 1) easy accessibility of the stem cell pool, 2) sufficient concentration of stem cells at the site of tissue regeneration and 3) appropriate signals from the damaged site to direct exogenous stem cells to the site where they are needed. It is not yet clearly understood how to manipulate the microenvironment surrounding the area of tissue regeneration actively and signal exogenous stem cells to participate in tissue regeneration *in vivo* (Korbling and Zeev Estrov 2003).

II.4.5. ROLE OF BONE MARROW CELLS IN MYOCARDIAL REGENERATION

Despite the increasing evidence that the adult myocardium was not a post mitotic and terminally differentiated tissue, but was able to produce new myocytes, the potential regenerative capacity was not sufficient to change the course of adverse myocardial remodeling and the development of cardiac failure following the loss of a significant number of ventricular myocytes, as a result of myocardial infarction. Thus the question arose on how to complement the endogenous myocardial regenerative capacity to replace the significant fraction of the myocytes lost after an MI. There had been several reports indicating the role of bone marrow derived cells in regenerating the injured myocardial tissue upon transplantation following MI. In addition to giving rise to blood cells, these cells could also differentiate into several other cell types, particularly in response to tissue damage (Ferrari *et al.* 1998; Legasse *et al.* 2000; Brazelton *et al.* 2000; Mezey *et al.* 2000; Krause *et al.* 2001). When genetically tagged lineage negative (LIN^{neg}) bone marrow cells (BMCs) expressing the stem cell factor receptor, ckit, was injected into the border zone of acute MIs, the cells could adopt myocardial phenotype (Orlic *et al.* 2001). The injected BMCs regenerated most

of the necrosed myocardium with biochemically and anatomically differentiated myocytes, arterioles and capillaries. The number of regenerated cells was more than three orders of magnitude higher than the number of injected cells, demonstrating that they were not only able to differentiate into myocytes, endothelial and smooth muscle cells but were also able to amplify until a number slightly higher than the cells lost had been regenerated. These new cells restored contractility to the infracted area and improved the ventricular performance of the affected hearts. More recent results demonstrated that $ckit^{pos}$ bone-marrow derived cells are highly efficient in the regeneration of the post-infarcted myocardium (Kajstura *et al.* 2005). Even though the injected cells contribute to physiologically meaningful myocardial regeneration, the precise identity of the regenerating cells was left unanswered. Additionally, the results have been recently challenged by groups claiming their inability to obtain similar results (Balsam *et al.* 2004; Murry *et al.* 2004). All these experiments suggest the possibility that the regenerating cells have a strong tropism for the damaged tissue since they colonize only the ischemic myocardium and are not found in the neighboring healthy tissue.

There were reports of intra coronary infusions of autologous bone marrow cells. Two trials involved patients with acute myocardial infarction and one trial involved patients with chronic left ventricular dysfunction and dysfunction and history of myocardial infarction. The three trials together had 376 patients. Small significant but clinically uncertain improvements in ventricular function were seen in bone marrow treated groups in two studies. In the third study, no significant difference was observed in left ventricular function and infarct size between control and bone marrow treated groups (Lunde *et al.* 2006; Schachinger *et al.* 2006).

In another study, direct homing of bone-marrow derived cells to the post ischemic myocardium by increasing their concentration in the peripheral circulation by stimulating the bone marrow was attempted. To test this, post-infarcted mice were injected with stem cell factor (SCF) and granulocyte-colony-stimulating factor (G-CSF) using a protocol known to increase the number of circulating stem cells from ~29 in the non-treated controls to ~7,200 in the cytokine treated mice (Quaini *et al.* 2002). This cytokine stimulation protocol resulted in a significant degree of tissue regeneration 27 days later. The cytokine induced myocardial regeneration decreased mortality by 68%, infarct size by ~40%, cavitary dilation by ~26% and diastolic wall stress by ~70%. Ejection fraction progressively increased and hemodynamics improved as a consequence of the formation of $\sim 15 \times 10^6$ new myocytes with arterioles and capillaries connected to the coronary circulation. The results strongly suggested that ischemic myocardium can be colonized by circulating cells whose number can be increased by the proper cytokine stimulation of the bone marrow. But during the normal lifespan of an organism, there exists a low level of myocardial colonization from these circulating cells when there is myocardial damage. Two clinically relevant cases of human chimerism: cases of sex mismatched cardiac and bone marrow transplants test this hypothesis. The most relevant observation that dramatically challenged the old paradigm of the heart as a postmitotic organ was the sex mismatched cardiac transplantation, in which female heart was transplanted into a male recipient (Quaini *et al.* 2002). The colonization and differentiation of host cells could be identified by the presence of a Y chromosome in the transplanted female heart. A few days after the transplantation, the female hearts contained a significant number of male (Y-chromosome positive) myocytes, as well as endothelial and

smooth muscle cells organized into arterioles and capillaries. The stimulus for the colonization of the circulating cells is the dispersed areas of myocardial damage in the donor heart secondary to the ischemic and physical trauma of the transplant procedure. These observations have been further strengthened by the data obtained from sex-mismatched bone marrow transplants where a similar phenomenon of myocardial colonization from the donor bone marrow was observed. The transplanted donor bone marrow cells (BMCs) produced myocytes endothelial and smooth muscle cells organized into arterioles and capillaries (Deb *et al.* 2003) similar to the cardiac transplants.

These observations in humans strongly support the notion that the myocardium can be colonized by bone marrow derived cells that, most likely, reach the myocardium through the circulation. These cells are also capable of differentiating into three main myocardial cell types: myocytes, endothelial and smooth muscle cells. In the case of sex-mismatched cardiac transplants, cells with the immunological and morphological characteristics of stem cells were identified in the host myocardium (Quaini *et al.* 2002). These cells were of both donor and recipient origin, and some of them could be seen in the process of commitment to the myogenic phenotype because of their expression of myocyte transcription factors and initial accumulation of sarcomeric proteins. The identification of these undifferentiated/early differentiating cells suggested the presence of stem cells in the myocardium and also provides a satisfactory explanation for the existence of the small population of cycling myocytes.

II.4.6. CARDIAC STEM CELLS FOR THERAPY

The finding of the presence of small dividing cells in the heart expressing cardiac contractile proteins with stem cell properties and the promise of cellular therapy of the diseased heart using a variety of different cell types have created a new field in cardiac research. Valuable knowledge has been obtained from the large number of animal studies and a number of small clinical trials that have utilized a variety of adult stem cells for regenerating infarcted hearts. However, contradictory reports exist regarding the regenerative potential of the cardiac progenitor cells (CPCs) and the mechanisms behind the underlying hemodynamic effects. Hence a better understanding of CPC biology is pivotal for progressing therapeutic cardiac regeneration employing resident cardiac stem cells. This includes an extended knowledge of the molecular mechanisms behind their mobilization, differentiation, survival and integration in the myocardium.

CSCs or their progeny have now been found in hearts of multiple species including mouse (Messina *et al.* 2004; Urbanek *et al.* 2005), rat (Beltrami *et al.* 2003; Dawn *et al.* 2005), dog (Linke *et al.* 2005), pig and human (Messina *et al.* 2004; Urbanek *et al.* 2005; Urbanek *et al.* 2005). Results suggest that in response to diverse forms of cardiac injury, these cells undergo mitosis (Kajstura *et al.* 1998; Urbanek *et al* 2003) and differentiate into all the constituting cells of the heart (Beltrami *et al.* 2003; Messina *et al.* 2004), thus making them theoretically capable of repairing injuries in the heart. In animal studies conducted, administration of *in vitro* expanded CSCs substantially improved cardiac performance (Lyngbak *et al.* 2007). Even though these cells can differentiate into cardiac myocytes, endothelial and vascular smooth muscle

cells (Beltrami *et al.* 2003; Dawn *et al.* 2005; Linke *et al.* 2005; Urbanek *et al.* 2005), they do not *in natura* heal larger structural injuries in the heart. Several issues need to be addressed before CSCs be applied in human trials. These include establishing methods for isolation of cells from biopsies, culturing and multiplication of the isolated cells *in vitro*, standardizing the methods for delivery to the heart and finding out how to control their growth and maturation once given back to the heart. The knowledge can in turn lead to therapeutic principles where CSCs already residing in the heart could be recruited by stimulating their proliferation and differentiation. Since it is still an evolving field, the potential mechanisms underlying the effect on therapy with these cells have to be extrapolated from studies performed with bone marrow derived stem cells.

Ideally, CSCs could be used to ameliorate cardiac function in two principally different ways. Either cells isolated from the myocardium can be cultured and propagated *ex vivo* before re-adminstration or resident stem cells neighbouring the infarct zone can be boosted to proliferate and differentiate by the injection of survival factors both leading to partial cardiac regeneration and functional improvement. A series of animal experiments analyzed the effect of administering CSCs or stimulating the local CSCs after cardiac injury (Beltrami *et al.* 2003; Linke *et al.* 2005). Beltrami *et al* first conducted studies on functional competence of injected CSCs in a rat model of myocardial infarction (Beltrami *et al.* 2003). In another model of ischemia, intravenously administrated CSCs homed to the injured myocardium and turned into capillaries, arterioles and cardiac myocytes. The treated animals exhibited significant improvements in cardiac function validated by echocardiographic and hemodynamic parameters (Linke *et al.* 2005).

II.4.7. CARDIOSPHERES CONTAIN CLUSTERS OF c-KIT$^+$ CELLS AND HAVE CELL THERAPY POTENTIAL

Cardiospheres are small clusters of primitive ckit$^+$ cells that have been isolated from both murine and human hearts (Messina *et al.* 2004). The cells isolated from cardiospheres are clonogenic and are capable of long-term self-renewal, which makes them a possible candidate for cell-based regeneration therapy. Soon after isolation, a minor percentage of cardiosphere cells express both stem (c-kit, Sca-1 and CD-34) and endothelial cell (flk-1 and CD-31) markers. After 6 days in culture, the expression of c-kit marker is mainly preserved indicating that cells expressing this marker might be responsible for the continued proliferation of cells. Murine cardiosphere-derived cells (CDCs) spontaneously beat under *in vitro* conditions whereas human cells require co-culture with rat cardiomyocytes to acquire this trait. But human cardiosphere-derived cells differentiated into cardiac myocytes, endothelial and smooth muscle cells after injection into the infarcted hearts of immunodefecient mice (Messina *et al.* 2004; Barile *et al.* 2007). Smith *et al* showed that human CDCs injected into the border zone of myocardial infarcts engrafted and migrated into the infarct zone (Smith *et al.* 2007). After 20 days, the percentage of viable myocardium within the infarct zone was greater in the CDC-treated group than in the control. Left ventricular ejection fraction was also significantly higher in the CDC-treated group. The study had shown the feasibility of generating human cardiospheres and expanding stem cells from routine endomyocardial biopsy specimens. Thus cardiac stem cell therapy may well change our fundamental approach to the treatment of heart diseases.

II.5. ORIGIN OF CARDIAC STEM CELLS

Following the recognition of a tissue-specific stem cell in the adult heart, the question concerned whether this primitive cell population originates, lives, and dies within the myocardium or whether other organs continuously replenish the heart with an undifferentiated stem cell pool that subsequently acquires cardiac characteristics. The bone marrow constitutes the main reservoir of primitive cells in the organism, and these cells can migrate from the marrow niches, enter the systemic circulation, and repopulate the heart and other organs (Grove *et al.* 2004; Quesenberry *et al.* 2004; Theise and d'Inverno 2004; Lakshmipathy and Verfaillie 2005). However, this possibility has never been definitely proven. Bone marrow homeostasis is primarily maintained by the division of HSCs *in situ* (Weissman *et al.* 2001; Shizuru *et al.* 2005) and in the absence of myocardial injury, circulating HSCs do not home to the heart (Wagers *et al.* 2002; Wagers and Weissman 2004). A model of heterochronic parabiosis (McCay *et al.* 1957; Tauchi and Sato 1980) has confirmed that rejuvenation of aged muscle mass depends on circulating molecules contained in the peripheral blood of young animals and not on young HSCs homed to old skeletal muscle (Winn *et al.* 2002; Conboy *et al.* 2003; Conboy *et al.* 2005; Conboy and Rando 2005). This argues against the bone marrow as the source of primitive cells in the heart. The contribution of bone marrow cells to cardiac chimerism was investigated (Korbling *et al.* 2003) by a comparative analysis made between the degree of chimerism in cardiac allografts and in hearts of patients who received allogeneic bone marrow transplantation (Thiele *et al.* 2004). In the latter case, only 2–5% chimeric myocytes were detected, while 14–16% of chimeric myocytes and endothelial cells were found in transplanted hearts. These observations suggest the

intracardiac origin of the recipient cells in the donor heart and the extracardiac origin of chimeric cells in the resident heart following bone marrow transplantation. In the first case, host cells may have migrated from the residual atrial stumps to the donor heart (Quaini *et al.* 2002) and, in the second, donor cells may have reached the myocardium because of the high level of blood chimerism (Thiele *et al.* 2004). The blood-borne cardiac cells were detected exclusively when the peripheral blood contains a large number of HSCs. But whether HSCs represents the precursors of CSCs during the regenerative response of the damaged heart remains an unresolved issue. The striking discrepancy between the incidence of heart failure and bone marrow failure and the lack of comorbidity of these disease states in the same patient indicates that HSCs do not typically migrate from the bone marrow and repopulate the decompensated heart. If the bone marrow continuously replenishes the heart with new functionally competent HSCs, the decline in myocyte number with cardiac diseases would not occur, and the poorly contracting myocytes would be constantly replaced by a bone marrow-derived progeny. Current knowledge supports the notion that primitive cells are present in the heart during embryonic life and regulate heart morphogenesis and postnatal development.

Organogenesis begins with the specification of a progenitor cell population (Pandur 2005). The pool size of embryonic cardiac progenitors conditions the final dimension of the organ. For example, retinoic acid reduces the number of prenatal cardiac progenitors leading to a hypoplastic heart (Keegan *et al.* 2005). Heart morphogenesis is a complex process that results from the assembly of subsets of myocardial cells that exert contractile function but express different genes (Solloway and Harvey 2003; Sieber-Blum 2004; Wessels and Perez-Pomares 2004). Initially, it was believed that

distinct unipotent progenitors give rise to the myocytes that populate the different parts of the heart (Mikawa and Fischman 1996). More recently, the early segregation of two lineages of myocytes from a common precursor has been documented (Meilhac *et al.* 2004; Meilhac *et al.* 2004). According to this theory, the primitive left ventricle and the outflow tract are derived from a single lineage, while the other regions including the primitive atria and the right ventricle are colonized by both lineages. The differential expression of transcription factors, Nkx2.5, Tbx5, Isl1, eHAND, and dHAND in distinct regions of the heart supports this possibility (Solloway *et al.* 2003; Pandur 2005). The studies finally led to the unequivocal documentation of the intracardiac origin of adult myocytes and have excluded the possibility that a secondary immigration of stem cells from distant organs occurs in adulthood under physiological conditions.

II.6. CARDIAC STEM CELLS AND THEIR NICHES

CSCs are undifferentiated cells that express on the membrane the stem cell-related antigens, c-kit, MDR1 and Sca-1 in variable combinations (Linke *et al.* 2005). Although these stem cell antigens have been unequivocally detected in CSCs, there is no single marker capable of providing an absolute identification of these cells *in vivo*. It has been reported that stem cells are stored in niches that are located deep in the tissue for protection from damaging stimuli (Palmer *et al.* 2000; Watt and Hogan 2000; Palmer 2002). The niche constitutes the microenvironment in which primitive cells divide, differentiate, and die. The recognition of stem cells within their natural milieu is of crucial importance and they do not exist in the absence of supporting cells within the niche (Spradling *et al.* 2001).

Stem cells play a crucial role in the organization and specification of niches (Fuchs *et al.* 2004). The structure of the niche appears to be specifically tailored to suit the particular needs of its resident stem cells. Niches control signal transduction pathways that regulate the slow-cycling, self-renewing, undifferentiated state of their resident cells (Allman *et al.* 2002; Berdnik *et al.* 2002; Campos *et al.* 2004). Niches in various self-renewing organs may differ in architectural organization and cellular composition.

Stem cell replication is mostly confined to the niches (Spradling *et al.* 2001). Stem cells undergo symmetric or asymmetric division (Jan and Jan 2001; Estivill-Torrus *et al.* 2002; Jafar-Nejad *et al.* 2002). When stem cells divide symmetrically, two self-renewing daughter cells are formed, and the purpose of this mechanism of growth is the expansion of the stem cell compartment (Watt and Hogan 2000). When stem cells engage in asymmetric division, one daughter stem and one daughter amplifying cell are obtained. The objective of this division is cell differentiation, i.e., the production of a committed progeny. Stem cells can also divide symmetrically into two committed amplifying cells, decreasing the number of primitive cells (Watt and Hogan 2000). The developmental choice made by stem cells at any given time has a direct impact on the stem cell pool size, the number of progenitors and precursors and, ultimately, the number of mature cells (Fuchs E *et al.* 2004; Lai 2004). The direct physical interactions between stem cells and their non-stem cell neighbors in the niche are critical in keeping stem cells in this specialized compartment and in maintaining stem cell characteristics (Lin 2002; Calvi *et al.* 2003; Arai *et al.* 2004). For example, in organs that possess an epithelial lining, such as the skin niches, the supporting cells or

nurse cells which maintain the niche are the dermal papilla cells and the mesenchymal cells underlying the crypt (Kim and Shibata 2002; Tumbar *et al.* 2004).

The niche characteristics are applicable to all organs whether bone marrow, brain, or heart. Recently CSC clusters have been found in the adult heart (Nadal-Ginard *et al.* 2003a; Chimenti *et al.* 2003; Urbanek *et al.* 2005). Although CSC clusters are scattered throughout the myocardium, their distribution appears to be conditioned by distinct levels of wall stress. The frequency of CSC clusters is inversely related to the hemodynamic load sustained by the anatomical regions of the heart: they accumulate in the atria and apex and are less numerous at the base and midportion of the left ventricle. Physical forces, mechanical deformation, and high wall stress can be transduced in intracellular responses that regulate cell behavior and fate. In a fashion similar to chemical cues, i.e., cytokines and hormones, local forces modulate cell migration, proliferation, differentiation, and death (Cheng *et al.* 1995; Sadoshima and Izumo 1997; Leri *et al.* 1998; Estes *et al.* 2004) CSCs nested in the atrial microenvironment are implicated in the preservation of the CSC pool through symmetric division and the formation of self-renewing daughter cells. Conversely, the very high degree of stress at the base and midregion of the LV wall may condition the turnover of mature progeny and CSC commitment. Ventricular niches are mostly involved in the formation of transient amplifying cells while apical niches exert a dual function.

In a CSC niche, lineage negative cells expressing stem cell antigens have been clustered together separately or in combination, with cardiac progenitors and precursors. In close proximity to lineage negative cells, myocytes and early

differentiating cells are present. The structural organization of a CSC niche is different from that of niches in epithelium (Braun *et al.* 2003), seminiferous tubes of the testis (Shinohara *et al.* 2001), and gut crypts (Potten *et al.* 2003). In contrast, similarities in the spatial arrangement of candidate stem cells and committed cells can be found with mouse bone marrow (Yoshimoto *et al.* 2003) and rat brain (Palmer 2002; Doetsch 2003) niches. However, nurse cells in these organs have been characterized while they remain to be identified in the heart.

Stem cells and supporting cells in the niches interact structurally and functionally through specialized gap and adherent junctional proteins (Fuchs *et al.* 2004). The passage of small molecules and signals that are involved in cell-to-cell communication are mediated by the gap junction channel proteins, connexins (Goldberg *et al.* 2004). These gap junctions are involved in the activation, commitment, and migration of stem cells out of the niches (Montecino-Rodriguez and Dorshkind 2001; Paraguassu-Braga *et al.* 2003; Fuchs *et al.* 2004). For example, survival factors and mitogens traverse gap junctions to oppose cell death and favor cell growth. The identity of these molecules is largely unknown. Ca^{2+}, ATP, adenosine, and cyclic nucleotides can translocate from one cell to another via gap junctions (Goldberg *et al.* 2004).

The molecular glue that holds stem cells within the niches is represented by the cadherin family of proteins, which, together with β-catenin, participate in the formation of specialized intercellular junctions, called adherens junctions (Yap and Kovacs 2003). When differentiating stem cells lose contact with the neighboring cells, they proliferate more easily and change location within the niche. Cadherins are

calcium-dependent transmembrane adhesion molecules (Yap and Kovacs 2003; Perez-Moreno *et al.* 2003), which have a dual function; they anchor stem cells to their microenvironment and promote interaction between stem cells and between stem cells and supporting cells. Stem cell anchorage depends also on integrin receptors that bind cells to a basal lamina composed of extracellular matrix (Jalali *et al.* 2001). Elevated levels of integrins are often characteristic of stem cells. Both integrins and adherent junctions play a critical role in the maintenance of adhesiveness and quiescent status of stem and early committed cells within the organ (Perez-Moreno *et al.* 2003).

The recognition of cardiac niches and the identity of the supporting cells constitute a major challenge for the definitive proof that the heart is a self-renewing organ in which cardiac homeostasis is regulated by a stem cell compartment. The niches are expected to control the physiological turnover of myocardial cells and the growth, migration, and commitment of primitive cells leaving the niches to replace old dying cells in the myocardium.

II.7. CHALLENGES OF CELL BASED THERAPY

Living cells are far more complex than most other biological products & hence evaluation may need to be more involved than for other types of products. Current understanding of cellular & developmental biology does not allow definitive conclusions regarding a cell-based product to be based on analytical procedures alone. A major challenge in the routine use of cell therapeutics in the clinic is the development of quality controls for efficacy on the basis of clinically relevant potency assays, with prospective validation in human clinical trials (De Bari and Dell'accio 2008).

Major challenges in stem cell research include:

1. Difficulty in identifying stem cells in tissue culture conditions
2. Once identified and isolated, the appropriate biochemical solution must be developed to differentiate these cells into the desired cell type
3. Ethical controversy surrounding the use of ES and EG cells and
4. More basic research must be done in order to fully understand the events that lead to cell specialization in humans.

II.7.1. CHALLENGES TO CELLULAR CARDIOMYOPLASTY (CCM) EMPLOYING CARDIAC STEM CELLS

Several challenges remain to successfully repair infarcted or failing myocardium with any type of cell and they are complicated by the extreme heterogeneity of cardiovascular disease. The criteria for reproducible engraftment of large numbers of cells may be very different in the early post-myocardial infarction patient versus the patient with end-stage cardiac dysfunction. Similarly, the method of delivering cells (surgical versus endovascular), the concentration of cells delivered and a host of other criteria ranging from age to co-existing disease states have to be considered as cell transplantation emerges into clinical trials (Taylor 2001). The most significant challenge in myocardial cell repair is to halt the progression of cardiovascular deterioration. Preclinical studies suggest that administering growth factors may help improving diastolic dysfunction (Atkins *et al.* 1999; Hutcheson *et al.* 2000). To improve the contractility of the infarcted heart, we must implant cells that can

electrically couple with the existing cells of the heart and the implanted cells should survive for extended periods of time. Hence it is advantageous to depend on engineered grafts rather than simple cell engraftments, in which the nutrient delivery and blood supply in the infarcted region is guaranteed. Additionally the graft will protect the surrounding myocardium from mechanical remodeling and decompensation secondary to grafting. However, more research has to be done in this area to bring the idea to the forefront.

Another attempt was to establish feeder layers to support the growth of stem cells (Majka *et al.* 2005). Stem cells are able to proliferate and differentiate in long-term cultures in the presence of feeder layers (Sutherland *et al.* 1989; Dexter *et al.* 1977). Recently in order to further increase the stem cell promoting potential of the feeder layer cells, genetic modification has been introduced. The approach is based on introduction of genes that inhibit differentiation of target cells. The Notch pathway which controls different cellular processes including proliferation, differentiation or apoptosis is responsible for maintaining various cell types in the undifferentiated state (Karanu *et al.* 2000; Maillard *et al.* 2005). Another way is to directly modify stem cells based on our understanding of the molecular pathways responsible for stem cell self-renewal and proliferation. The experimental data so far obtained show that controlled manipulation of gene expression by stem cells could lead to development of new therapeutic strategies.

II.7.2. GROWTH FACTORS AND CARDIAC STEM CELLS

Another major challenge in employing cardiac stem cells for therapy is the difficulty in obtaining sufficient number of cells for treatment. Because of that, since their

discoveries there have been a growing number of different approaches to increase their number *in vitro*. Several different cytokines and growth factors were attempted to increase the stem cell numbers in culture. The goal was that they would induce proliferation but not differentiation in the culture (Luskey *et al.* 1992; Bryder & Jacobsen 2000). It has been reported that paracrine secretion of survival and angiogenic factors contribute to improvement in cardiac function at the molecular level (Hamano *et al.* 2002; Tang *et al.* 2004; Tang *et al.* 2005). The role of vascular endothelial growth factor (VEGF) in bringing out angiogenic responses in infarcted myocardium has been well studied (Fazel *et al.* 2006). Similarly, paracrine secretion of multiple arteriogenic cytokines from MSCs has been shown to enhance proliferation of endothelial and smooth muscle cells and increase collateral flow and remodeling in a murine hind limb ischemia model (Kinnaird *et al.* 2004; Kinnaird *et al.* 2004). This attenuated muscle atrophy and fibrosis by the release of factors like VEGF and fibroblast growth factor 2 (FGF2) (Kinnaird *et al.* 2004; Kinnaird *et al.* 2004). Paracrine secreted hormones act as survival factors by working directly on the constituting cells of the myocardium or by mobilizing resident CSCs for myocardial repair. In another study reported, injection of survival factors like hepatocyte growth factor (HGF) and insulin-like growth factor 1 (IGF-1) into the border zones of the infarcted myocardium boosted the repair of the damaged cardiac tissue (Urbanek *et al.* 2005). Intramyocardial injection of these growth factors (GFs) promoted the translocation of CSCs-EECs (early committed cells) to the damaged area which in turn activated their growth and differentiation eventually resulting in the formation of functionally competent myocardium (Wollert and Drexler 2005). Hence, the growth,

proliferation and differentiation of stem cells require the presence of additional factors whose controlled action promote efficient myocardial repair.

II.7.3. SIGNALING MECHANISMS IN CARDIAC STEM CELLS

The quiescent state is thought to be an indispensable property for the maintenance of stem cells. Interaction of stem cells with their particular microenvironments, known as the stem cell niches, and some soluble factors is critical for maintaining the stem cell quiscence. A classic example is TGFβ, which plays a major role in maintaining hematopoietic stem cells (HSCs) in quiescent state (Fortunel *et al.* 2000). TGFβ is a prototypical growth factor that affects hematopoietic stem cells very differently depending upon the state of differentiation of the cells. For myeloid committed progenitor cells, TGFβ induces differentiation. For HSCs, it helps to maintain them in a quiescent undifferentiated state. In both cases, there is an inhibition of cell cycle activation, and the cell-specific effects are due to the different relative expression levels of multiple transcription factors. Stem cells themselves have intrinsic characteristics that differ from other cells including specific combinations of nuclear factors, chromosomal modifications, and mitotic clocks (Cheung *et al.* 2000). These intrinsic clocks determine the frequency and number of cell divisions. This includes telomerase and cyclin dependent kinase inhibitors which regulates the cell cycle. Cyclin dependent kinases (CDKs) regulate the cell cycle at different check points. The CDK inhibitors include p21, p27 and p18. These inhibitors play distinct roles in the regulation of stem cell renewal and differentiation. The CDK inhibitor p21 acts to maintain a stem cell in quiescent state while p27 and p18 more specifically inhibit cycling of committed progenitor cells (Cheng *et al.* 2000).

The mechanisms controlling CSC self-renewal or differentiation remains a mystery. The important players retaining the quiescent state of CSCs have to be identified. It is unclear if this cell fate decision is controlled by a purely stochastic mechanism or is the result of environmental cues mediated through specific receptor – ligand interactions. It is likely that cell fate is influenced both by the stochastic nature of gene expression and by soluble factors and cell-cell interactions. The presence of specific combinations of growth factor receptors will prime the cells for induction when they are exposed to specific growth factor combinations present in the microenvironment.

The proliferation of CSCs appears to be dependent on the capacity of the cells to undergo cell cycle progression through the phosphorylation of Akt in response to EGF and bFGF stimulation, as observed in neural stem cells (Groszer *et al*. 2006). Nuclear-targeting of Akt leads to the rapid expansion of cKit$^+$ CSCs in the postnatal heart (Gude *et al.* 2006). The functional improvement of damaged myocardium after CSC transplantation was attenuated by knock down Sca-1 gene, in which new vessel formation and inhibition of myocardial apoptosis by release of angiogenic growth factors and myocyte regeneration by grafted CSCs were severely impaired (Tateishi *et al.* 2007). This implicates the role played by Sca-1 in CSC maintenance and function. Sca-1-mediated signaling is important in CSC development in normal circumstances and its beneficial effect might be involved the responses to hypoxic and ischemic conditions. The cardioprotective effect of CSC transplantation indicates that Sca-1-mediated ligand responses may participate in the production of angiogenic and antiapoptotic paracrine effectors (Tateishi *et al.* 2007). Hence it will be of interest to assess the gene expression profile in CSCs by targeting Sca-1 transcripts to identify

the factors responsible for optimizing CSC therapy in heart failure. The ILK signaling pathway is also used as a means for cardiac stem cell proliferation and self-renewal.

II.7.4. CAD RISK FACTORS AND CARDIAC STEM CELLS

In patients with more number of CAD risk factors, or those having a severe form of the disease, culture of the primary progenitor cells is limited by increased senescence and/or decreased proliferation. Hence, appropriate strategies such as *ex-vivo* transfection of cells with relevant genes may be followed before using them as therapeutic tools. The identification of resident stem cells in the heart provides hope for an adult heart derived stem cell therapy in patients with ischemia and chronic heart failure. However, it is not yet clear whether in patients with chronic CAD, the yield of CSCs would be influenced by disease severity and risk factors for CAD or the presence of individual risk factors would influence the yield of cells that can be isolated from cardiac biopsies. The issue remains to be clarified before the clinical utility of heart-derived stem cells is accepted.

II.8. REGULATORY CONSIDERATIONS IN MANUFACTURING, PRODUCT TESTING AND PRECLINICAL DEVELOPMENT OF CELLULAR PRODUCTS FOR CARDIAC REPAIR

The successful initiation of a clinical trial with an investigational cellular therapy for cardiac repair requires not just a thorough knowledge of clinical medicine and clinical trial design, but also a working knowledge of the other inherent disciplines namely manufacturing and preclinical testing.

II.8.1. PRODUCT MANUFACTURING AND TESTING

II.8.1.1. Product considerations for cardiac cellular therapies

The mechanism of cardiac regeneration involves a combination of events including cardiomocyte generation/regeneration, angiogenesis/revascularization, production of cytokines or other secreted factors or a combination of these actions. During the initiation of a clinical investigation, product safety is the primary concern. During manufacturing, safety has to be ensured through the implementation of a controlled process combined with testing of the cellular product before administration to the subjects. It is important to confirm that all the devices and materials used for processing or administration of the cellular product are compatible with the cell suspension components. In addition, parameters to product characterization such as source, phenotype, purity and the number and proliferative potential of the cells should also be considered. Appropriate identity and potency tests must also be done and validated so that when the product is shown to be safe and effective, each commercial lot of that product should have the same safety, purity and potency.

II.8.1.2. Sources and collection of cellular products for cardiac repair

Different sources employed for cardiac repair includes: -

1. bone marrow-derived cellular products
2. muscle-derived cellular products
3. embryo-derived cellular products and
4. tissue stem cells

To participate in the regeneration of cardiac function, these cells must be able to involve in a variety of activities which are not usually associated within their tissue of origin including revascularization, muscle regeneration and electrical conduction. Although the cells may not normally perform all these activities, they can be induced to expand and differentiate into a variety of cell types when cultured *ex vivo* with cytokines and growth factors (Gunsilius *et al.* 2001). For example, unfractionated bone marrow cells, which do not normally secrete measurable amounts of VEGF can do so after 4 weeks in culture indicating the existence of a cell population with the potential to facilitate angiogenesis when introduced into myocardium (Fuchs *et al.* 2003).

II.8.2. Testing of cellular products for cardiac repair

Regardless of the type of the product and the manufacturing process, a variety of parameters must be evaluated by adequate and appropriate testing to ensure administration of a safe product. This includes: -

1. infectious disease testing
2. microbiological safety
3. cell dose and viability and
4. product characterization which consists of

- product identity
- product potency and
- product purity

II.8.3. Product testing during clinical development

In all investigational phases, product safety testing must be performed on each lot of cellular product. Testing and screening of the donors should also be performed. Microbiological safety testing is necessary to ensure administration of a sterile product and the risk of contamination should be avoided maximum. The cell dose and viability should be measured to ensure safety and consistency. Fully validated assays are required until the product reach phase 3 clinical trials.

II.9. Preclinical studies

II.9.1. General toxicological principles applied to cellular products

Cellular products are complex and preclude a standard design of preclinical studies. The major sources of cellular product's complexity include: the inherent biological heterogeneity, potential safety concerns posed by the different routes of administration, cell-device interactions and the effects of immune responses to the product. The animal models of disease are to be frequently modified to generate the preclinical toxicity data needed for regulatory decisions on trials of cellular therapies. The endpoints to collect sufficient toxicology data will vary depending on the animal models used, the product tested and the clinical trial design. Knowledge of a product's mechanism of action acquired during preclinical trials is beneficial in the design and conduct of clinical trials. It can also guide clinical trial design in terms of dosage regimens, monitoring modalities, monitoring intervals *etc.* Clinical trial designs based on hypothesis supported by *in vitro* data alone results in anecdotal experiences. Another concern is the immunological complications underlying the use of allogenic cell types. The immunological reaction to human products in animals often necessitate

that preclinical studies be performed with animal cellular products that are analogous to the intended clinical product, rather than the actual human product. The degree of understanding of the relationship between an animal cell and its human correlate is an important factor in determining human risk assessment (Areman *et al.* 2006).

II.9.2. Preclinical testing of cellular products for cardiac repair

Cellular products presently used in clinical trials for cardiac repair are the bone marrow cells (hematopoietic and mesenchymal lineages) and the skeletal muscle cells. The manufacturing processes of the harvested cells to produce the ultimate cellular product for administration represent a very broad range of processes. Several routes of administration are currently under investigation. They are (1) direct, syringe- and –needle injection through the exposed epicardial surface during the concomitant thoracic surgery; (2) infusion of the cellular products into the coronary arteries; and (3) intramyocardial injection of cell suspensions through cardiac catheters (Areman *et al.* 2006). The diversity inherent in the combination of a specific cellular product, route of administration and clinical indication results in an extensive array of combinational possibilities which are to be explored.

III. MATERIALS AND METHODS

III.1. MATERIALS

III.1.1. Fine Chemicals

Iscove's Modified Dulbecco Medium, Dulbecco's Modified Eagle's Medium-Ham F12 mix, fetal bovine serum, β-mercaptoethanol, bovine serum albumin, trypsin, ethylene diamine tetra acetic acid, monoclonal anti-vimentin antibody, anti-human von Willebrand antibody, L-glutamine, gelatin, TRI reagent, Tris base, thrombin and agarose were purchased from Sigma Aldrich, MO, USA. Collagenase type IV and B27 were purchased from Gibco Invitrogen Corporation, NY, USA. Epidermal growth factor, basic fibroblast growth factor, vascular endothelial growth factor, insulin-like growth factor, hepatocyte growth factor, transforming growth factor β and platelet derived growth factor were procured from US Biologicals, USA. Anti-human antibodies against ckit, MDR1, CD34, cTN1 and MHC were obtained from Santa Cruz Biotechnology, Inc., CA, USA. FITC labeled anti-rabbit secondary antibody and rodamine red X labeled anti-mouse secondary antibody were purchased from Molecular Probes, Netherlands. RT-PCR reagents were procured from Promega Corporation, Madison, WI, USA. Mac-1, Gr-1 and B220 markers, poly-D-lysine and fibronectin were procured from BD biosciences, USA. PD098059, SB 203580 and Wortmannin were obtained from Cell Signaling Technology, Inc, MA, USA. RT-PCR reagents were procured from Promega Corporation, Madison, WI, USA.

III.1.2. Routine Chemicals

Potassium chloride, potassium dihydrogen phosphate, sodium bicarbonate, sodium chloride, sodium dihydrogen phosphate, sodium hydroxide, tricholoroacetic acid,

concentrated hydrochloric acid and ethanol were purchased from SISCO Research Laboratories, India.

III.1.3. ELISA Kit

PathScan Phospho-p38 MAPK Sandwich ELISA Kit was purchased from Cell signaling technology, Inc., MA, USA)

III.1.4. MACS microbead kit

CD117 microbead kit was procured from Miltenyi Biotech, Germany.

III.1.5. Cell proliferation kit

Rapid cell proliferation kit was purchased from Calbiochem, Inc., Darmstadt, Germany.

III.1.6. Cell culture wares

35mm and 100mm cell culture dishes, T25 cell culture flasks, 4 well plates, 24 well plates an 96 well plates were purchased from Nunc, Denmark. Cell culture filter ware was procured from Millipore, USA.

III.1.7. Equipments used

ELISA reader (Bio-Tek instruments, USA), UV-visible spectrophotometer (Shimadzu, Japan), high speed refrigerated centrifuge (Hitachi, Japan), weighing balance (Sartorius, Germany), water bath (LKB, Sweden), ice making machine (Hoshizaki, Japan), pH meter (Labindia, India), CO_2 incubator (Sanyo, Japan), phase-contrast microscope (Nikon, Japan), laminar flow hood (Clean Air, USA), magnetic

concentrated hydrochloric acid and ethanol were purchased from SISCO Research Laboratories, India.

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activated cell sorting system (Miltenyi Biotech, Germany), fluorescence activated cell sorting machine (BD FACS Aria, USA), magnetic stirrer (Schott, Germany), EASY pure UV/UF compact reagent grade water system (Barnstead, USA), transilluminator, submarine electrophoresis unit (Bangalore Genei, India), and programmable thermal cycler (MJ Research Inc, USA).

III.2. COMPOSITION OF MEDIA, REAGENTS AND BUFFERS

III.2.1. Agarose Gel for DNA (1%)

200 mg agarose in 20 ml 0.5X TBE.

III.2.2. Complete explant medium

Iscove's Modified Dulbecco Medium containing 10% FBS, 50 U/mL benzyl penicillin, 50 μg/mL streptomycin, 2 mmol/L L-glutamine and 0.1mmol/L 2-mercaptoethanol.

III.2.3. Cardiosphere growing medium

35% complete IMDM/65% DMEM Ham F-12 mix containing 2% B27, 0.1mmol/L 2-mercaptoethanol, 10ng/mL epidermal growth factor, 20ng/mL fibroblast growth factor, 40nmol/L thrombin, 2 mmol/L L-glutamine, 50 U/mL benzyl penicillin and 50 μg/mL streptomycin.

III.2.4. Diamino benzidine

6 mg diamino benzidine in 10 ml Tris (pH 7.6) containing 10 μl of 30% H_2O_2.

III.2.5. Diethyl Pyrocarbonate (DEPC) treated water

0.1% DEPC in deionised water.

III.2.6. DNA/RNA gel-loading dye

Bromophenol blue (0.25%); xylene cyanol FF (0.25%); EDTA (1mM); glycerol (50%) in DEPC-treated deionized water.

III.2.7. Ethidium bromide (Stock solution)

1mg in 1ml deionized water; 5μl of this stock solution was added to 20ml of 1% agarose gel for DNA/RNA electrophoresis.

III.2.8. Lysis buffer

1x buffer containing 1mM PMSF.

III.2.9. MACS buffer

PBS of pH- 7.4 containing 0.5% BSA and 2mM EDTA.

III.2.10. Phosphate Buffered Saline (PBS) (pH 7.4)

Sodium chloride 137 mM; Potassium chloride 2.7 mM; Disodium hydrogen phosphate 10.14 mM; Potassium dihydrogen phosphate 1.76 mM.

III.2.11. Tris Borate EDTA Buffer (TBE) 5X (pH 8.3)

54 g Tris base; 27.5 g boric acid; 20 ml 0.5 M EDTA (pH 8.0) per liter.

III.2.12. Trypsin/EDTA solution

PBS of pH 7.4 containing 0.25 mg/ml trypsin and 0.2 mg/ml EDTA.

III.3. ISOLATION, CULTURE AND CHARACTERIZATION OF CARDIAC STEM CELLS

III.3.1. Isolation of ckit ^{pos} stem cells

All experiments had the approval of the Institutional Ethics Committee. Right atrial appendages were obtained from patients with ischemic heart disease who underwent coronary artery bypass grafting at our institute.

Two different isolation methods were attempted at for the isolation of ckit ^{pos} cells – an enzymatic method and a non-enzymatic method.

In the enzymatic method, ckit ^{pos} cells were isolated from right atrial samples using the method described by Messina *et al.* (2004).

Right atrial samples were collected immediately after cannulation in ice cold Ca^{2+}-Mg^{2+} free PBS (Ref III.2.10) containing 50 U/mL benzyl penicillin and 50 µg/mL streptomycin. The atrial tissue was cut into 1-2mm^3 pieces and washed with PBS several times to remove fat and contaminating red blood cells. The tissue fragments were digested three times for 5 minutes at 37°C with 0.2% trypsin and 0.1% collagenase IV. The obtained cells were discarded, and the remaining tissue fragments were washed in complete explant medium (Ref III. 2. 2) and cultured as explants in CEM at 37°C and 5% CO_2 in 2% gelatin coated culture dishes. Medium change was done every two days without disturbing the adherent explants. After a period of

incubation, ranging from 1 to 3 weeks, a layer of fibroblast-like cells was generated from adherent explants over which small phase-bright cells migrated.

In the non-enzymatic isolation method, after sufficient washing with Ca^{2+}- Mg^{2+}-free PBS, the sample tissue was minced into 1–2-mm^3 pieces and cultured as explants in gelatin-coated culture dishes in the above-mentioned culture medium (Urbanek *et al.* 2005). Every 2 days, the medium was changed without disturbing the loosely adherent explants.

III.3.2. Processing of sphere forming cells

Cells that migrated from explants of both procedures were similarly processed. They were collected by pooling 2 washes with Ca^{2+}- Mg^{2+}-free PBS, one wash with EDTA, and then with trypsin/EDTA solution (Ref III. 2. 12) at 37°C. After passing through a 50-micron nylon mesh, the cells were re-suspended in cardiosphere growing medium (Ref. III. 2. 3). The cells obtained were seeded at ~0.5 to 2 $\times 10^5$ cells/mL in poly-D-lysine-coated culture dishes. Within 24 hours of culture, the seeded cells formed loosely adherent small round clusters (cardiospheres).

Isolation of the cardiosphere-forming cells could be performed at least 4 times at 6- to 10 day intervals from the same explant.

III.3.3. Characterization of the isolated stem cells

III.3.3.1. Morphology

The cells were routinely viewed under an inverted phase contrast microscope to analyze their morphology and growth pattern.

III.3.3.2. Immunocytochemistry

Migrated cells were washed thrice with PBS and fixed in 100% ice-cold methanol for 10 minutes. After two washings in PBS, endogenous peroxidase was quenched with two drops of 3% hydrogen peroxide for 5 minutes. Cells were again washed with PBS. Nonspecific binding was blocked with 2% BSA in PBS for 10 minutes. Two drops of primary antibody diluted in PBS containing 1% BSA was added and incubated for 60 minutes. The cells were washed thrice with PBS and incubated for 30 minutes with diluted secondary antibody conjugated with either HRP. Cells were washed thrice with PBS and incubated with either the substrate reagent diaminobenzidine (Ref. III. 2. 4) for up to 10 minutes and observed under a microscope. After sufficient color development, the cells were washed in deionized water for 5 minutes. The cells were counterstained with hematoxylin, mounted in glycerol and observed under a microscope.

Monoclonal anti-human vimentin was diluted 1:50 and anti-human von Willebrand factor was diluted 1:800. Immunostaining was done using a commercially available kit in which the all reagents were provided ready-to-use.

III.3.3.3. Immunofluorescence

Migrated phase bright cells were stained for the expression of stem cell marker ckit. Positive control used for the experiment was gastrointestinal stromal tumor tissue (GIST). For immunohistochemical staining, the sections were dewaxed in xylene and rehydrated in descending grade series of alcohol followed by treatment with 3% hydrogen peroxide for blocking endogenous peroxidase. Target antigen was retrieved

by immersing the slides in 0.01M sodium citrate buffer (pH-6.0) followed by heating for 5 minutes in microwave oven. The sections were blocked with 3% BSA and incubated with primary antibody overnight at 1:50 dilution. After washing off unbound primary with phosphate buffered saline, incubated for 30 minutes with Alexa 350- conjugated anti-rabbit secondary antibody diluted 1:100. After washing off unbound secondary antibody, sections were mounted in glycergel and observed under fluorescent microscope.

III.3.3.4. Magnetic activated cell sorting analysis (MACS)

Characterization of the isolated small round cells for ckit expression was performed by MACS using an anti-human CD117 micro-bead kit, according to the manufacturer's manual. Briefly, the migrated phase-bright cells were collected by trypsin-EDTA treatment, pooled and incubated with monoclonal anti-human ckit antibody-coated micro-beads. The cell suspension was passed through a magnetic column placed in a strong magnetic field. The unlabeled cells ($ckit^{-}$) were washed out using MACS buffer (Ref III. 2. 9), and the labeled ($ckit^{+}$) cells were retained in the MACS column. The column was separated from the magnetic field, and the $ckit^{+}$ cell fraction was collected. The $ckit^{+}$ cells sorted by MACS were stained with Hoechst 33258, which stains the nucleus blue, and examined under a fluorescent microscope. MACS sorted $ckit^{+}$ cells were further stained with fluorescent labeled secondary antibody (CD117-PE) and analyzed by flow cytometer.

III.3.3.5. Fluorescence activated cell sorting (FACS)

For characterization of isolated phase-bright cells by FACS, the migrated cells (both phase-bright cells and the feeder cells) were first washed with PBS to remove the growth media completely, followed by dissociation with trypsin-EDTA (1X) solution for 1–2 min at 37°C. The cells were collected and centrifuged at 2,000 rpm for 5 min. After sufficient PBS washes, the cells were incubated with primary antibodies against CD117, MDR-1, CD34 and CD45 markers with a dilution of 1:50 at 4°C for 30 min. After incubation, the cells were washed with cold PBS to remove unbound primary antibodies, incubated with fluorescent-labeled secondary antibodies (anti rabbit – FITC for ckit & CD34 monoclonals, anti-mouse-PE for CD45 & MDR1 monoclonals) for 30 min at 4°C. Cells were washed with cold PBS to remove unbound antibodies, fixed with 4% paraformaldehyde, and stored at 4°C until analysis. The samples were analyzed with a 4-color multiparameter flow cytometer.

III.4. Analysis of cardiosphere formation under different growth and substrate conditions and characterization of cardiosphere cells

III.4.1. Cardiosphere formation

Cardiosphere formation under different growth and substrate conditions was analyzed. The growth conditions employed were IMDM with/without growth factors and CGM with/without growth factors. The substrate conditions used include gelatin, laminin, fibronectin and poly-D-Lysine.

III.4.2. Characterization of cardiosphere cells

For characterization of cardiosphere cells by fluorescence-activated cell-sorting analysis, the cardiospheres were first washed with PBS to remove the growth media completely, followed by dissociation with trypsin-EDTA (1X) solution for 1–2 min at 37°C. Undissociated clusters were subjected to mechanical trituration. The cells were collected and centrifuged at 2,000 rpm for 5 min. After sufficient PBS washes, the cells were incubated with primary antibodies against CD117, MDR-1, CD34, von Willebrand factor, cardiac troponin I and myosin heavy chain markers with a dilution of 1:50 at 4°C for 30 min. After incubation, the cells were washed with cold PBS to remove unbound primary antibodies, incubated with fluorescent-labeled secondary antibodies for 30 min at 4°C. Similarly conjugated isotype-matched monoclonal antibodies were used as controls. Cells were washed with cold PBS to remove unbound antibodies, fixed with 4% paraformaldehyde, and stored at 4°C until analysis. The samples were analyzed with a 4-color multiparameter flow cytometer.

III.5. Comparison between the enzymatic and non enzymatic isolation methods

Right atrial biopsy samples were obtained from 10 patients (2 women and 8 men) aged 42 to 72 years, with ischemic heart disease. The objective of the study was to identify an easy and cost-effective method for the isolation and expansion of human adult cardiac-resident stem cells. The cell yield and cluster formation was compared. The number of cardiospheres obtained per isolation were calculated based on the formula –

$$\text{No: - of CSs/cm}^2 = \frac{\text{Average No: - of CSs in 50 fields x100}}{1.13}$$

where 1.13 = Area of a field at 10X magnification

A minimum of 50 randomly selected fields were counted in each culture dish, and the number of cardiospheres per cm^2 was calculated.

III.6. Effect of disease severity and coronary artery disease risk factors on the number of migrating cardiac stem cells

III.6.1. Patient Characteristics: The study group comprised of 30 patients (M: F = 26:4; age: 38-72 years; 54.87 $\pm$ 8.9). Prevalence of cardiovascular risk factors such as hypertension, smoking, diabetes and dyslipidemia among the patients were noted based on the following definitions: Smokers as those having a history of smoking for more than 2 years; hypertensives (those with a systolic pressure above 140mmHg and a diastolic pressure above 90mmHg) for > 1year; diabetics: those who had the need for oral antidiabetic drug therapy or insulin use; presence of dyslipidemia: (those with serum levels of LDL >130 mg/dl, HDL <40 mg/dl, triglycerides >150 mg/dl and total cholesterol >200 mg/dl). The total vascular risk score was calculated for each patient by considering the risk factors, such as hypertension, diabetes, smoking, and dyslipidemia as reported earlier (Vasa M *et al, 2001).* Patients were grouped depending on the presence or absence of the individual risk factors as well as according to severity of coronary artery occlusion (the number of coronary arteries affected as assessed during coronary angiography), NYHA functional class, mitral regurgitation rates, wall motion abnormalities, total vessel blockage, involvement of right coronary artery and intake of drugs.

Patient profiles were collected from the Medical Records Division of our institute. The patient proforma prepared for data collection is given in appendix III.

III.7. Effect of growth factors on cardiosphere formation

Cardiosphere formation was analyzed under different growth factor conditions. The growth factors employed includes epidermal growth factor (EGF), basic fibroblast growth factor (bFGF), vascular endothelial growth factor (VEGF) and insulin-like growth factor (IGF) with/without 3.5% FBS at a concentration of 10ng/mL. The control used was cardiosphere growing medium (IMDM/DMEM HamF12 mix) supplemented with 3.5% FBS.

Effect of growth factors on cardiosphere formation was analyzed by

- determining cardiosphere size and
- cell proliferation assay.

III.7.1. Determination of cardiosphere size

Cardiospheres were cultured in 4-well culture dishes to determine the size. Four brightfield images were randomly captured from each well and the size of each cardiosphere was analyzed using image pro plus software. The size of the cardiospheres was defined as an equivalent circle diameter (D). Cardiospheres of D < 30 μm were excluded from the analysis because they were mainly single or paired cells.

III.7.2. Cell proliferation assay

Cardiosphere forming cells were seeded onto 96 well culture wells supplemented with 100μL of respective culture medium. Cells were incubated for 24hrs for sphere formation at 37^0C. Non radioactive cell proliferation assay was performed using WST cell proliferation kit according to manufacturer's manual. 10μL WST-1 reagent was added onto each well and incubated for 2hrs at 37^0C. WST was bioreduced by metabolically active cells, which was quantified by ELISA at 450nm.

III.8. Cardiosphere culture and characterization

III.8.1. Culture of isolated cardiospheres

Cardiosphere-forming cells were seeded at 2-3 x 10^4 cells/mL on poly-D-lysine coated dishes in cardiosphere growing medium. Several days later, cells that remained adherent to the poly-D-lysine–coated dishes were discarded, whereas detached cardiospheres were plated on fibronectin-coated culture dishes in 10% FCS containing medium and expanded as monolayers to form cardiosphere derived cells (CDCs). CDCs were subsequently passaged by trypsinization and splitting at a 1:2 ratio. Single cells were counted under phase microscopy with a hemocytometer.

III.8.2. Characterization of cardiosphere derived cells

III.8.2.1. Fluorescence activated cell sorting analysis

For characterization of cardiosphere-derived cells by fluorescence-activated cell-sorting analysis, the cells were first washed with PBS to remove the growth media completely, followed by dissociation with trypsin-EDTA (1×) solution for 1–2 min at 37°C. The cells were then collected and centrifuged at 2,000 rpm for 5 min. After

sufficient PBS washes, the cells were incubated with primary antibodies against CD117, CD31, CD34, CD45, CD133, cardiac troponin I, myosin heavy chain, Lin^{neg} markers (B220, Gr-1, Mac-220) markers with a dilution of 1:50 at 4°C for 30 min. After incubation, the cells were washed with cold PBS to remove unbound primary antibodies and then incubated with fluorescent-labeled secondary antibodies for 30 min at 4°C. Cells were washed with cold PBS to remove unbound antibodies, fixed with 4% paraformaldehyde, and stored at 4°C until analysis. The samples were analyzed with a 4-color multiparameter flow cytometer.

III.8.2.2. RNA isolation

Total RNA was isolated from cultured CDCs grown in 35 mm culture dishes using TRI reagent as per the instructions of the manufacturer. The purity of RNA was determined from the 260/280 ratio. The yield of RNA was calculated from the formula

$$\text{RNA in } \mu g/\mu l = \frac{OD260 \times 40 \times DF}{1000}$$

Where, OD_{260} is the OD at 260 nm

DF is the dilution factor and

40 is included assuming that OD of 1 ≡ 40 μg/μl of RNA

III.8.2.2.1. RNA gel

To determine whether the isolated RNA is intact, the 3 μl of RNA samples were run on 1% agarose gel (Ref. III. 2. 1) containing ethidium bromide (Ref. III. 2. 7), by submarine electrophoresis using 1X TBE buffer (Ref. III. 2. 11). The sample run was

tracked by adding 3 µl of the loading dye (Ref. III. 2. 6) along with the sample. The gel was run at 65 mA for 15 minutes.

III.8.2.3. cDNA synthesis

cDNA reaction mix (30 µl)

5X RT buffer 6.0 µl

dNTPs 2.5 µl

Oligo dT 3.0 µl

RNAse inhibitor 0.5 µl

RT 2.0 µl

RNA 10.0 µl

DEPC water 6.0 µl

The cDNA mix was incubated in a 37^{0}C water bath for 1 hour. The cDNA was stored at -20^{0}C till PCR reaction was performed.

III.8.2.4. RT PCR analysis for epidermal growth factor receptor

The primer sequences were:

EGFR

Forward primer: 5'-GAG AGG AGA ACT GCC AGA A-3'

Reverse primer: 5'-GTA GCA TTT ATG GAG AGT G-3'

PCR mix (50 µl)

10X Taq buffer	5.0 µl
$MgCl_2$	3.0 µl
dNTPs	3.0 µl
Forward primer	1.0 µl
Reverse primer	1.0 µl
Taq polymerase	0.5 µl
cDNA	3.0 µl
Sterile water	33.5 µl

PCR was done in a MJ thermal cycler and the cycling conditions followed were: denaturation at 94^0C for 1 min, annealing at 57^0C for 30s, extension at 72^0C for 90s. The cycle was repeated 30 times and the PCR products were final extended at 72^0C for 7 minutes. PCR products were analyzed by electrophoresis (Ref. III. 2. 11) on a 1% agarose gel and stained with ethidium bromide.

III.8.2.5. Proliferation assay

III.8.2.5.1. Evaluation of the effect of growth factors on cardiosphere derived cell proliferation

CDCs were seeded on 96-well plates in 100µl 10% FCS medium. Following overnight attachment, cells were washed with PBS & serum starved for 2hrs. After

washing with PBS, cells were incubated with 100μl of experimental conditions and incubated at 37^0C for 24hrs. The different growth factors employed for the assay includes –

- Epidermal growth factor (EGF)
- Basic fibroblast growth factor (bFGF)
- Vascular endothelial growth factor (VEGF)
- Insulin-like growth factor-1(IGF-1)
- Transforming growth factor β (TGF β)
- Hepatocyte growth factor (HGF)
- Platelet derived growth factor (PDGF)

Non radioactive cell proliferation assay was performed using WST-1 rapid cell proliferation kit. The protocol is same as that followed for cardiosphere proliferation studies as described in Section III. 6. 2. Concentration of the growth factors employed was 10ng/ml and the control media used was supplemented with 10% FBS.

III.8.2.6. Analysis of the effect of EGF on wound homeostasis

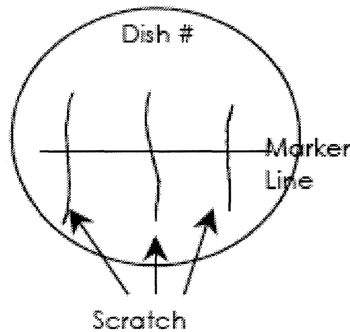
The effect of EGF on the migratory activity of cardiosphere derived cells was analyzed by (1) trans-well migration assay and (2) wound healing assay.

III.8.2.6.1. Trans-well migration assay

The migration assay was performed according to previously described protocol. The test agent EGF (10ng/mL) in 0.2% BSA containing medium was prepared and 600µl was added onto single wells of a 24-well plate. Polycarbonate filters were then positioned above the wells. CDCs were trypsinised and washed and 10^5 cells/ml suspended in 200 µl of 0.2% BSA containing medium was added to the upper chamber of the filter. The top half of the multi-well plate was reattached, and incubated at 37°C in 95% air-5% CO_2 for 24 h to allow a uniform cell attachment to the filter. After incubation, the filter was removed from the culture plate and fixed with methanol and counterstained with crystal violet stain. The control used was medium without the test agent. The number of migrated cells on the lower surface of the filter was counted in six randomly chosen fields and averaged. Experiments were performed in duplicate and were repeated three times.

III.8.2.6.2. Wound healing assay

CDCs were trypsinized and plated (1.5×10^5 cells) on 35-mm fibronectin coated culture dishes. After cell attachment, the medium was aspirated out and the cells were washed with PBS and maintained in serum deprived (2%) conditions and incubated overnight at 37^0C. On the day of the assay, medium was aspirated, and using a sterile 200 µl pipette tip, three separate wounds were made through the cells as shown.



Immediately after wounding, the cells were rinsed very gently with PBS and replaced with 1.5 ml of media. (Control -2% FCS containing media without any additives and test – media supplemented with 10ng/ml EGF). Phase contrast image was taken immediately with 4x objective as the 0hr reading. Images were then taken at 6, 12 and 24 hours subsequently. The images acquired for each sample were analyzed quantitatively using the Image Pro-Plus computing software. For each image, distance between one side of scratch and the other was measured (in microns). By comparing the images from time 0 to the last time point (24hr), distance of each scratch closure were obtained.

III.8.2.7. Identification of signaling pathway involved in epidermal growth factor induced proliferation of cardiosphere derived cells

III.8.2.7.1. Effect of inhibitors on EGF induced proliferation of cardiosphere derived cells

To delineate the signaling pathways involved, direct effects of pathway specific pharmacological inhibitors –such as those specific for p42/44 MAPK [PD098059, 10μM], p38 MAPK [SB203580, 10μM], and Wortmannin which inhibits ERK1/2, p38 MAPK and PI3K/Akt pathways respectively on EGF induced CDC proliferation were studied. Control was CDCs grown in EGF supplemented medium with out any

inhibitor. CDCs were pre-treated with inhibitors for one hour and then supplemented with growth medium containing 10ng/mL EGF. The cultures were then incubated at 37^{0}C and cell proliferation was assayed using WST-1 rapid kit. The protocol is same as described in Section III. 6. 2.

III.8.2.7.2. Assay of p38 MAPK activation in EGF induced cardiosphere derived cells

Activation of p38 MAPK in CDCs upon EGF treatment was assayed by PathScan Phospho-p38 MAPK Sandwich ELISA Kit according to the manufacturer's manual. CDCs were treated with medium containing regulator (EGF-10ng/mL) with/without the pharmacological inhibitor SB203580 for desired time periods (2 mins and 10 mins). Medium was removed immediately and cells were washed with ice cold PBS. Cells were harvested under nondenaturing conditions, and 0.1 ml ice-cold 1X Cell Lysis Buffer (Ref III. 2. 8) was added and incubated on ice for 5 minutes. Cells were aspirated in and out for sufficient time period and centrifuged for 10 minutes at 4°C. The supernatant was collected and assayed for MAPK activation by ELISA.

III.9. STATISTICAL ANALYSIS

For experiments with more than two variables, ANOVA was employed for the comparisons. Differences between experimental groups were assessed by Student's t-test. The values are expressed as mean ± SD. $p<0.05$ was considered statistically significant.

IV. RESULTS

IV.1. Isolation of ckit$^+$ human adult cardiac stem cells

Right atrial biopsy samples were obtained from patients who had undergone bypass surgery (Figure 3) and cultured as explants. After a period of incubation ranging from 1–3 weeks, a layer of fibroblasts was generated from well-adherent explants, over which small round phase-bright cells were seen to be migrated. Majority of the migrated phase-bright cells were seen in the vicinity of the explants. Cell migration was observed in both enzymatic and non enzymatic isolation methods (Figures 4 & 5). The maximum numbers of phase-bright cells were observed during the 3rd week of explant culture (Figures 6 & 7). Cell isolation could be performed at least 4 times at 6- to 10-day intervals from the same explant.

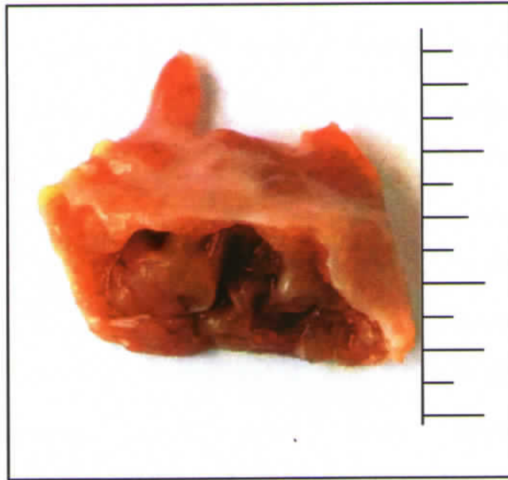


Figure 3: Gross photograph of a sample of right atrial appendage collected from a patient with coronary artery disease

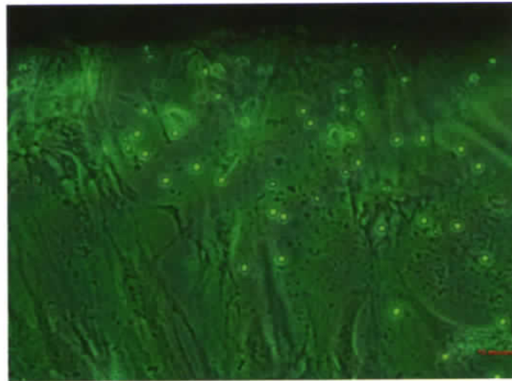


Figure 4: Photomicrograph of migrated phase-bright cells over fibroblast-like cells, Day 7 (Enzymatic method)

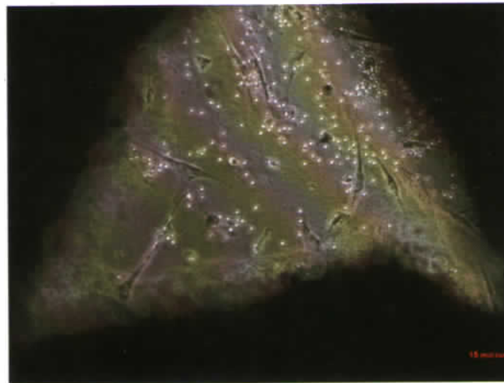


Figure 5: Photomicrograph of migrated phase-bright cells over fibroblast-like cells, Day 7 (Non-enzymatic method)

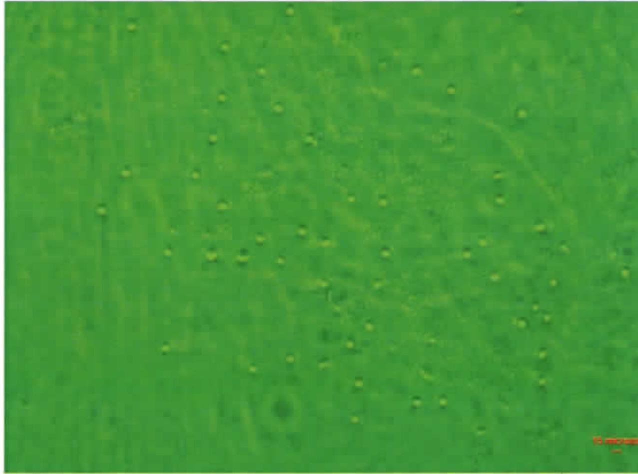


Figure 6: Photomicrograph of migrated phase-bright cells over fibroblast-like cells, 3rd week of explant culture (Enzymatic method)

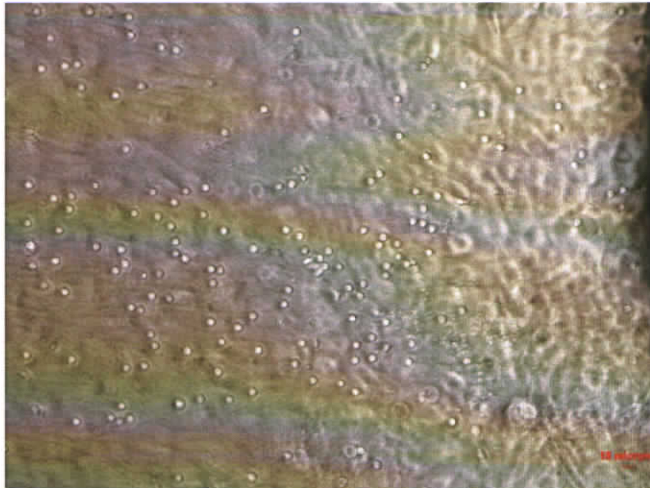


Figure 7: Photomicrograph of migrated phase-bright cells over fibroblast-like cells, 3rd week of explant culture (Non-enzymatic method)

IV.2. Characterization of migrated phase-bright cells

IV.2.1. Immunocytochemistry

The cells grown on culture dishes were tested for immunoreactivity to anti-Factor VIII related antigen and anti-vimentin. The cells stained negative for anti-Factor VIII-related antigen and vimentin (Figure 8A & B). Nuclei stained blue with haematoxylin. Since migrated cells were fewer in number and not well adherent to the culture surface, the number of positively stained cells per field is limited. The feeder cells stained positive for vimentin (Figure 8C).

IV.2.2. Immunofluorescence

Migrated phase-bright cells stained positive for the stem cell marker ckit (Figure 8D). Positive control used was GIST tissue (Figure 8E).

IV.2.3. Magnetic activated cell sorting (MACS)

MACS sorted ckit^{+} cells were stained with Hoescht 33258 which stained the nucleus blue (Figure 8F). Approximately 20% purity was obtained after MACS procedures. In order to further purify the sorted cells, MACS sorted cells were stained with PE-labeled anti-human CD117 antibody and analyzed by FACS. 87% purity was obtained after the MACS and FACS sorting steps. (Figure 9).

IV.2.4. Fluorescence activated cell sorting (FACS)

Fluorescence-activated cell-sorting analysis of primary cells derived from the explants revealed cells stained positive for CD117, MDR1, CD34 and CD45 markers (Figure 10).

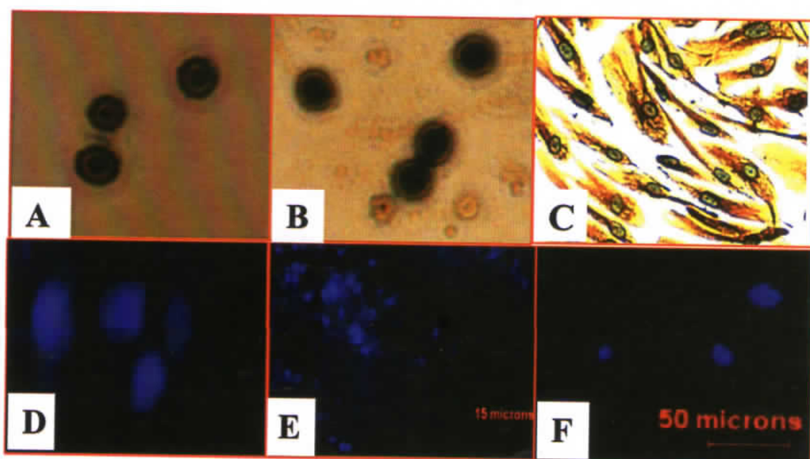


Figure 8: Photomicrographs of migrated phase-bright cells. A & B – stained negative for Factor VIII (200x) & vimentin (200x), C - feeder cells stained positive for vimentin. Fluorescent micrographs of D - phase - bright cells stained positive for ckit, E - GIST tissue stained positive for ckit and F - ckit^{+} cells stained with Hoescht 33258

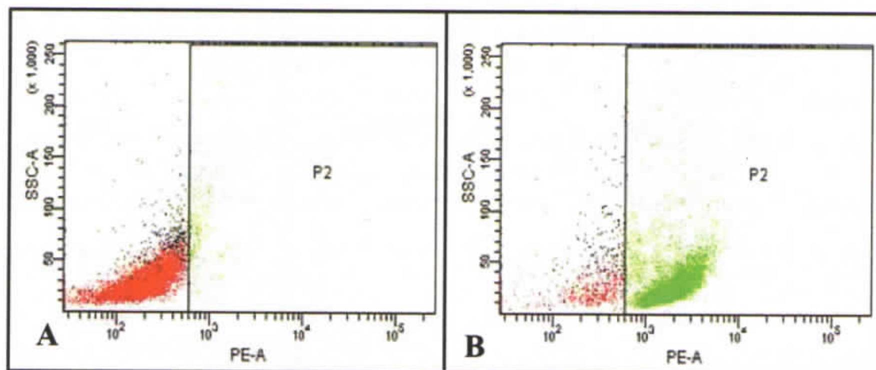


Figure 9: FACS analysis of MACS sorted ckit$^+$cells. (A) Control and (B) Positive fraction – ckit$^+$ cells

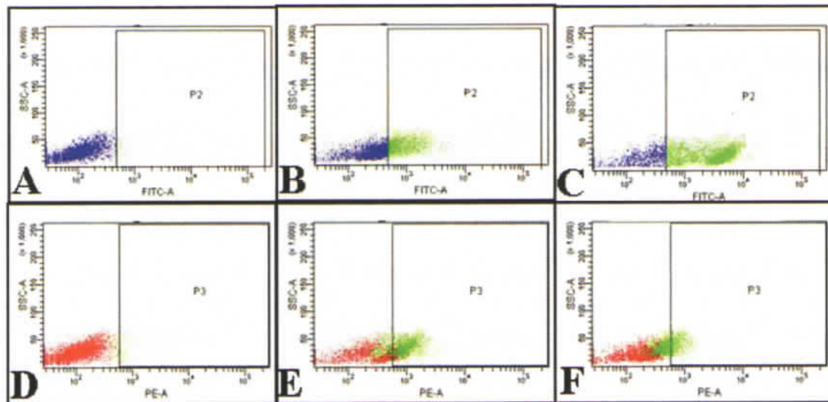


Figure 10: FACS analysis of primary culture cells.
A & D – Control FITC & PE. Phenotypic profile of migrated cells stained positive for (B) ckit (C) CD34 (E) CD45 (F) MDR1

IV.3. Expansion of sphere generating cells

The isolated cells were seeded in poly-D-lysine-coated culture dishes at a density of 0.5 to $2x10^5$cells.mL^{-1}. Most cells became loosely adherent in culture, whereas others remained in suspension. Some contaminating fibroblast-like cells attached firmly to the poly-D-lysine coat. After 10-12 hours of incubation, small round clusters of cells, termed cardiospheres were formed. Cadiospheres were obtained both in enzymatic and non-enzymatic isolation methods (Figures 11 & 12). Within 24 to 36 hours of culture, cardiospheres increased in size and some of them detached from the culture surface (Figure 13). The size of the cardiospheres varied from 20 to 172 microns. Larger spheres contained dark zones towards the interior.

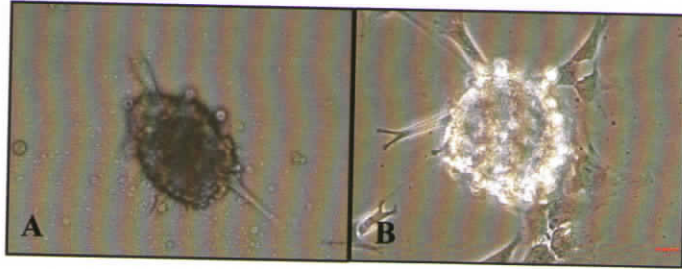


Figure 11: Photomicrograph of adherent cardiospheres obtained by enzymatic method

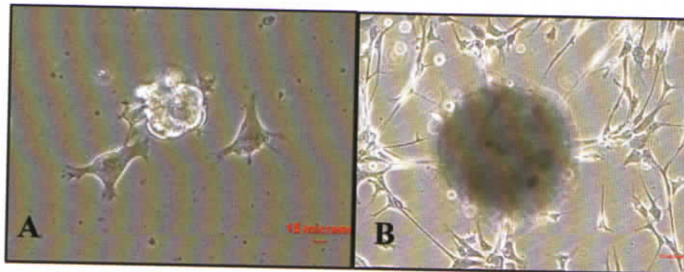


Figure 12: Photomicrograph of adherent cardiospheres obtained by non-enzymatic method

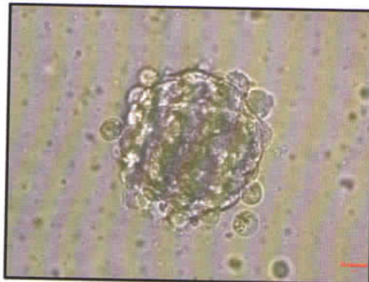


Figure 13: Photomicrograph of a floating cardiosphere

IV.3.1. Analysis of cardiosphere formation under different growth and substrate conditions

Among the different growth conditions (IMDM with/without growth factors and CGM with/without growth factors) and culture substrates (gelatin, fibronectin, laminin and poly-D-lysine) studied, cardiosphere formation was observed only in poly-D-lysine-coated culture dishes supplemented with growth factors. With other substrates, only fibroblasts were confluent; cardiosphere formation was not observed (Figure 14).

IV.3.2. Characterization of cardiosphere cells

After expansion in a medium supplemented with growth factors, the cardiosphere cells stained positive for the markers ckit (Figure 15B), MDR-1 (Figure 15C), CD34 (Figure 15D) and CD45 (Figure 15F). The cardiosphere cells stained positive for the expression of endothelial differentiation marker von Willebrand factor (Figure 15E), and cardiac differentiation markers - myosin heavy chain (Figure 15G) and troponin 1 (Figure 15H).

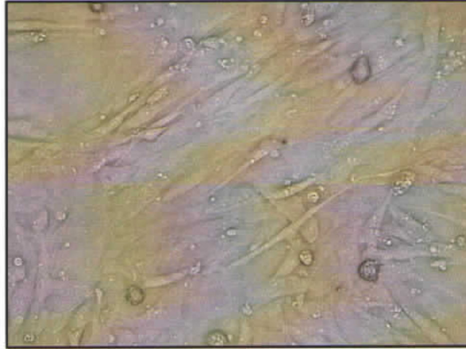


Figure 14: Photomicrograph of confluent fibroblasts

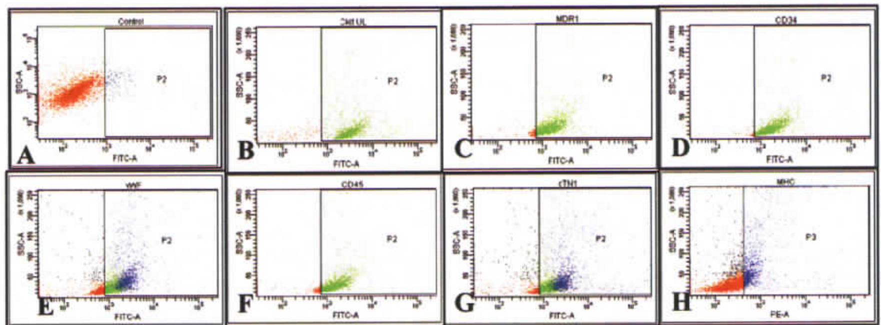


Figure 15: FACS analysis of cardiosphere cells. (A)– Control. Phenotypic profile of CS cells stained positive for (B) ckit (C) MDR1 (D) CD34 (E) vWF (F) CD45 (G) cTN1 and (H) MHC expression

IV.4. Comparison between enzymatic and non-enzymatic isolation methods

The clinical characteristics of 10 patients from whom right atrial appendage were taken are summarized in Table 1. With both the isolation methods, after a period of incubation ranging from 1–3 weeks, a layer of fibroblasts were generated from well-adherent explants over which small round cells migrated. The isolated cells formed clusters of dividing cells termed cardiospheres. The results of a comparative analysis between enzymatic and non-enzymatic isolation methods for the initial cell count and number of cardiospheres formed after culture enrichment are given in Table 2. The cell yield and cluster formation were not significantly different with either of the techniques used.

Variables	No. of Patients (10)
Age (years)	58 ± 10
Male sex	8
Hypertension	5
Smoker	7
Diabetes mellitus	6
Dyslipidemia	5
1-vessel disease	0
2-vessel disease	5
3-vessel disease	5
NYHA functional class	
I	0
II	8
III	2
IV	0

NYHA = New York Heart Association

Table 1: Clinical characteristics of 10 patients with ischemic heart disease, from whom right atrial appendage tissues were obtained

Non-enzymatic		Enzymatic	
Initial cell count *(cells·mL^{1}· g^{-1} ×10^{5})*	**Cluster count** *(clusters·cm^{-2} ×10^{2})*	**Initial cell count** *(cells·mL^{-1}. g^{-1} ×10^{5}*	**Cluster count** *(clusters·cm^{-2} ×10^{2})*
6.46	206	7.91	321
4.59	580	5.00	407
2.66	489	5.09	389
5.08	257	3.33	367
3.45	531	2.47	310
Mean 4.45 ± 1.5*	Mean 4.12 ± 169 †	Mean 4.76 ± 2.08*	Mean 3.59 ± 42†

* Initial counts nonenzymatic vs. enzymatic, $p > 0.5$
† Cluster count nonenzymatic vs. enzymatic, $p > 0.5$

Table 2: A comparison of initial cell counts and later cluster counts following non-enzymatic and enzymatic isolation procedures

IV.5. Effect of disease severity & CAD risk factors on the number of migrating cardiac stem cells

The clinical profile of patients studied is given in Table 3. The cardiovascular risk factors and the other clinical parameters did not seem to affect the number of ckit positive cells migrated from explanted atrial tissue samples. The number of migrated cells was also not affected by any of the drugs taken by the patients (Table 4). Among the various clinical parameters analyzed, only age of the patient had a statistically significant relationship with the stem cell number ($p \leq 0.05$; $r = -0.419$; Figure 16).

Characteristics	Patient group (n= 30)
Age (years) mean $\pm$ SD	54.87 $\pm$ 8.9
Male: Female	26:4
Risk factors	
Smoking -- no. (%)	40
Hypertension -- no. (%)	60
Diabetes mellitus -- no. (%)	70
Dyslipidemia -- no. (%)	53
Extent of coronary artery disease-- no. (%)	
One vessel	3
Two vessels	30
Three vessels	66
NYHA class -- no. (%)	
1	0
2	83
3	16
Mitral Regurgitation rates -- no. (%)	
0	26
1	46
2	26
Right Wall Motion Abnormality-- (RWMA) no (%)	46
Total blockage of the vessels -- no. (%)	46
Right coronary artery blockage-- no. (%)	23
Statin users – no. (%)	40
Beta Blockers	90
Calcium channel blockers	16
Nitrates	80

Table 3: Clinical characteristics of the patients, in whom the relationship between coronary artery disease risk factors and yield of ckit^{+} cells from right atrial tissue was studied

Variables	N	Counts of ckit+ cells from right atrial tissue		P value
		Mean	SD	
Sex				
Male	26	7.76	6.42	0.84
Female	4	8.46	6.72	
Smoking status				
Smokers	12	6.94	4.55	0.52
Non smokers	18	8.47	7.36	
Hypertension status				
Hypertensives	18	8.33	5.6	0.62
Non-hypertensives	12	7.15	7.49	
Diabetes status				
Diabetic	21	7.52	7.00	0.67
Non-diabetic	9	8.63	4.72	
Dyslipidemia status				
Dyslipidemic	16	7.68	5.75	0.87
Non-dyslipidemic	14	8.06	7.17	
TVRS				
1	7	9.36	9.20	
2	9	6.64	5.11	0.69
3	10	9.26	6.12	
4	3	4.27	2.87	
NYHA				
II	25	7.96	6.36	0.84
III	5	7.32	6.93	
Disease severity				
Double vessel disease	9	4.76	4.02	0.15
Triple vessel disease	20	9.44	6.83	

RWMA				
Present	14	8.90	6.42	0.41
Absent	16	6.94	6.33	
MR grades				
0	8	9.33	9.42	
1	14	5.41	3.14	0.12
2	8	10.67	6.00	
Totally blocked vessel				
Present	14	6.70	6.13	0.36
Absent	16	8.86	6.54	
Involvement of RCA				
Present	7	6.61	6.05	0.56
Absent	23	8.23	6.51	
Nature of drugs administered				
Statins				
Users	12	6.16	5.08	0.23
Non-users	18	8.99	6.97	
β-blockers				
Users	27	8.52	6.34	0.08
Non-users	3	1.89	0.75	
Nitrates				
Users	24	6.88	5.12	0.09
Non-users	6	11.77	9.47	
Ca-channel blockers				
Users	5	3.80	1.93	0.12
Non-users	25	8.66	6.63	

N=No: - of patients, NYHA= New York Heart Association, RWMA=Right wall motion abnormality, MR=Mitral Regurgitation.

Table 4: ckit^{+} cell counts from atrial tissue of patients with coronary artery disease and the relationship with coronary artery disease risk factors

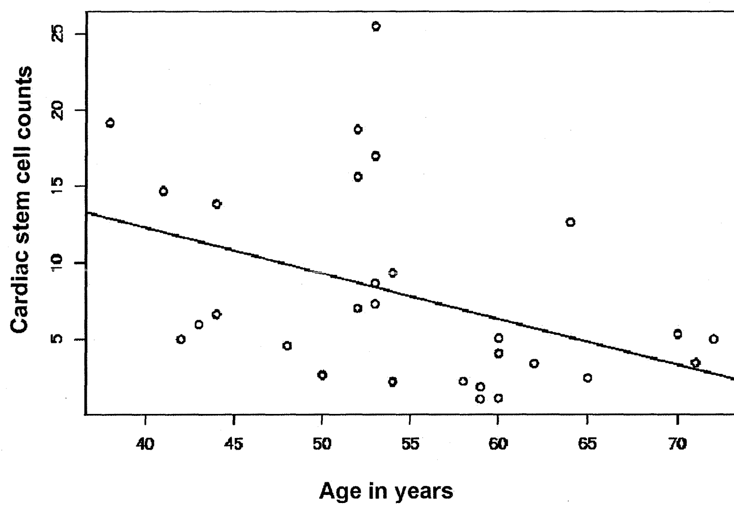


Figure 16: Regression plot of age vs. cardiac stem cell counts

IV.6. Effect of growth factors on cardiosphere formation

Among the growth factors analyzed, maximum enhancement of cardiopshere formation was obtained in medium supplemented with epidermal growth factor (EGF) ($p < 0.05$). EGF regardless the presence/absence of serum in the medium accelerated cardiosphere formation as confirmed by the sphere count (Figure 17) and proliferation assays (Figure 18). The presence of serum in the medium accelerated the cell proliferation rate by five times (Figure 19).

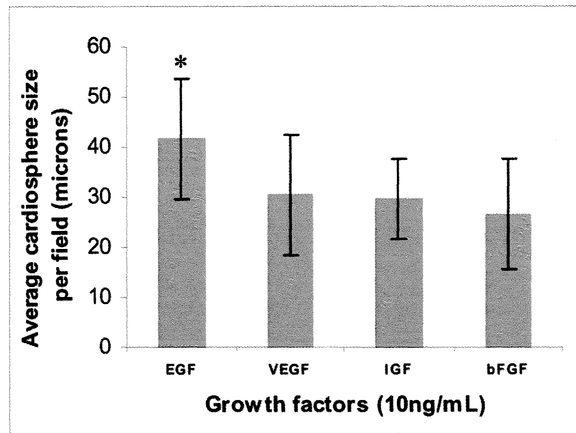


Figure 17: Effect of growth factors on cardiosphere formation. The values are mean ± SD (n = 4; *p<0.05 by ANOVA)

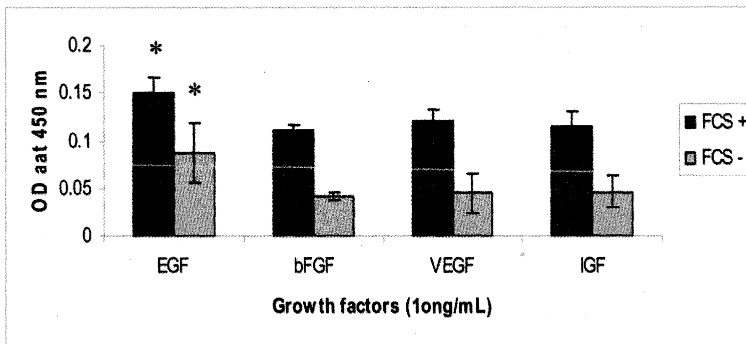


Figure 18: Effect of growth factors on cardiosphere formation in serum reduced/deprived conditions. Results of WST proliferation assay. The values are mean ± SD (n = 4; *p<0.005 by ANOVA)

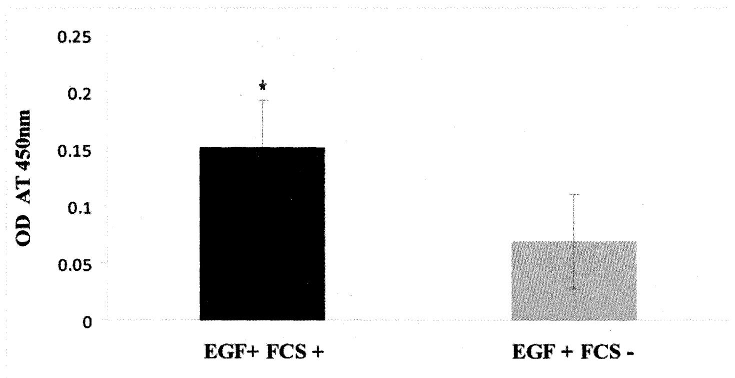


Figure 19: Effect of serum in cardiosphere formation. The values are Mean ± SD (n = 4; *p<0.001 by student's t-test)

IV.6.1. Cardiosphere culture and characterization

Cardiospheres plated on fibronectin coated culture dishes were expanded into monolayers termed cardiosphere-derived cells (CDCs) (Figure 20). CDCs had antigenic and cytochemical similarities to cardiospheres. After expansion, CDCs were positive for the markers CD34 (Figure 21B), c-Kit (Figure 21C), CD31 (Figure 21H), cTN1 (Figure 21G) and MHC (Figure 21J). These cells were largely $CD133^-$ (Figure 21E) and $CD45^-$ (Figure 21D), as well as negative for a cocktail of blood lineage markers (B220, Mac-1 & Gr1). CDCs contain subpopulations that phenotypically resemble endothelial cells ($CD34^+$ $CD31^+$). Phenotypic profile of CDC population is shown in Figure 22.

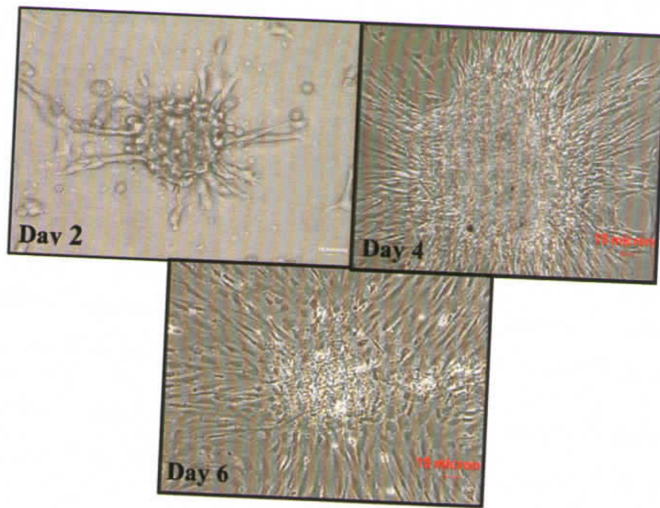


Figure 20: Photomicrograph of cardiospheres plated on fibronectin coated culture dish for expansion

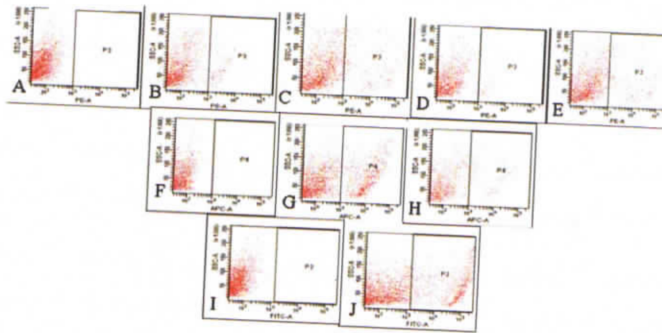


Figure 21: FACS analysis of cardiosphere-derived cells. A, F & I - Control - PE, APC & FITC respectively. Phenotypic profile of CDCs stained for B - CD34, C- ckit, D-CD45, E-CD 133, G- cTN1, H – CD 31 and J- MHC

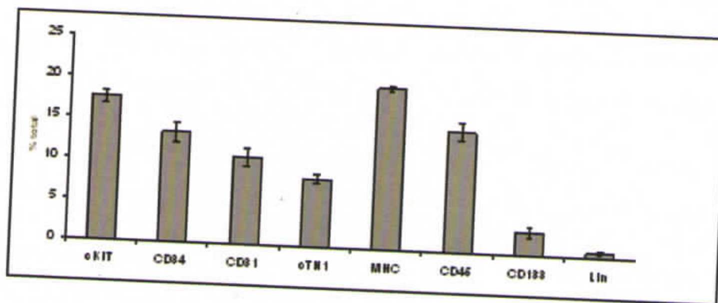


Figure 22: Phenotypic profile of cardiosphere-derived cells

IV.6.1.1. RT-PCR for epidermal growth factor receptor (EGFR)

The RNA yield from the cardiosphere-derived cells was in the range of 35-60 μg/100 mm dish. The 260/280 ratio was ≥ 1.7 and the RNA was confirmed to be of good quality. The RNA samples gave clear and distinct bands on the gel indicating that the isolated RNA is intact, not degraded or sheared and are without protein or DNA contamination (Figure 23). The CDCs expressed EGFR (Figure 24).

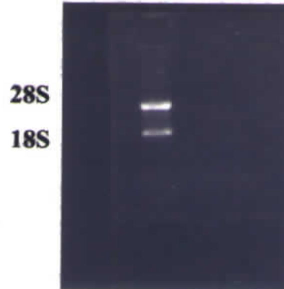


Figure 23: mRNA isolated from cardiosphere-derived cells

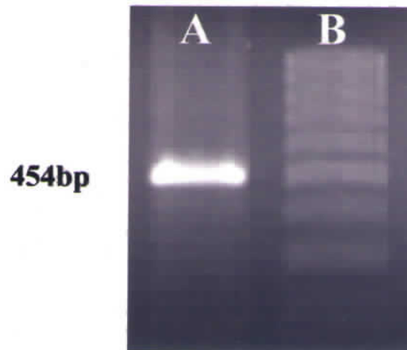


Figure 24: RT-PCR analysis of epidermal growth factor receptor mRNA expression.
Lane A - EGFR expression in CDCs
Lane B –DNA ladder (100bp)

IV.6.1.2. Evaluation of the effect of the growth factors on cardiosphere-derived cell proliferation

Maximum enhancement of cardiopshere-derived cell proliferation was obtained in medium supplemented with epidermal growth factor (EGF) ($p < 0.005$, Figure 25).

IV.6.1.3. Effect of EGF in cardiomyocyte differentiation in cardiosphere-derived cells

EGF treated CDCs were positive for the markers CD34 (Figure 26B), c-Kit (Figure 26G), CD31 (Figure 26C), cTN1 (Figure 26H) and MHC (Figure 26I). Percentage of cells expressing CD133 (Figure 26E) and CD45 (Figure 26D) markers were less. Considerable plularities expressed endothelial markers $CD34^+$ and $CD31^+$. However, EGF treatment seemed to enhance the expression of myocyte markers in cultured CDCs. Majority of the cells cultured in EGF supplemented medium were $cTN1^+$ and MHC^+ where as the percentage of $ckit^+$ cells remain the same in treated and untreated controls. Phenotypic profile of EGF treated CDC population and untreated CDCs is shown in Figure 27.

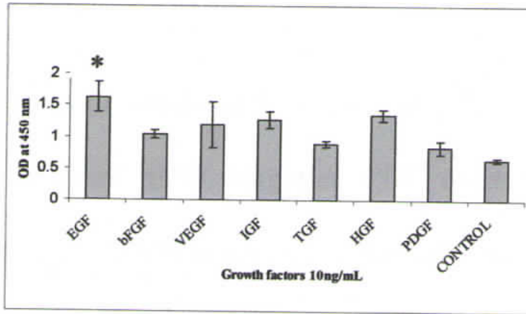


Figure 25: Effect of growth factors on cardiosphere-derived cell proliferation. The values are mean ± SD ($n = 4$; *$p<0.005$ by ANOVA)

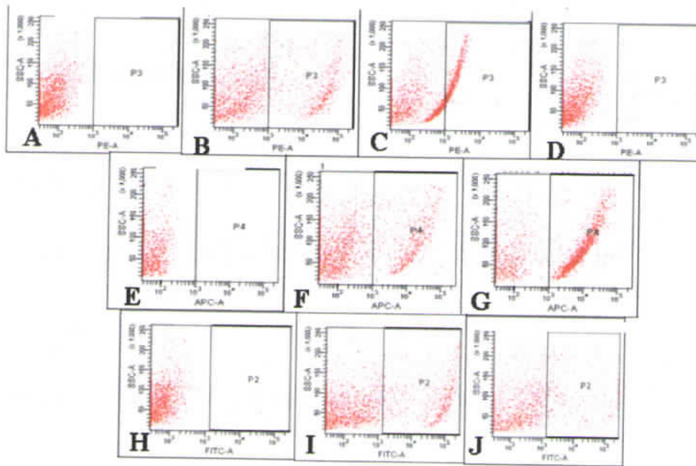


Figure 26: Fluorescent activated cell sorting analysis of epidermal growth factor treated cardiosphere-derived cells. A, E & H - Control - PE, APC & FITC respectively. Phenotypic profile of CDCs stained for B – CD34, C- CD31, D- CD45, F-ckit, G- MHC, I- ckit and J-CD133

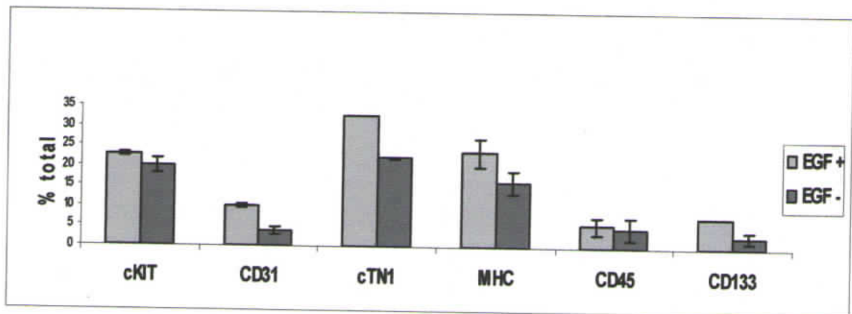


Figure 27: Phenotypic profile of epidermal growth factor treated and untreated cardiosphere-derived cells

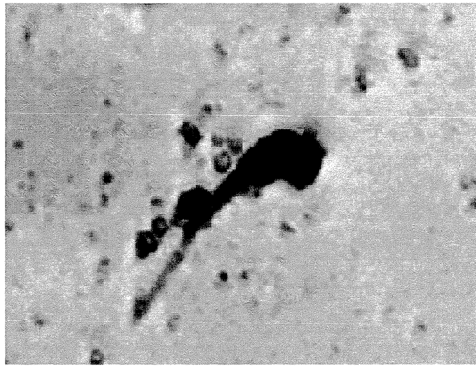


Figure 28: Photomicrograph of cell migration in epidermal growth factor free medium

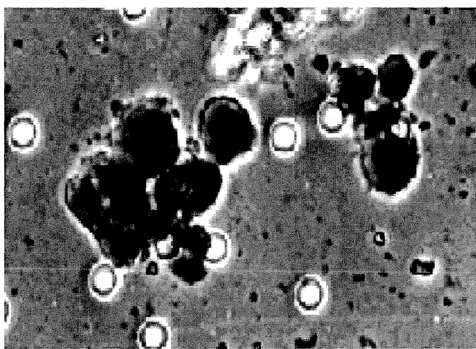


Figure 29: Photomicrograph of migrated cardiosphere-derived cell clusters on treatment with 10g/mL epidermal growth factor

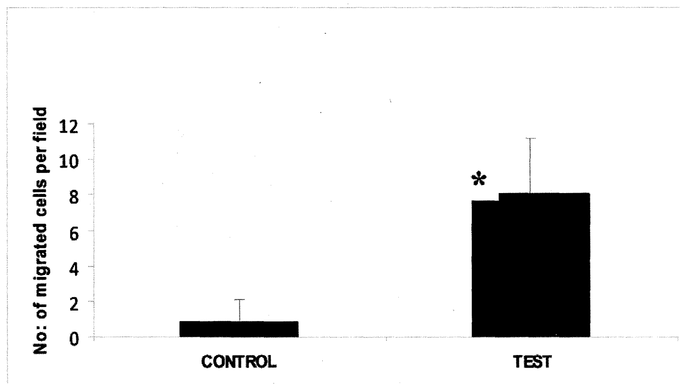


Figure 30: Effect of EGF on cardiosphere-derived cell migration. The values are mean ± SD (n = 4; *p<0.0005)

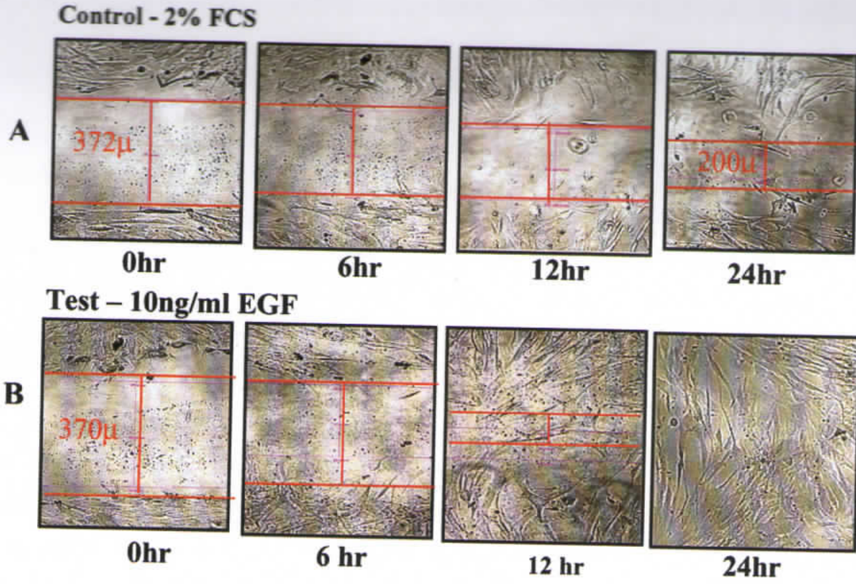


Figure 31: Photomicrograph showing the effect of epidermal growth factor in wound healing activity of cardiosphere-derived cells over the hours.
A - cardiosphere-derived cells without epidermal growth factor treatment and
B - cardiosphere-derived cells treated with epidermal growth factor

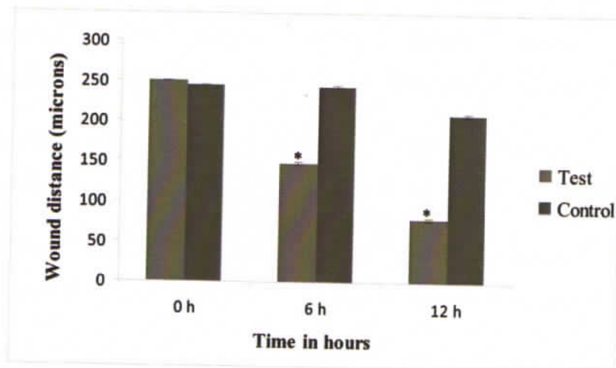


Figure 32: Wound distance over time in cardiosphere-derived cells with and without epidermal growth factor treatment. The values are mean ± SD (n = 3; *p<0.0001 in both 6hrs and 12hrs)

IV.6.1.5. Delineation of signal transduction pathway involved in epidermal growth factor induced proliferation of cardiosphere derived cells

IV.6.1.5.1. Effect of inhibitors on EGF induced proliferation of CDCs

Proliferation induced in CDCs by EGF supplemented medium was abolished on addition of the MAPK inhibitor SB203580. p42/44MAPK inhibitor and PI3K/Akt inhibitor did not decrease the CDC proliferation rate on treatment with EGF. Incubation of cardiosphere-derived cells with EGF supplemented medium, after pretreatment with SB 203580 decreased the cell proliferation rate by 42% compared to control conditions ($n=4$; $p<0.005$) (Figure 33). The results suggest that p38MAPK pathway may be involved in EGF induced CDC proliferation.

IV.6.1.5.2. Assay of p38 MAPK activation in EGF induced cardiosphere-derived cells

To determine the role of p38MAPK pathway in EGF induced CDC proliferation, endogenous levels of phospho-p38α MAP kinase in the cells was determined by ELISA. Since the protein concentration obtained was less, the levels of phospho-p38 MAPK protein were undetectable.

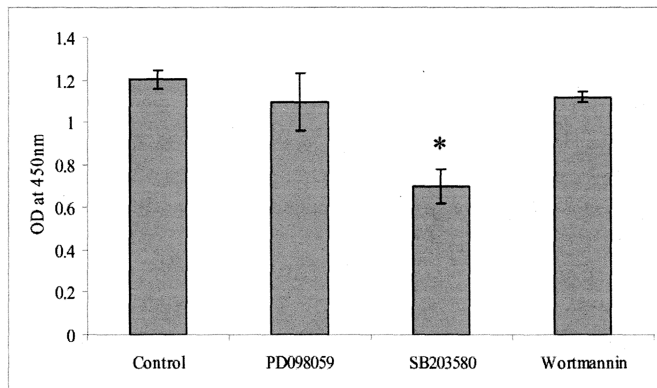


Figure 33: Cell proliferation rate as measured by WST proliferation assay in cardiosphere-derived cells incubated with EGF in the presence of p42/44MAPK inhibitor PD098059, p38 MAPK inhibitor SB203580, PI3K/Akt inhibitor Wortmannin and in the absence of inhibitors (control). The values are mean ± SD (n=5; control vs. SB203580, *p<0.005 by ANOVA)

V. DISCUSSION

Currently, there is increasing interest in developing cell transplantation technology (cellular cardiomyoplasty) and gene therapy to regenerate functional muscle and blood vessels in previously infarcted, scarred and dysfunctional myocardium (Anversa *et al.* 2003). Multiple cell types including fetal cardiomyocytes, autologous skeletal myoblasts, immortalized myoblasts and bone marrow derived adult hematopoietic stem cells have been transplanted and have yielded promising results though certain deficiencies remain to be redressed (Soonpaa and Field 1994; Koh *et al.* 1995; Zhang *et al.* 2001; Reinecke *et al.* 2000). Autologous skeletal myoblasts is the best studied for myocardial repair (Taylor 2001). The procedure involves harvesting skeletal muscle, expanding cells in a laboratory and reinjecting into patient's heart. The use of auotologous skeletal myoblasts could overcome the major limitations associated with other cell-based treatments. These include shortage of donor tissue, need for immunosuppression and the ethical issues associated with the use of allogenic or embryonic cells. The use of primary skeletal myoblast cells decreased the likelihood of tumor formation after transplantation. (Tremblay *et al.* 1991). The promising effects following skeletal myoblast CCM includes adequate survival, engraftment and improvement in myocardial functional performance.

Current attempts are to develop technology, which employs patient derived (autologous) adult cardiac stem cells (Nadal-Ginard *et al.* 2003a). Following the reported possibility of a pool of adult clonogenic stem cells in postnatal human heart, which was enhanced after myocardial infarction and attenuated during chronic heart failure, these cells were successfully isolated and cloned from animal hearts (Beltrami *et al.* 2001; Anversa and Nadal-Ginard 2002; Hughes 2002; Beltrami *et al.* 2003; Urbanek *et al.* 2003; Messina *et al.* 2004; Urbanek *et al.* 2005). The resident cells or

their clonal progenies can reconstitute a well-differentiated myocardial wall when injected into infarcted ventricle (Mendez-Ferrer *et al.* 2006). Adult cardiac-resident stem cells thus provide effective means of regenerating damaged myocardium. Compared to other cell types (such as bone marrow-derived cells), they are believed to be faster in achieving the structural and functional characteristics favorable for regenerating damaged myocardium as well. Nonetheless, CSC therapy for myocardial regeneration is not without potential limitations. The use of autologous cells necessitates sufficient time between injury and re-injection, to grow cells in the laboratory. This waiting period may be in line with nature's demand in that injecting cells soon after an infarction, seems to limit the number of cells that survive presumably due to the inflammatory response that occurs after injury.

The origin of SCs in post-natal human hearts is highly debated. It has been postulated that cardiac progenitor cells (CPCs) might have originated from stem cells in the bone marrow capable of homing and differentiating into cardiac myocytes (Deb *et al.* 2003). Genetic tagging experiments showed that ckit$^+$ cells from the bone marrow homed to the myocardium after acute ischemia secreted VEGF resulting in angiogenesis (Fazel *et al.* 2006). No differentiation into myocytes was found. Likewise, labeled side population (SP) cells from the bone marrow constituted upto 25% of the cells in the infarcted myocardium (Mouquet *et al.* 2005). In addition to this, several reports have documented that various bone marrow derived stem cells can transdifferentiate into functional cardiac tissue when injected into the infarcted heart (Jackson *et al.* 2001; Orlic *et al.* 2001; Kawada *et al.* 2004). Be that as it may, CSC therapy has undoubtly arrived.

Isolation and *in vitro* enrichment of the CSCs is one of the major challenges in myocardial repair therapy. The regenerative capacity of CSCs could be exploited in two different ways, either by boosting of surviving CSCs after injury or by *ex vivo* enrichment of progenitor cells obtained directly from the heart by means of a biopsy before re-application. The need for an invasive procedure is potentially hazardous and delays treatment, increases scar formation and thus reduces the efficacy of stem cell mediated regeneration. Also, no published reports have yet determined if early resident CSCs have the same immunological tolerance as MSCs. Hence, it is important to understand the factors necessary for *in vitro* enrichment and optimization of the subsequent cardiac differentiation *in vivo* (Lynbak *et al.* 2007). The major challenges include: How can culture conditions be optimized in order to obtain a homogenous and multipotent stem cell population? Should CSCs be differentiated *in vitro* and to what degree before re-administration? Can the extent of regeneration be optimized by the concomitant injection of different subsets of stem cells with different lineage potential? What is the maximum number of cells that will give the maximal amount of regeneration? When is the optimal time point for the injection of CSCs after MI? In addition, a complicated ensemble of agonistic and antagonistic factors of autocrine and paracrine origin and the necessity for direct intercellular coupling wih myocytes, all seem to be important for cardiac differentiation.

While utilizing enriched CSCs *in vivo*, the route of delivery to the ischemic heart becomes important in order to ensure the greatest number of viable donor cells. No consensus about the preferred method has yet been obtained. Although intravenous administration has demonstrated the ability to home to the heart in animal studies, this has only lead to inadequate regeneration (Oh *et al.* 2003; Dawn *et al.* 2005).

Intramyocardial injection of stem cells will most likely result in only a few surviving donor cells (Suzuki *et al.* 2004). Application of survival and homing factors into the myocardium has shown to increase survival of resident CSCs after infarction and boost regeneration (Urbanek *et al.* 2003; Linke *et al.* 2005). The admintration of factors such as IGF-1 has increased regeneration when utilized with embryonic stem cells (Kofidis *et al.* 2004).

Recent studies suggest that heart derived stem cells could be isolated and grown *in vitro* from small biopsy samples. Considering the clinical plausibility in treating patients with end-stage cardiac disorders, the present *in vitro* study was aimed at identifying an easy and cost effective method for the isolation and expansion of heart derived stem cells from right atrial tissue samples. The present study confirmed that the yield of the isolated cardiac stem cells is independent of the severity and risk factors for coronary artery disease. The effect of EGF on the mitogenic, migratory and healing activity of cardiosphere-derived cells were confirmed, after which an attempt was made to examine the possible signaling pathways involved in EGF mediated cardiosphere-derived cell proliferation.

V.1. Isolation, characterization and expansion of adult cardiac stem cells

V.1.1. Isolation and characterization of resident cardiac stem cells

The current best characterized group of stem cells resident in the heart constitutes a pool of cells that express the receptor for stem cell factor known as ckit (Linke *et al.* 2005). This receptor is widely used as a cell surface marker of pluripotent long-term reconstituting murine stem cells and has been used to define a population of cells

from the bone marrow that can regenerate the dysfunctional myocardium. Among the progenitor cells resident in the heart, only the ckit$^+$ cell fraction fulfill all criteria for being stem cells (Beltrami *et al.* 2003). The composition of ckit$^+$ cell population in the heart is heterogenous. Only a minor percentage of the ckit$^+$ cells show cardiomyogenic potential. Bone marrow derived progenitor cells expressing ckit receptor are chemoattracted in relation to inflammatory stimuli, but their cardiomyogenic potential is limited (Wojakowski and Weissman 2004; Fazel *et al.* 2006). The ckit$^+$ cells from the heart with stem cell abilities do not express cell surface markers of other lineages, making them lineage negative. These cells are clonogenic, able to self-renew, multipotent and have been shown to reconstitute the heart after an acute ischemic event by differentiating into cardiac, smooth muscle and endothelial lineages. Freshly isolated ckit$^+$ cells express transcription factors from early myocyte lineages as well.

The differentiation of stem cells in the myocardium or culture is based on the presence or absence of cell surface markers, transcription factors and cytoplasmic proteins. An undifferentiated stem cell would express stem cell markers and do not express transcription factors or cytoplasmic proteins of cardiac cells. On the other hand, differentiated cells would have lost their stem cell antigens and express cytoplasmic proteins specific for cardiac cells such as myocytes, endothelial and smooth muscle cells. The expression of cardiac transcription factors and sarcomeric proteins during CSC maturation *in vitro* or *in vivo* does not necessarily reflect the activation of the developmental program of the stem cell. It might be an intrinsic property of undifferentiated cells in the myocardium to acquire a phenotype that resembles the environment surrounding them and therefore express certain cardiac

transcription factors and their translated proteins (Lyngbak *et al.* 2007). Another concern is regarding the identification of the stem cell candidates employing technical procedures such as immuonohistochemistry, FACS *etc* which are highly dependent on the specificity of the applied antibody. Hence a meticulous approach is highly important for stem cell isolation and characterization procedures.

In the present study, resident cardiac stem cells were isolated from right atrial biopsy samples by both enzymatic and non-enzymatic methods. Morphologically, these cells are small and round with a large nucleus surrounded by a thin rim of cytoplasm. Morphological examination under phase contrast microscope established the uniqueness of the migrated cells from the other cell types of the heart. Migrated cells stained positive for ckit, and negative for vimentin and von Willebrand factor. Negative immunostaining for vimentin and von Willebrand factor ruled out the possibility of both fibroblasts and endothelial cells. Furthermore, MACS and FACS analysis of the migrated phase-bright cells confirmed the presence of ckit, a stem cell marker, which is absent in other cell types of the heart. Fluorescence-activated cell-sorting analysis of primary cells derived from the explants showed 31% positivity for CD117 marker. The primary culture cells stained positive for the other stem cell markers – MDR1 and CD34 as well. When the MACS sorted cells were analyzed by FACS, 86% purity was obtained. Given that the isolated cells stained positive for the stem cell markers (ckit, MDR1 and CD34) and were obtained from heart biopsy samples, they are likely to be stem cells resident in adult human hearts – the cardiac stem cells.

Be that as it may, there is considerable debate as to whether resident pool of stem cells exists in the heart and whether it is possible to isolate these cells based on specific marker expression.

V.1.2. Expansion of the isolated phase bright cells to cardiospheres and characterization

The isolated ckit-positive cells formed clusters of dividing cells in a growth factor supplemented medium termed cardiospheres. Cardiospheres consisted of a mixture of cardiac stem cells and differentiating progenitors confirmed by FACS analysis. CSs consist of vascular cells depending on the size of the sphere and time in culture (Messina *et al.* 2004). Death, differentiation, and responsiveness to growth factors of the different cells within the CSs could depend on the three-dimensional architecture and on localization within the CSs (Layer *et al.* 2002). Proliferating ckit positive cells are found primarily in the core and differentiating cells expressing cardiac and endothelial cell markers in the periphery of the cardiosphere. These sphere-forming cells can be isolated at repeated intervals from the same explant (biopsy specimen), and once they are established, the CSs would beat in culture spontaneously (embryonic murine CSs) or after coculture with rat neonatal cardiomyocytes (human CSs) (Messina *et al.* 2004) CSs exposed to extremely low frequency magnetic fields modulate their differentiation potential, turning on cardiogenesis and turning off vasculogenesis. Data obtained from GFP-c-kit transgenic mice suggest that adult CSC is c-kit^{+} (Messina *et al.* 2004). Green fluorescen protein (GFP)-positive cells were present from the beginning of the formation of CSs isolated from transgenic mice expressing GFP under the control of the c-kit promoter and no double-positive cells

were observed in the CSs isolated from a double heterozygote mouse model (GFP-cKit/MLC3F-nLacZ or GFP-cKit/TnI-nLacZ) suggesting that c-kit$^+$ cells were contributing to the maintenance of proliferation. The delivery of human CSs or CDCs into the injured heart of the SCID mouse resulted in engraftment, myocardial regeneration and improvement of left ventricular function, with respect to control groups receiving PBS and/or an alternative cell source (fibroblasts), at least within 1 month after cell transplantation (Messina *et al.* 2004). Although the intracardiac origin of adult myocytes has been unequivocally documented and an extracardiac origin of CSCs in adulthood has been excluded under physiological conditions, GFP$^+$ CSs were obtained 3-4 weeks after heart injury in lethally irradiated mice whose bone marrow was repopulated by bone marrow from a syngenic GFP-c-kit animal suggesting that the bone marrow could repopulate the heart.

Cardiospheres resemble neurospheres in that they are derived from primary explant culture and contain many proliferating cells that express stem cell–related antigens as well as other cells undergoing spontaneous cardiac differentiation (Reynolds and Weiss 1992; Messina *et al.* 2004; Vasa *et al.* 2001b). The spontaneous formation of spheres is a known prerogative of neural stem cells, some tumor cell lines (LIM) (Bates *et al.* 2000), endothelial cells (Korff and Augustin 1998), and fetal chicken cardiomyocytes (Armstrong *et al.* 2000). All these models that mimic the true three-dimensional architecture of tissues consist of spheroids of aggregated cells that develop a two-compartment system composed of a surface layer of differentiated cells and a core of unorganized cells that first proliferate and then disappear over time (perhaps through apoptotic cell death). As well-documented in fetal chick cardiomyocytes and endothelial cell spheroid culture, three-dimensional structure

affects the sensitivity of cells to survival and growth factors. In particular, central spheroid cells do not differentiate and are dependent on survival factors to prevent apoptosis, whereas the cells of the surface layer differentiate beyond the degree that can be obtained in two-dimensional culture and become independent of the activity of survival factors. Furthermore, cell–cell contact and membrane-associated factors known to be important for the division of neural precursor cells (Svendsen *et al.* 1998), could be involved in the maintenance of CSs also. This is in accordance with the notion that stem cells will only retain their pluripotency within an appropriate environment, as suggested by the "niche" hypothesis (Nilsson and Simmons 2004).

In the context of cardiac stem cell therapy, cellular subfractionation would probably delay transplantation and appears unnecessary to achieve regeneration. Because both c-kit-positive cells and cells that express mesenchymal markers are present in the cardiosphere, they have the potential to improve function through several mechanisms (Barile *et al.* 2007). Indeed, in addition to new cardiomyocyte formation, improvements in cardiac function and increased regeneration within the infarct area could be attributed to cellular paracrine effects, increased mobilization of endogenous cardiac stem cells, and formation of new blood vessels. Hence CSs could be a better candidate for myocardial repair therapy.

Adult cardiac stem cells undergo lineage commitment, and they divide and differentiate into myocytes, smooth muscle cells, and endothelial cells, under appropriate stimulus (Anversa *et al.* 2003; Nadal-Ginard *et al.* 2003b). The cardiosphere cells as obtained in my study stained positive for the endothelial marker-von Willebrand factor and cardiac differentiation markers - myosin heavy chain and

cardiac troponin I which demonstrated the ability of these cells to differentiate into distinctive lineages under appropriate stimuli.

V.2. Comparison between the enzymatic and non enzymatic isolation methods

Several structural and physical factors play critical roles in the distribution of CSCs in the heart. Wall stress is experienced higher at the base and midregion of the ventricle. It is reduced further at the atrial region (Hughes 2002). Therefore, the probability for finding a CSC niche in the atrial region of the heart is relatively high. Thus, atrial biopsy samples were collected for the present study. Several groups have initiated trials on CSC isolation from human samples as well as from animals such as mice and pigs (Beltrami *et al.* 2001; Messina *et al.* 2004; Smith *et al.* 2007). The number of stem cells that can be obtained from a given amount of sample tissue is relatively very less. Hence, the method adopted for isolation should be selective in providing maximum cell yield and viability.

The results of the present study suggest that $ckit^{+}$ cells can be isolated and grown into cardiospheres by simple explant culture methods. The initial plating density of the phase-bright cells and the number of active cells present in the culture strictly determined cluster formation. It is not clear whether all the migrated ckit positive cells in the primary explant culture retain their inherent property of stemness, subsequently in culture for longer periods. The cell yield and cardiosphere count obtained in the non-enzymatic method were comparable with those of the enzymatic method. The time lag in sample preparation is less, and costly enzymes (collagenase and trypsin) are not required in the nonenzymatic method. As the digestion step is bypassed in this approach, there is less chance of tissue damage as well.

V.3. Effect of disease severity & CAD risk factors on the number of migrating cardiac stem cells

Despite the evidence for myocardial regeneration by the activation of resident cardiac stem cells, the factors that influence cardiac stem cell numbers are not yet identified. Cardiovascular risk factors play a major role in the progression of atherosclerotic leisons, which ultimately results in myocardial infarction and sudden cardiac death. This includes age, sex, hypertension, diabetes, dyslipidemia, smoking etc (Vasa *et al.* 2001a). CAD risk factors induce endothelial injury, impair endothelial function and predict the risk of subsequent cardiovascular events (Schachinger *et al.* 2000; Suwaidi *et al.* 2000; Gokce *et al.* 2002; Halcox *et al.* 2002). Endothelial damage represents a balance between the magnitude of injury and the capacity for repair. EPCs contribute to ongoing endothelial repair by providing a circulating pool of cells that can home to denuded parts of the artery after balloon injury (Griese *et al.* 2002; Walter *et al.* 2002). Impairment of this repair capacity may affect atherosclerotic disease progression. Classic risk factors for atherosclerosis are associated with reduced number and function of circulating EPCs (Hill *et al.* 2003; Fadini *et al.* 2005; Wang *et al.* 2005; Kaur *et al.* 2007). Levels of circulating EPCs were shown to correlate with endothelial vasodilator function, cerebral infarction (Taguchi *et al.* 2007) and coronary collateral support in patients with CAD (Lambiase *et al.* 2004). Furthermore, there is evidence that progenitor cell number and function declines with age and such age related alterations correlate directly with the degree of endothelial dysfunction (Heiss *et al.* 2005).

Stem cells are involved in the repair process of damaged organs and changes in stem and progenitor cells may be of great importance in the aging process as well. Decline in the number and the functional integrity of stem cells could potentially lead to progressive deterioration of functional and proliferative homeostasis in organs. Results of the present study suggest that coronary artery disease and cardiac remodeling in chronic ischemia may not affect the yield of ckit positive cells from cardiac tissue. There is a significant inverse correlation between patient age and cardiac stem cell yield from atrial tissue samples. This suggests that in older patients, stem cell isolation from cardiac biopsies may not succeed, and such cells may not be available to them for cell therapy. The findings support the current pursuits in developing cardiac regenerative therapy in patients with coronary artery disease using autologous stem cells isolated and grown from cardiac biopsies.

V.4. Effect of EGF on the mitogenic, migratory and healing activities of cardiac stem cells

Myocardial regeneration comprises of parenchymal cells and coronary vessels. It provides additional evidence in support of the notion that the adult heart possesses an extraordinary growth reserve capable of restoring tissue loss commonly considered fatal in animals and humans. Local administration of growth factors (GFs) may become a novel powerful therapeutic strategy for the acutely decompensated infarcted heart. Intramyocardial injection of GFs promotes the translocation of CSCs and early committed cells (ECCs) to the damaged area and activates their growth and differentiation, resulting in the formation of functionally competent myocardium (Urbanek *et al.* 2005a). The blunted response to HGF or IGF-1 emphasizes the need

for a dual stimulation of CSCs and ECCs. The survival and growth is provided by IGF-1 together with the chemotactic effects of HGF. These results leave unanswered the question of why ischemia does not activate stem cells locally, favoring their homing to the damaged area and whether the effects of GFs on ischemic myocytes influence cell viability?

Myocardial ischemia is a potent inducer of IGF-1 (Cheng *et al.* 1996) and HGF (Ueda *et al.* 2001). The locomotion of CSCs occurs through the myocardial interstitium independently from the coronary circulation. CSCs traverse *in vitro* a 3-dimensional substrate of matrigel and dense barriers of collagen type I and III *in vivo* (Urbanek *et al.* 2005a). MMP-2 and MMP-9 appear to be the proteolytic enzymes responsible for the invasive phenotype of CSCs and their trafficking across the myocardial interstitium. MMP-9 can also trigger the release of stem cell factor (Heissig *et al* 2002) from CSCs, although stem cell factor has no effects on the invasive properties of these cells *in vitro* (Linke *et al.* 2005). Ischemia is a potent inducer of the chemoattractants- HGF, stromal cell-derived factor-1 and vascular endothelial growth factor as well. Although these cytokines may favor cell movement, it is only with HGF, CSCs engages in directional migration toward the injured myocardium. A stop signal may also be present to promote the accumulation of cells within the infarct. CSCs adhere to the wall of fibronectin that delimit the interstitial channels. Importantly, fibronectin is implicated in the migration of c-kit$^+$ cells as well (Takano *et al.* 2002).

The progressive nature of ischemic cardiomyopathy appears to be dictated by the inability of CSCs to translocate and home to the infarcted myocardium rather than by

limitations in the growth reserve of the damaged heart. The resident stem cells distributed in the infarcted region do not survive the ischemic event and die by apoptosis and necrosis in a manner identical to that of myocytes and coronary vessels. CSCs and ECCs are not capable of opposing the death signals activated by permanent coronary occlusion and initiate regenerative growth within the infarcted myocardium (Urbanek *et al.* 2003). Moreover, these primitive cells cannot escape replicative senescence with severe telomeric shortening and activation of the death program in end-stage failure or premature myocardial aging in humans. Hence designing strategies favoring activation and migration of the resident stem cells to the site of injury are an exciting area of research. Further, designing procedures enhancing the stem cell number could be ideal for utilizing these cells for therapy.

V.4.1. Effect of EGF on cardiosphere formation

The present study investigated the effect of different growth factors in inducing cardiosphere formation. Among the different growth factors analyzed, maximum enhancement of cardiosphere formation was observed in EGF supplemented medium. EGF significantly enhanced the cell proliferation rate confirmed by the sphere count and the proliferation assays ($p<0.05$). Results suggest that EGF has a potent role in CSC proliferation.

V.4.1.1 Cardiosphere culture and characterization

Lack of sufficient cell numbers hinders the clinical utility of heart derived stem cells. In a recent study, cardiosphere expansion step was done in order to obtain reasonable numbers of cells for transplantation studies from small biopsy specimens (Smith *et al.*

2007). The cells thus derived termed cardiosphere derived cells (CDCs) when injected into the border zone of myocardial infarcts engrafted and migrated into the infarct zone and the left ventricular ejection fraction was subsequently improved. This indicates that CDCs could be better candidates for myocardial cell therapy. CDCs as obtained in my study comprises of a mixture of cell populations in addition to the genuine cardiac progenitor cells as confirmed by the FACS data. Previous studies suggested the use of a mixed cell population as advantageous for tissue regeneration and possibly for regenerative efficacy.

CDCs obtained in our study were largely $CD133^-$, and $CD45^-$, as well as negative for a cocktail of blood lineage markers (Mac1, B220 & Gr1). This indicates that CDCs are phenotypically distinct from bone marrow–derived or endothelial progenitor stem cell populations that populate the heart via the circulation which are $CD133^+$ $CD34^+$ and $CD45^+$ (Smith *et al.* 2007). CDCs contain subpopulations that phenotypically resemble endothelial cells $CD34^+$ $CD31^+$ (Pusztaszeri *et al.* 2006), which suggests that these cells can cause sufficient angiogenesis following delivery to the infarct site. CDC subpopulations consist of cells positive for the stem cell markers (ckit and MDR1) as well.

V.4.1.2. Effect of EGF on cardiosphere-derived cell proliferation

The significant rise in cell proliferation rate when incubated with EGF supplemented medium connotes that EGF has a profound mitogenic effect on CDC proliferation. When compared to other widely used growth factors, cardiac stem cells respond to EGF more efficiently, which in turn suggests that EGF treatment yields adequate cell numbers for transplantation studies.

V.4.1.3 Effect of EGF on CDC phenotypes

In the present study, EGF treatment enhanced the percentage of cells positive for the myocyte markers (cTN1 & MHC) when compared with the untreated control. Cells positive for the stem cell markers remain almost the same in both the cases which indicates that EGF treatment resulted in the differentiation of the CDC subpopulations towards the myocyte lineages. The findings imply that CDCs are suitable candidates for myocardial regeneration. The EGF mediated effect on CDCs is likely to be of significance in clinical trials.

V.4.2 Effect of EGF on the migratory and healing activities of CDCs

V.4.2.1 Effect of EGF on CDC migration

Earlier studies by other investigators have revealed that CSCs express HGF receptor c-Met and IGF-1 receptor (IGF-R) (Urbanek *et al.* 2005a). Under the treatment with their ligands, CSCs secrete HGF/IGF-1. When myocardial infarction was produced and human HGF/IGF-1 was locally injected, expression levels of murine mRNA and proteins for HGF/IGF-1 in infarcted tissue were increased. Activation of these growth factor signals was confirmed by phosphorylation of c-Met, IGF-R, and their downstream targets. Migration studies demonstrated that HGF promoted motogenic and invasive activity of CSCs, whereas IGF-1 had little effect. Conversely, IGF-1 showed more antiapoptotic and proliferative effects on CSCs compared with HGF (Urbanek *et al.* 2005a).

Urbanek *et al* examined whether HGF/IGF-1 stimulate migration, proliferation, and differentiation of CSCs in the infarcted heart (Urbanek *et al.* 2005a). They found that

cycling CSCs exist in the atrioventricular groove. They further injected retrovirus expressing enhanced green fluorescent protein (EGFP) in this region. After EGFP was integrated into the CSCs, myocardial infarction was made and subsequently HGF/IGF-1 were injected into the predicted pathway of migrating cells. This resulted in migration of EGFP-positive cells toward the infarcted area through the interstitium of the heart. Furthermore immunohistochemical analysis showed that the locomotive EGFP-positive cells possess the characteristics of CSCs. Consistent with their *in vitro* data, HGF but not IGF-1 had locomotive effects on CSCs. Regenerated myocardium after the HGF/IGF-1 combined treatment was identified as BrdU positive cardiomyocytes and vessels. HGF/IGF-1 treatment increased the number of newly-formed cells, resulting in an increased volume of myocardium, improvement of cardiac function, and better survival.

Since maximum enhancement of cardiosphere and cardiosphere-derived cell proliferation was obtained with EGF, the present study looked into the effect of EGF on cell migration and wound healing in CDCs. Earlier studies have indicated the chemotactic effect of EGF on fibroblasts, smooth muscle cells and keratinocytes (Elenius *et al.* 1997). Results of the present study show the chemotactic responses of CDCs to EGF. EGF at a concentration of 10ng/mL could significantly favor CDC migration. Conversely, in control conditions, the rate of cell proliferation, migration and wound closure remained significantly low. The study provided evidences that adequate concentrations of EGF encourage directed migration of transplanted CDCs.

V.4.2.2. Effect of EGF on wound healing responses of CDCs

My observations suggest that EGF can induce CDC migration, proliferation and wound healing process. The significant increase in CDC migration *in vitro* may be comparable to those *in vivo* following ischemic insults.

V.5. EGFR signaling in CDCs

CSCs in the normal myocardium remain quiescent or cycles very slowly. At any one time, only a small fraction of CSCs are amplifying and differentiating to replace the parenchymal cells lost by normal wear and tear. This mechanism has been hard to measure because of the low cell turnover in the normal adult myocardium. In response to stress, the myocytes produce growth factors and cytokines, for which CSCs have receptors, and autocrine activation of growth-factors is simultaneously triggered in the CSCs (Lynbak *et al.* 2007). The findings from human and animal studies led to test whether *in situ* activation of CSCs by growth factors would be effective in regenerating myocardium after ischemia.

Eventhough several studies shed light on intrinsic stem cell therapy by local administration of growth factors, it also raises several important questions that should be addressed by future studies. First, what determines the CSC quiescence in 'cardiac niche'? In bone marrow niche, angiopoietin-1/Tie-2 signaling is critical for the maintenance of hematopoietic stem cell quiescence (Arai *et al.* 2004). Although it is not clear whether the similar mechanism is present in the heart, it is possible to antagonize signals maintaining CSCs quiescence state ulilizing the receptor/ligand interactions (Nagai *et al.* 2005). CSCs coexpress HGF/IGF-1 and c-Met/IGF-R, and

there is a positive feedback loop of HGF/IGF-1 signaling in CSCs. CSCs may exit from the quiescence when challenged by higher doses of HGF/IGF-1. Identification of the mechanism that maintains CSC quiescence is of particular importance. Inhibition of CSC quiescence can be a novel strategy to promote myocardial regeneration by enhancing CSC proliferation. Another matter of concern is how does HGF induce migration of CSCs to the infarct area? Among several signaling molecules activated by HGF, PI3-kinase (PI3K) seems to be critical for HGF-mediated cell migration, because PI3K is required for HGF-induced lamellipodia formation and subsequent migration (Khwaja *et al.* 2008; Royal *et al.* 2000). Whether PI3K is also critical in CSCs–ECCs migration should be determined. In addition, inhibition of negative regulators can be another strategy to promote myocardial regeneration.

Another major signaling pathway activated by a broad range of stimuli that modulates cell growth is the EGF receptor and associated kinases, including the ERK1/2 pathway (Vaidya *et al.* 2003; Zhuang and Schnellmann 2006). The role of EGF in maintaining repair and proliferation of renal epethial cells has been well studied. EGF receptor activation documented to induce migration in these cells (Lash 2008). The role of EGF in cardiac stem cell proliferation has not been studied so far.

Epidermal growth factor (EGF) is a small mitogenic protein that plays important role in regulation of cell growth, proliferation, differentiation, wound healing and oncogenesis (Herbst 2004). EGF acts by binding with high affinity to epidermal growth factor receptor (EGFR) on the cell surface and stimulates the intrinsic protein-tyrosine kinase activity of the receptor. The signal is soon transmitted across the entire cell by various receptor tyrosine kinases (RTKs) which activate the downstream

signal transduction pathways. EGF has diverse role in inducing stem cell differentiation. It has been reported that treatment of hMSC with EGF resulted in stimulation of osteoblast differentiation (Kratchmarova 2005). Likewise EGFR signaling is crucial for the maintenance of human brain tumor stem cells (Soeda 2008). In addition to numerous cancers, EGFR may be involved in the progression of vascular diseases.

Results from the present study suggest that EGF has a potent role in CSC expansion and migration. Also the pathway responsible for EGF mediated expansion of cardiosphere-derived cells is suggested to be p38MAPK dependent. Additional experiments examining the activation status of p38 MAPK are required to ascertain the role played by this pathway in mediating EGF induced CDC proliferation. Given that p38MAPK is a stress induced kinase, presence of the p38 MAPK inhibitor, SB203580 in the culture might have probably suppressed the CDC proliferation rate.

V.6. SIGNIFICANCE OF THE STUDY

The findings confirm the existence of $ckit^{pos}$ cardiac stem cells in post natal human hearts. Except in older patients, isolation of stem cells from adult heart tissue could provide an avenue for developing regenerative cell therapy in patients with coronary artery disease. The study provided evidences suggesting role for EGF in encouraging migration of transplanted CDCs directed towards sites of wound and stimulating proliferation of thus attracted cells. The study further provided evidences that CDCs expanded with EGF could supply reasonable cell numbers for transplantation studies.

V.7. LIMITATIONS OF THE STUDY

Although several lines of evidence support the change in marker expression profile of cardiac stem cells in culture, there are reports suggesting the use of clonal progenies as advantageous for clinical delivery. In that sense, inability to retain cardiac stem cell clones to evaluate the effects of EGF is a limitation of this study. Lack of an appropriate control population and small sample size in the comparative study between CAD risk factors and CSC yield is another limitation. Collection of control tissue samples from healthy individuals however is considered unethical. Attempts to determine the phosphorylation status of p38 MAPK protein was not successful on account of several problems commonly encountered in ELISA and Western blot analysis, including low concentration of proteins from sub-confluent cultures, low protein transfer and binding, non-specific staining and high background, and inconsistency in the protein profile between samples of the same group.

VI. SUMMARY AND CONCLUSIONS

The documentation of the existence of resident adult cardiac stem cells has created great expectations concerning their utilization as a strategy for the management of heart diseases. One of the goals of the present study was to establish easy and cost-effective protocols for the isolation and expansion of human adult resident CSCs. CSCs were isolated from right atrial biopsy samples obtained from patients with chronic coronary artery disease and were characterized by their morphology and positive staining for stem cell markers ckit, MDR1 and CD34.

CSCs were expanded in a growth factor supplemented medium to form cardiospheres. The cardiosphere cells were found to retain the markers of stemness as well as consisted of cardiac and endothelial lineage differentiation markers. Cardiosphere formation was found to enhance in the presence of EGF in the growth medium. The influence of EGF on cardiac stem cells is of considerable interest because EGF, as a potent mitogen can play an important role in cardiac stem cell expansion. The study examined the role of disease severity and CAD risk factors in determining the yield of the stem cells isolated from biopsy samples. The yield of stem cells was not found to be influenced by any of the clinically relevant factors except for age.

Isolated cardiospheres were expanded to form dividing manolayers termed cardiosphere-derived cells. The phenotypic profile of CDCs in the presence and absence of EGF was studied. EGF treatment seemed to enhance cardiomyocyte differentiation in CDC populations.

An additional goal of the study was to examine the role of EGF in the mitogenic, migratory and healing activities of CDCs. EGF significantly favored migration, proliferation and healing processes. The study concluded that cardiac stem cells

respond to EGF more efficiently when compared to other widely used growth factors. Preliminary studies suggested that p38MAPK signaling cascade is involved in EGF mediated CDC expansion.

Together, the findings underscore the influence of EGF on CDCs that could contribute to the development of therapeutic strategies favorable for myocardial regeneration.

VI.1. FUTURE DIRECTIONS

The present study established the presence of $ckit^{pos}$ stem cell populations in adult human hearts and the role of EGF in CSC expansion. It would be interesting to further explore

1. The cell cycle regulation in CSCs
2. The role of niche in CSC maintenance and recruitment upon appropriate stimulus
3. The crucial signaling pathwy involved in CSC expansion
4. The underlying signaling mechanism involved in CSC quiescence in post natal human hearts
5. The relative advantages and disadvantages of antigenically purified progenitor cells, CDCs, cardiosphere-forming cells, and cardiospheres themselves as products for regenerative therapy
6. The detailed signaling pathway involved in EGF mediated CSC expansion

7. The role of EGF in encouraging directed migration and proliferation of transplanted CDCs towards the site of myocardial infarcts *in vivo*.

8. Identification of optimal culture and differentiation conditions for CSCs devoid of xenogenic growth supplements.

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VIII. LIST OF PUBLICATIONS/AWARDS

1. Aghila Rani KG, Jayakumar K, Srinivas G, Nair RR, Kartha CC. Isolation of ckitpos cardiosphere forming cells from human atrial biopsy. *Asian Cardiovasc Thorac Ann* 2008;16:50-56.

2. Aghila Rani KG, Jayakumar K, Sankara Sarma P, Kartha CC. Clinical determinants of ckit-positive cardiac cell yield in coronary disease. *Asian Cardiovasc Thorac Ann* 2009;17:1-4.

3. Aghila Rani KG, Jayakumar K, Kartha CC. Effects of epidermal growth factor receptor signaling in cardiosphere-derived cell migration and proliferation. (Manuscript under preparation).

4. Young Scientist Award at the '20th Kerala Science Congress' meeting held during 28th-31st January 2008 at Trivandrum for the presentation entitled "Disease severity and risk factors do not alter the yield of ckitpos cells obtained from right atrial biopsies from patients with coronary artery diseases".

APPENDIX I

SREE CHITRA TIRUNAL INSTITUTE FOR MEDICAL SCIENCE & TECHNOLOGY
THIRUVANANTHAPURAM-695 011, INDIA
(An Institute of National Importance under Govt. of India)
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Institute Ethics Committee
Technical Advisory Committee
Hospital Wing

Members
Prof. C.C. Kartha
Dr. Lissy K. Krishnan
Dr. Harikrishnan. S
Dr. Girish Menon R

Certificate of Clearance-Technical Advisory Committee

Title of the Proposal: Adult human resident cardiac stem cells & endothelial progenitor cells: detection of optimum condition for their therapeutic use.

TAC-Registration No. : SCT-S/ 2004/ 007

Name (s) of Investigators and affiliations:

Principal investigator : Prof C. C. Kartha

Designation and Affiliation: Prof & Head, Div of Cellular & Molecular Cardiology, SCTIMST

Co-Investigator (s) : 1. Name: Dr. K. Jayakumar
Designation and Affiliation: Prof. CVTS, SCTIMST

2. Name:
Designation and Affiliation

Date of TAC meeting 22.1.2005

Prof. C.C. Kartha

Dr. Lissy K. Krishnan

Dr. Harikrishnan. S

Dr. Girish Menon R

The above mentioned proposal has been found satisfactory from a perspective of safety, study design and analysis.

Signature of Member Secretary
Date : 22.1.05

Dr. GIRISH MENON R
Associate Prof.
Dept. of Neurosurgery
Sree Chitra Tirunal Institute for Medical Sciences and Technology, Trivandrum-11

APPENDIX II

CONSENT FORM FOR USE IN COLLECTION OF TISSUE TO BE USED IN HUMAN STEM CELL RESEARCH

Project Title	:	
Name and Complete Address of the Project Implementing Agency	:	
Name, Address and Telephone Number of the Principal Investigator	:	
I have been explained the purposes of the research being undertaken, and I have understood them	:	Yes/ No
I have had the opportunity to ask questions and am satisfied with the answers provided to me	:	Yes/ No
I have been informed of the risks of participation and donation	:	Yes/ No
I have been informed of the compensations to be provided to me in case of any physical injury to me resulting from the tissue collection	:	Yes/ No
I have been informed of the steps to be implemented for protecting my privacy and confidentiality and I am satisfied with them	:	Yes/ No
I have been informed that certain screening tests may be perfomed on the tissue samples donated by me and that I will not be provided with the results of these tests	:	Yes/ No
I have been informed that no	:	Yes/ No

identifiable results pertaining to me that are generated during the course of this research will be provided to me

I have been informed that I will not derive any direct benefits resulting from this research	:	Yes/ No
I have been informed that I have the right to instruct destruction of my tissue samples at any stage of this research provided that my tissue samples are identifiable at that stage	:	Yes/ No

I am willingly donating my sample for the purpose of this research study and I confirm that I have not been coerced, directly or indirectly, to donate my sample.

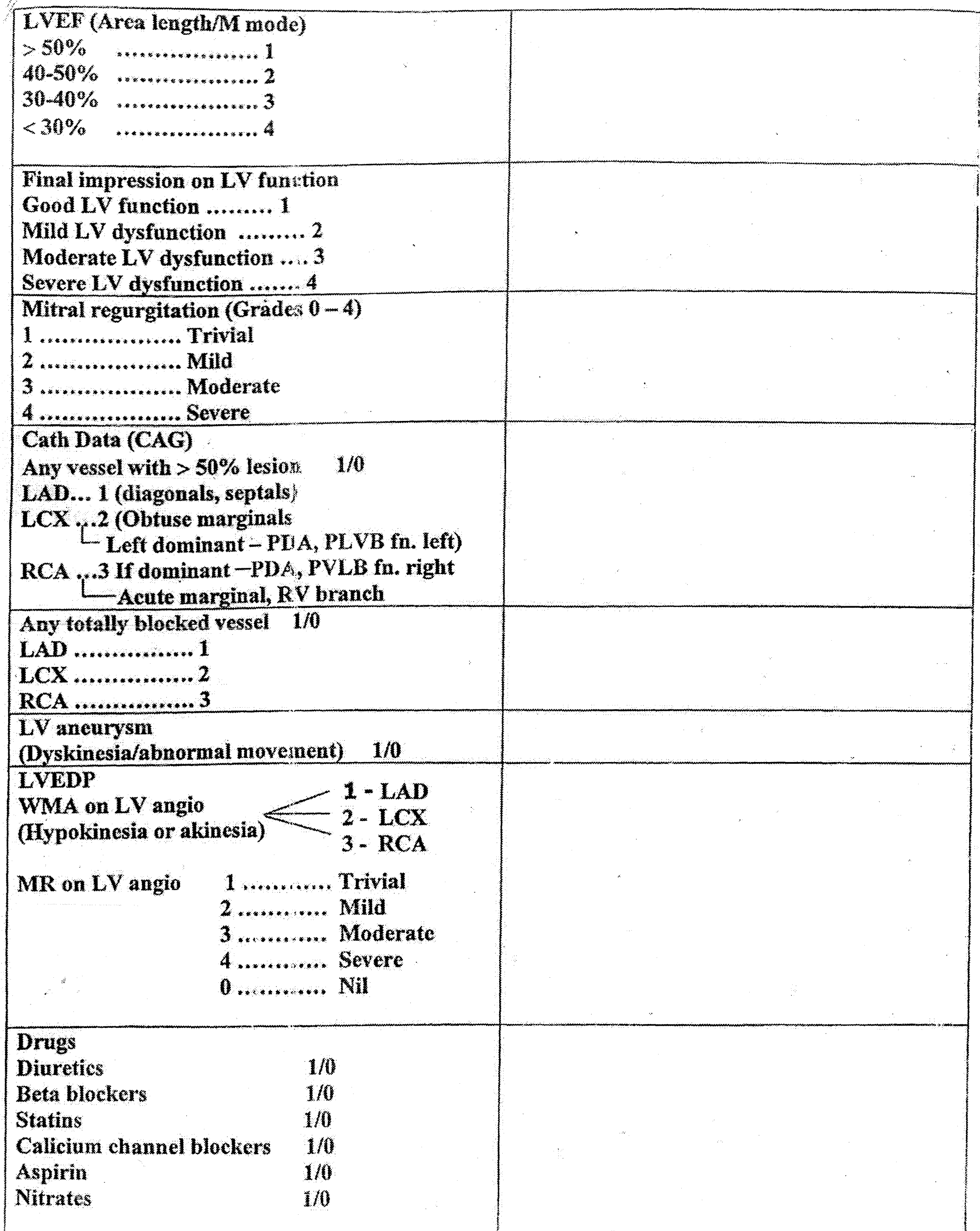
Donor's Signature ____________________ **Date** ____________________

Witnessed by ____________________ **Date** ____________________

APPENDIX III

PROFORMA

Hospital Number	
Age	
Sex 1/0	
Diagnosis Post myocardial infarction........... 1 Chronic stable angina .... 2 Unstable angina 3	
Smoking 1/0	
Hypertension 1/0 (Either a past history or a recorded BP of >140/90)	
Diabetes Mellitus 1/0 (FBS >126, PPBS >180, or a past history)	
Insulin requiring/not requiring 1/0	
IFG (Impaired Fasting Glucose) FBS 110-126mg%	
Dyslipidemia (↓HDL) 1/0 < 40 Low HDL 1/0 >100 High LDL 1/0 > 180 High TG 1/0 > 240 High TC 1/0	LDL= TC-[TG/5 + HDL]
Other cardiac diseases	
Other medical diseases	
NYHA functional class I / II / III / IV	
Presence of angina 1/0	
Presence of CHF 1/0	
TMT 1/0 Exercise time minutes/seconds Angina 1/0 Recovery prolonged >3mins 1/0 Stage ≥ +ve 1/0 avr ST elevation 1/0 Strong +ve 1/0	
Echo Data Presence of Wall motion 1/0 RWMA in LAD territories ------ 1 (AW, IVS) LCX .... 2 (LW) RCA .... 3 (IW)	



LVEF (Area length/M mode) > 50% .... 1 40-50% .... 2 30-40% .... 3 < 30% 4	
Final impression on LV function Good LV function .... 1 Mild LV dysfunction .... 2 Moderate LV dysfunction .... 3 Severe LV dysfunction 4	
Mitral regurgitation (Grades 0 – 4) 1 .... Trivial 2 .... Mild 3 .... Moderate 4 .... Severe	
Cath Data (CAG) Any vessel with > 50% lesion 1/0 LAD... 1 (diagonals, septals) LCX ...2 (Obtuse marginals └ Left dominant – PDA, PLVB fn. left) RCA ...3 If dominant –PDA, PVLB fn. right └ Acute marginal, RV branch	
Any totally blocked vessel 1/0 LAD .... 1 LCX .... 2 RCA 3	
LV aneurysm (Dyskinesia/abnormal movement) 1/0	
LVEDP WMA on LV angio (Hypokinesia or akinesia) → 1 - LAD, 2 - LCX, 3 - RCA MR on LV angio 1 .... Trivial 2 .... Mild 3 .... Moderate 4 .... Severe 0 .... Nil	
Drugs Diuretics 1/0 Beta blockers 1/0 Statins 1/0 Calicium channel blockers 1/0 Aspirin 1/0 Nitrates 1/0	