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Ciclo: XX

ISOLATION, EXPANSION AND DIFFERENTIATION OF HUMAN BONE MARROW CD133+ CELLS: PLASTICITY AND CARDIAC REGENERATION

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SOMMARIO

L'uso di cellule staminali adulte per rigenerare tessuto danneggiato non ha le implicazioni di tipo morale e tecnico associate invece all'uso di cellule di origine embrionale.
Le cellule staminali mesenchimali adulte (MSCs) possono essere isolate da vari tessuti, più comunemente dal midollo osseo, e sebbene siano presenti in una piccola percentuale, sono facilmente espandibili.
Recenti studi hanno mostrato come l'uso di MSCs possa portare benefici clinici a pazienti con osteogenesi imperfecta, malattia da reazione di rigetto, e infarto al miocardio.
Accanto a queste cellule staminali, è noto che cellule CD133+ dal midollo osseo umano (BM) hanno un ruolo importante nel compartimento ematopoietico. Le cellule infatti possono prendere parte alla ricostituzione vascolare quando diventano cellule endoteliali (EC), alla rigenerazione di fibre muscolari scheletriche quando sono precursori muscolari e a cardiomiociti quando differenziano in cellule simil-cardiomiocitarie. Mentre il ruolo in ematopoiesi e vasculogenesi delle cellule CD133+ è ben conosciuto, la loro capacità a differenziare verso linee non endoteliali non è ancora stata ben definita.
Scopo di questo studio è di provare se cellule CD133+ derivate da BM, paragonate alle MSCs, sono in grado di differenziare in vitro non solo in cellule del sangue ma anche in linee cellulari che appartengono al foglietto germinale del mesoderma.
A tal fine cellule CD133+ sono sono state isolate utilizzando un metodo clinicamente approvato e si è paragonato il loro potenziale differenziativo a quello delle cellule ematopoietiche (HSCs) e delle MSCs ottenute dagli stessi campioni di midollo osseo umano.
Nelle condizioni di coltura utilizzate, l'espressione del marker CD133 è diminuita mantenendosi comunque ad un livello inferiore di espressione dopo il secondo passaggio in coltura, come pure l'espressione dei markers c-kit e OCT-4, mentre l'espressione di Stage Specific Embryonic Antigen 4 (SSEA4) è rimasta consistente in tutte le differenti

condizioni di coltura. CD133+ espanse sono risultate positive anche per HLA-ABC ma negative per HLA-DR in accordo con quanto riportato per le MSCs. Inoltre si sono dimostrate in grado di differenziare in adipociti, mioblasti, cellule endoteliali, osteociti, cardiomiociti, e cellule neuronali precursori. I risultati di questo studio hanno dimostrato l'ampio potenziale differenziativo delle CD133+ da midollo osseo umano, non solo in senso mesodermico ma anche ectodermico (neuronale).

In vivo, utilizzando un approccio di ingegneria tissutale, si è voluto studiare se un polimero di collagene biocompatibile e biodegradabile, chiamato cardiac patch, applicato nella zona infartuata di cuore di ratto nudo, possa dare ospitalità alle cellule staminali e renderle disponibili per migliorare la funzionalità cardiaca. Le cellule sono state iniettate sia sul patch (modello I) sia per via sistemica (modello II); anticorpi contro Actina muscolare liscia e Von Willebrand hanno evidenziato lo sviluppo di nuovi vasi nel patch e nella zona di cryoinury (come già dimostrato dal gruppo di ricerca); circa il 2% delle cellule ha vissuto e ha mostrato buona mobilità nel patch di collagene, è stata accertata migliore funzionalità cardiaca rispetto agli animali di controllo, anche se dopo quattro settimane non è stato osservato attecchimento delle cellule staminali a seguito di iniezione delle stesse sia a livello locale sia sistemico.

In conclusione, i due diversi modelli *in vivo* hanno voluto definire sia la resistenza del patch dopo iniezione delle cellule nel primo modello I sia l'effetto trofico del patch nel modello II.

Per trattare in modo approfondito i punti sopra menzionati, sarà necessario analizzare l'espressione bcl-2 in animali trattati e non trattati con le cellule, con e senza patch, per verificare se l'approccio di tissue engineering con questo polimero possa favorire l'effetto paracrino oppure sia necessario cambiare il polimero per migliorare la sopravvivenza cellulare.

SUMMARY

The use of adult stem cells to regenerate damaged tissue circumvents the moral and technical issues associated with the use of those from an embryonic source.

Mesenchymal stem cells (MSC) can be isolated from a variety of tissues, most commonly from the bone marrow (BM), and, although they represent a very small percentage of these cells, are easily expandable. Recently, the use of MSC has provided clinical benefit to patients with osteogenesis imperfecta, graft-versushost disease and myocardial infarction.

Beside these stem cells, it is known that the bone marrow $CD133^+$ cells play an important role in the hematopoietic compartment. The cells indeed can take part to vascular reconstitution when become endothelial cells (EC), to skeletal muscle fiber regeneration when switch in muscle precursors, and to cardiomyocytes phenotypic conversion when differentiate in cardiomyocytes like cells. While the role on hematopoiesis and vasculogenesis of the selected cells is well established, their ability to differentiate along multiple non-EC lineages has not yet been fully elucidated.

The goal of this study is to assert whether human $CD133^+$-BM derived cells, compared with MSCs, are able to differentiate in vitro besides to blood cells, to cell lineages pertinent to the mesoderm germ layers.

To this end $CD133^+$ cells have been isolated using a clinically approved methodology and their differentiation potential compared to that of hematopoietic stem cells (HSCs) and mesenchymal stem cells (MSCs) obtained from the same BM samples.

In adopted culture conditions, CD133 expression was consistently decreased after passage 2, as well as the expression of the stemnesss markers c-kit and OCT4, whereas expression of Stage Specific Embryonic Antigen 4 (SSEA4) remain consistent on all different conditions. Expanded CD133 were also positive for HLA-ABC, but negative for HLA-DR in accordance to what has been previously reported for MSCs. Moreover they were able to

differentiate into adipocytes, myoblasts, endothelial cells, osteocytes, cardiomyocytes and neuronal precursor cells.

The results of this study fully support the notion of a wide range differentiation potential of $CD133^+$-BM derived cells, encompassing not only mesodermal but also ectodermic (neurogenic) cell lineages.

CD133 antigen could be potentially used to select a cell population with similar characteristics to the MSCs ones; the obtained results remark the great potential of $CD133^+$ cells from BM, and support the existence of a broadly multipotent/pluripotent cell that persists in the adult, and justify the upcoming interest for possible therapeutic applications.

In vivo, using a tissue engineering approach, it has been investigated the possibility that a biodegradable and biocompatible collagen polymer, called cardiac patch, applied on the infarction area of nude rat, can give hospitality to stem cells and deliver them to improve cardiac functionality. Cells have been injected into the patch (model I) and systemically (model II); smooth muscle actina and Von Willebrand antibodies detected many new vases in patch and cryoinjury area (as already found by the research group); about 2% of cells survived and showed good mobility into the collagen patch; improved functionality was detected compared with control animals however no cell engraftment was seen after four week from cell injection either locally or systemically.

Summarizing, the two different *in vivo* models aimed at define both the patch strength after cell injection in model I and the patch trophic effect in model II.

Therefore to address these points it will be mandatory to analyze bcl-2 expression on cells treated and un-treated animals, with and without patch, to verify whether the tissue engineering approach with this specific polymer, could enhance the paracrine effect of human CD133+, or suggest a change of the polymer to ameliorate cell survival.

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Chapter 1 Introduction

Basic and clinical research accomplished during the last few years on stem cells has constituted a revolution in regenerative medicine and cancer therapies by generating multiple possible therapeutically useful cell types. Intense research on stem cells has provided important information on developmental, morphological and physiological processes that govern tissue and organ formation, maintenance, regeneration and repair after injury. More recently, significant advances in the understanding of stem cell biology have provoked great interest and hold high therapeutic promises based on the possibility of stimulating the ex vivo and *in vivo* expansion and differentiation into functional progeny that could regenerate the injured tissue /organ firstly in animals and then in humans (*Mimeault M et al, 2007*)

1.1 Stem Cells

Stem cells are primal cells found in all multicellular organism defined by two characteristics: they are unspecialized cells that renew for a lifetime by cell division to maintain the stem cell pool, and they can differentiate to cells with special functions under

specific physiologic or experimental conditions. To fulfill this dual function, they undergo symmetric and asymmetric divisions during development.

To begin with, we can distinguish between embryonic and adult stem cells;

Embryonic stem cells (ES cells) were first derived from mouse embryos in 1981 by Martin Evans and Matthew Kaufman and independently by Gail R. Martin.

ES cells are stem cells derived from the inner cell mass of an early stage embryo known as a blastocyst (4-5 days post fertilization); ES cells are pluripotent, therefore they are able to differentiate into all derivatives of the three primary germ layers: ectoderm, endoderm, and mesoderm. These include each of the more than 220 cell types in the adult body. Pluripotency distinguishes ES cells from multipotent progenitor cells found in the adult; these only form a limited number of cell types. When no stimuli is given for differentiation, (i.e. when grown *in vitro*), ES cells maintain pluripotency through multiple cell divisions. The presence of pluripotent adult stem cells remains a subject of scientific debate, however, research has demonstrated that pluripotent stem cells can be directly generated from adult fibroblast cultures (*Takahashi K and Yamanaka S, 2006*)**.** Because of their plasticity and potentially unlimited capacity for self-renewal, ES cell therapies have been proposed for regenerative medicine and tissue replacement after injury or disease. However, to date, no approved medical treatments have been derived from embryonic stem cell research mainly because they form teratoma.

Differently from ES, which are defined by their origin, it is unknown where adult stem cells come from in mature tissues (Figure 1).

Adul stem cells are undifferentiated cells found among differentiated cells in a tissue or organ; the primary roles of adult stem cells in a living organism are to maintain and repair the tissue in which they are found. They have thus far been the only stem cells used to successfully treat several diseases, such as a number of blood and immune-system related genetic diseases, cancers, and disorders; juvenile diabetes.

The history of research on adult stem cells began about 40 years ago. In the 1960s, Joseph Altman and Gopal Das present scientific evidence evidence of adult neurogenesis, in the same period it has been discovered that the bone marrow contains at least two kinds of stem cells. One population, called hematopoietic stem cells, forms all the types of blood cells in the body. A second population, called bone marrow stromal cells, was discovered a few years later.

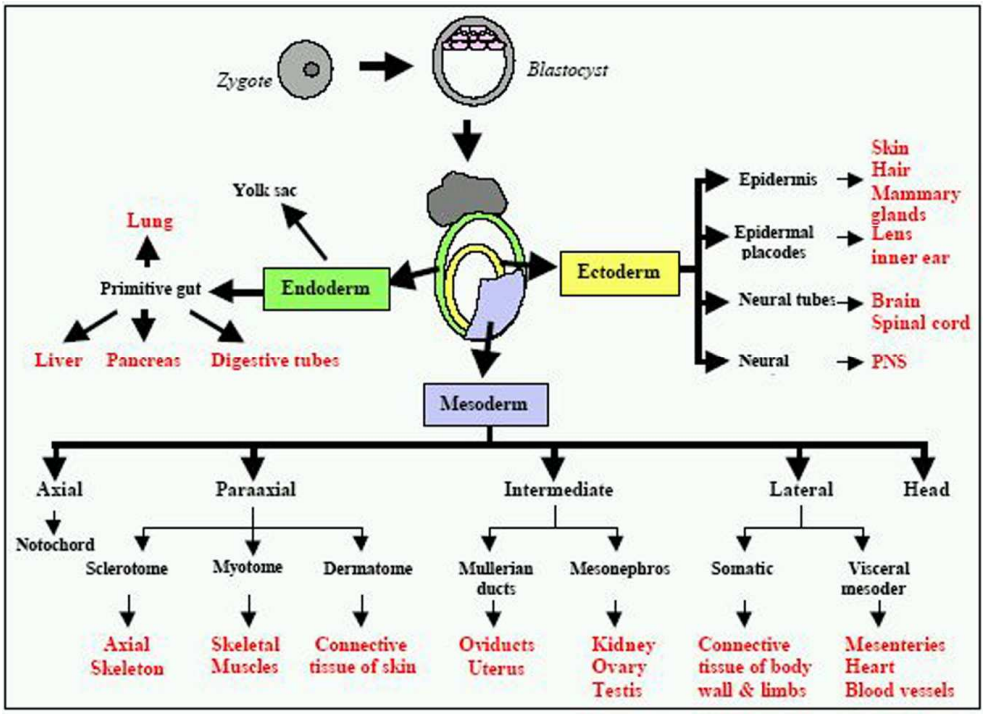


Figure 1:Development scheme of stem cells and organs. The pluripotent ESC types derived from blastocyst stage during embryonic development and multipotent tissue-resident adult stem cells arising from endodermal, mesodermal, and ectodermal germ layers are shown.

1.1.1 Mesenchymal Stem Cells and CD133+ population from human bone marrow

Bone marrow (BM) stromal cells were first identified by Friedenstein in 1976 (*Friedenstein AJ et al., 1976*), who described an adherent fibroblast-like population able to differentiate into bone that he referred to as osteogenic precursor cells . Subsequent studies demonstrated that these cells have the ability to differentiate into various other mesodermal cell lineages, including chondrocytes, tenocytes and myoblasts. Based on this multilineage differentiation capacity, Caplan introduced the term mesenchymal stem cells (MSCs). It was therefore recently proposed to use the term multipotent mesenchymal stromal cells (with the acronym MSCs) to describe fibroblast-like plastic adherent cells. Recently, Bonnet D et al. demonstrated that single cell-derived populations of murine BM-derived MSCs characterized by Stage Specific Embryonic Antigen-1 (SSEA-1) expression, were capable of differentiation in-vivo, thus showing their true stem cell properties. While originally isolated from BM, similar populations have been isolated from other tissues, including adipose tissue, placenta , amniotic fluid , and fetal tissues such as fetal lung and blood (*Zuk PA et al., 2001; In 't Anker PS et al., 2003*).

The capacity to differentiate into multiple mesenchymal lineages, including bone, fat and cartilage, is being used as a functional criterion to define human MSCs

Considering the phenotype of human MSC, at present no specific marker or combination of markers has been identified that specifically defines MSCs. Indeed, ex-vivo expanded MSCs express a number of non-specific markers (Table I), including Stro-1 which identify a non hematopoietic stromal cell precursors, CD105 (SH2 or endoglin), CD73 (SH3 or SH4), CD90, CD166 (activated leucocyte cell adhesion molecule, ALCAM), CD44 and CD29, SSEA4 and GD2 (*Gang EJ et al, 2007; Martinez C et al, 2007*). MSCs

are devoid of hematopoietic and endothelial markers, such as, CD11b, CD14, CD31 and CD45.Therefore, so far an unique protocol to isolate these cells cannot be agreed yet among different laboratories and represent an unsolved problem for standardized clinical applications.

Beside many issues remain to be solved regarding their characteristics, phenotype and behavior in culture, nonetheless MSCs have already demonstrated their efficacy in preliminary therapeutic applications, including osteogenesis imperfecta, hematopoietic recovery and bone tissue regeneration (*Horwitz EM et al, 2002; Petit H et al, 2000*).

Table I. Cell surface marker characterisation of human MSC. The common cell surface markers used to select or exclude MSC from a heterogeneous cell population. HLA: human leukocyte antigen. MHC: Major histocompatibility complex. From Fox JM et al, 2007

MSC selection markers	**MSC exclusion markers**
HLA-ABC (MHC Class I)	HLA-DR (MHC Class II)
CD90 (Thy-1)	CD4 (T-cell co-receptor)
CD105 (Endoglin/SH-2	CD4 (T-cell co-receptor)
CD106 (VCAM-1)	CD14 (LPSR)
CD73 (SH-3)	CD25 (IL-2R)
CD166 (ALCAM)	CD45 (Leucocyte antigen)
SSEA-4	CD34 (Haematopoietic marker)
Stro-1	
SH-4	

On a different ground, marrow isolated cells like Marrow Isolated Adult Multilineage Inducible cells (MIAMI; *D'Ippolito G et al, 2004*) and Multipotent Adult Progenitors Cells (MAPCs ;*Jiang Y et al, 2002*) have been described as pluripotent cells capable of differentiating *in vitro* into cell lineages derived from all three germ layers (mesoderm,

ectoderm and endoderm), and murine MAPCs were able to engraft into various tissues *in vivo*. Hence, these cells could represent a significant breakthrough for (cellular) therapy with respect to MSCs. Notwithstanding, MAPCs have been reported to be difficult to isolate and to maintain in culture, and as a consequence they have not been taken in consideration yet for cell therapy.

In the late Nineties, a new stem cell marker called prominin-1, CD133 was identified.

Prominin-1 is the first discovered member of a family of polytopic membrane protein conserved throughout the animal kingdom. It is a cholesterol binding protein of 115/120 kDa, selectively associated with plasma membrane protrusion which motivated the choice of the name prominin (from the latin word prominere); it has an unsual membrane topology containing 5 transmembrane domains and 2 large glycosilate extracellular loops.(Figure 2; *Fargeas CA et al., 2006*).

Prominin-1 was identified in two independent studies, both being reported in 1997, which concerned murine neuroepithelial cells (*Weigmann A et al, 1997*) and human hematopoietic stem and progenitor cells (*Miraglia S et al., 1997*) respectively.

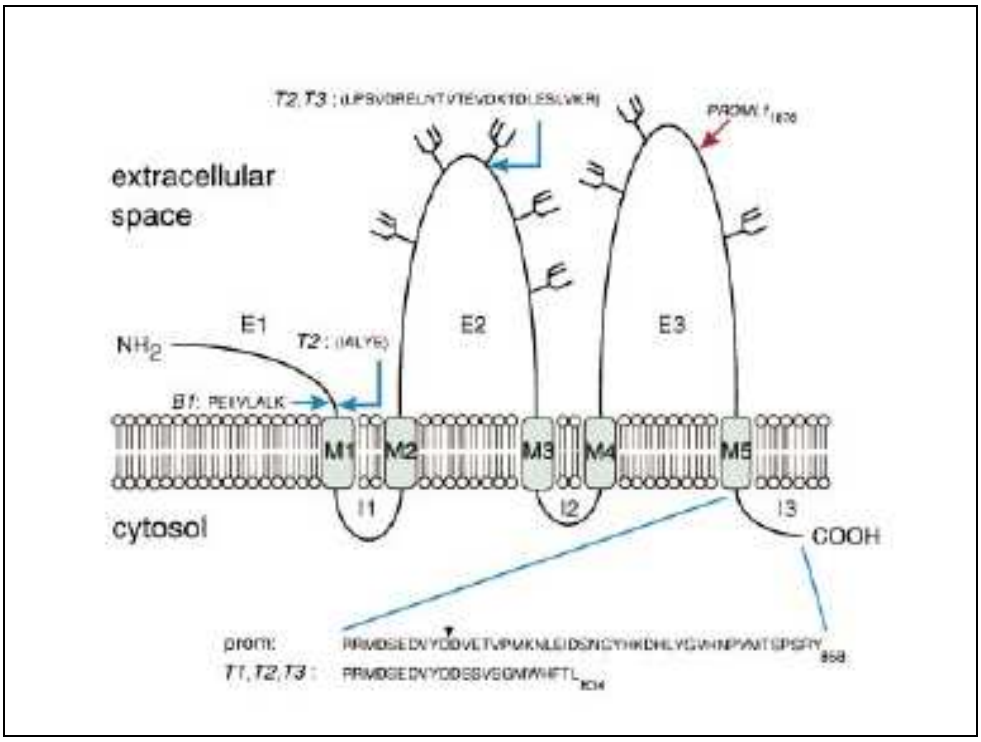


Figure 2: From Corbeil D et al., 2001. Membrane topology of prominin and prominin-related protein. Murine prominin. After cleavage of the signal sequence, prominin is predicted to consist of an extracellular N-terminal domain (E1), five membrane-spanning domains (M1–M5) separating two small intracellular domains (I1 and I2) and two large glycosylated extracellular domains (E2 and E3) and a cytoplasmic C-terminal domain (I3).

Regarding the first, studying the neuroepitelial cells of the ventricular zone rat monoclonal antibodies against mouse epithelial cells were raised. One antibody was found to label the apical plasma membrane of neuroepithelial cells, associated with microvilli.

In the hematopoietic system, several distinct cell lineages arise from rare pluripotent stem cells: since the total bone marrow- derived cell population is heterogeneous and the pluripotent hematopoietic stem cell constitute a small fraction, numerous effort have been

made toward their efficient isolation. Hence Yin and collegues generate antibodies against human hematopoietic progenitor cells. The mAb called AC133 identify the protein bearing the antigen AC133, a glycosilation-depentent epitope of CD133. The human protein expressing the antigen AC133 epitope is highly related to murine prominin in membrane topology, tissue distribution and subcellular localisation, the similarities in the topologies of human AC133 and mouse prominin suggested that these proteins were homologous , although their amino acid sequence showed only 60% identity and their tissue distribution was quite diverse.

Specifically, the expression of prominin-1 protein in adult humans is not limited to the stem and progenitor cells (*Miraglia S et al. 1997; Yin AH et al. 1997, see Table II*). Like murine prominin-1 (*Weigmann A et al. 1997; Maw MA et al,. 2000*), human prominin-1 is expressed in epithelial cells (*Florek M et al, 2005*) and cells derived therefrom, such as photoreceptor cells. The glycosylation-dependent AC133 epitope of human prominin-1 appears to specifically expressed in hematopoietic stem and progenitor cells (*Yin AH et al., 1997*), embryonic epithelia (*Corbeil D et al,. 2000; Uchida N et al,. 2000*), and cells dedifferentiating in the process of malignant transformation (*Florek M et al, 2005*), but not in differentiated adult epithelia, such as kidney (Figure 3; *Miraglia S et al., 1997*). Consistent with this, the AC133 epitope is present on undifferentiated human adenocarcinoma Caco-2 cells (*Corbeil D et al., 2000*) but is lost upon the differentiation of these cells, although the expression of the prominin-1 protein as such persists (*Florek M et al, 2005*). Therefore, the term “AC133 antigen” should not be used synonymously with prominin-1 or CD133 (*Fargeas CA et al., 2003*). Nonetheless, within the hematopoietic lineage, the human prominin-1 protein is exclusively expressed by primitive stem and progenitor cells and does indeed constitute a cell surface hematopoietic stem cell marker, as originally concluded from the expression of the AC133 antigen (*Miraglia S et al. 1997; Yin AH et al. 1997*).

Summarizing, human AC133 expression is restricted to non-differentiated cells, whereas upon cell differentiation and glycosylation, human and mouse CD133/prominin-1 expression is retained.

For example, in a multiple tissue expression array showed that human CD133 mRNA was stringly expressed in several tissues including adult kidney, mammary gland, trachea , salivary gland , placenta , pancreas, digestive tract and testes (*Florek M et al., 2005*).

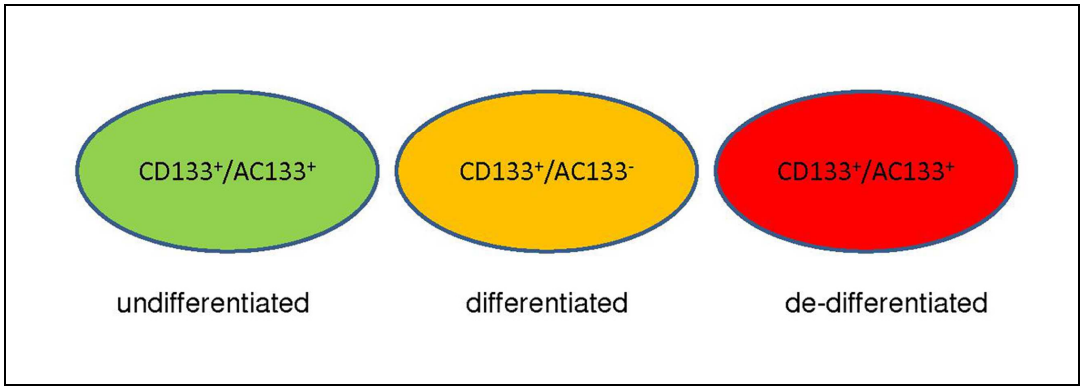


Figure 3:Cartoon of the expression of glycosylation-dependent AC133 epitope of prominin-1/CD133 according to the status of cell differentiation. From Florek M et al., 2005.

Table II: Tissue distribution of prominin-1: present in epithelial and non-epithelial cells. AC133 and 13A4 data refer to immunodetection analyses of human and mouse prominin using mAbAC13 (Weigmann A et al., 1997)and mAb 13A4(Yin H et al., 1997) respectively. n.d.=not determined. From Forek M, 2005.

Tissue	**Human**		**Mouse**	
	mRNA	AC133	mRNA	13A4
Embryonic				
Brain	n.d	+	n.d	+
Gut	n.d	+	n.d	+
Kidney	n.d	+	n.d	+
Stomac	n.d	n.d	n.d	+
Adult				
Bone marrow	+	+	n.d.	+
Brain	+	-	+	+
Colon	+	-	+	-
Heart	+	-	-	-
Kidney	+	-	+	+
Liver	+	-	-	-
Lung	+	-	+	-
Pancreas	+	-	n.d.	n.d.
Placenta	+	-	n.d.	n.d.
Skeletal muscle	+	-	+	n.d.
Small intestine	+	n.d.	+	-
Spleen	-	n.d.	-	-
Testis	n.d.	-	+	n.d.

In the last few years, research groups showed interest on CD133 marker. Indeed, it is proved that $CD133^+$ cells exhibit high proliferative potential and share the phenotypic (*Peichev M et al, 2000*) and functional properties with cells derived from the putative endothelial progenitor cells (angioblasts). As far as HSCs transplantation is concerned, preliminary clinical studies show slightly improved engraftments with $CD133^+$ cells compared to $CD34^+$ cells (*Lang P et al, 2004*). In such a clinical setting, highly purified $CD133^+$ peripheral cells have already been successfully used in autologous transplantation of a patient with ALL (*Koehl U et al., 2002*). More recently $CD133^+$-BM derived cells have also shown possible applications in regenerative medicine. In particular, it has been emphasized that they improve functionality of infarcted myocardium, possibly as result of the contribution of AC133+endothelial progenitors in blood vessels formation (*Stamm C et al., 2003*). Taking into account the broad spectrum of the AC133 epitope expression on stem cells of different tissues, a direct cardiomyogenic capacity of AC133+ cells could be a possibility (*Smelkov SV et al., 2005*). Moreover, they integrate in a regenerating skeletal muscle fusing with newly formed myofibres (*Torrente Y et al., 2004*) and differentiate into neural cells and astrocytes (*Lee A et al., 2005*).

Therefore, we can say that a role for CD133/AC133 as a marker of stem cells with the capacity to engraft and differentiate to form also functional non-hematopoietic adult lineages and contribute to disease amelioration via indirect (paracrine effect) or direct tissue regeneration, is emerging (Table III) and thus CD133/AC133 cell therapy may represent a promising treatment for many diseases.

Table III: Cells expressing prominin family members , often in combination with a repertoire of other markers, are widely expressed by multiple tissues and function as stem cells. BM:bone marrow; CB:cord blood. Adapted from Mizrak D et al., 2008.

Antigenic phenotype	Origin	Stem cells action	References
AC133 or CD133	Human adult blood, BM, CB and fetal liver	Human hematopoietic reconstitution	Yin AH, 1997
AC133	Human peripheral blood	Myogenesis in mouse model of Duchenne's muscular distrophy	Torrente Y, 2004
CD133	Human peripheral blood	Endothelial and cardiomyocytic diff *in vitro*	Bonanno G, 2007
CD133	Adult human kidney	Endothelial and epithelial differentiation, human renal regeneration	Bussolati B, 2005
CD133	Human BM	Human liver regeneration, mechanism unknown	am Esch JS, 2005
CD133	Human and mouse brain	Neural differentiation in mice	Barraud P, 2007
CD133 and SSEA4	Mouse embryonic forebrain	Neural differentiation *in vitro*	Corti S, 2007
AC133-2,β1 integrin	Human neonatal foreskin	Keratinocytes differentiation *in vitro*	Yu Y, 2002
AC133-2,β1 integrin	Human prostate basal cells	Prostatic differentiation *in vitro*	Richardson GD, 2004
CD133+CD34-CD45-Terl19-	Ductal epithelium neonatal mouse pancreas	Multiple lineage differentiation in mouse pancreas	Oshima Y, 2007
CXCR4,NGN-3,nestin, CD133,OCT-4,Nanog, ABCG2,CD117	Human pancreas	Islet differentiation *in vitro*	Koblas T, 2007

1.2 Cardiac environment :tissue engineering after miocardial infarction

1.2.1 Tissue engineering

Tissue engineering aims at restoring, maintaining or enhancing tissue and organ function. In particular scientists use combination of cells, engineering, materials, and suitable biochemical and physio-chemical factors to improve or replace biological functions. Tissue engineering plan the use of cells, capable of differentiating into the appropriate lineages; scaffold that provides a structural template for tissue development and in many cases bioreactor, that guides appropriate cell differentiation and tissue development by providing the necessary biochemical and physical regulatory signals .

Particularly the perspective to use engineered tissue as organ replacement in patients has intrigued scientists and clinicians alike. Independent of the scientific intellectual challenge, there is a clear necessity to develop alternatives to current organ transplantation.

Langer and Vacanti were the first to promote the concept that cells may be seeded on artificial matrices to support formation of functional tissue-like structures that may be used to repair diseased organs or to screen for wanted and unwated effects of drugs (*Langer R and Vacanti JP, 1993*).

A sound application is on cardiac environment. In fact, a dramatic hurdle to cardiac wound healing in rat and other mammals is represented by the low regenerating capacity of myocardium in face of the high regenerative ability of vascular endothelial cells and smooth muscle cells.

After death of the cardiomyocytes, macrophages, monocytes, and neutrophils migrate into the infarct area, initiating the inflammatory response. Then infarct expansion begins to occur because of the activation of matrix metalloproteases (MMPs), which degrade the extracellular matrix and results in myocyte slippage. This weakening of the collagen scaffold results in wall thinning and ventricular dilation. After the initial inflammatory phase, there is an increase in fibrillar, cross-linked collagen deposition, which resists deformation and rupture.

These characteristics, in combination with scar tissue formation, low blood supply, which resulted from the post-injury inflammatory response, induce a weak-to-severe left ventricular dysfunction, possibly culminating in heart failure (*Landmesser U et al, 2005*). In principle, replacement of damaged myocardium and vascular tissues with new cardiovascular units should avoid such an event and restores the contractile function or, at least, re-gains part of the original structural and functional cardiovascular properties (*Leri A et al, 2005*). Cardiovascular cell replenishment after acute myocardial infarction (AMI) or reduction/slowing down of progressive functional deterioration in case of prolonged ischemia are to be addressed by two different strategies: 1) implementation of angiogenesis and arteriogenesis along with cardiogenesis (via local activation or extra-cardiac mobilization of cell precursors, or exogenous administration of committed or precursor cells) (*Khurana R et al, 2005, Anversa P et al, 2006*) or 2) inducing angiogenesis/arteriogenesis followed by cardiogenesis if the local conditions can permit this sequential cell colonization (*Ertl G et al, 2005*). To this end, the choice of stem cell type to be used in therapeutic cardiovascular regeneration of acute or chronic myocardial ischemia could be of paramount importance when specific combinations of differentiated cell phenotypes must be obtained.

1.2.2 Cell source

Efforts to regenerate functional myocardial tissue are being pursued through cell grafting; fetal, neonatal and adult cardiomyocytes, skeletal myoblasts, bone marrow progenitors cells and embryonic stem cells have been considered for cardiac repair (Figure 4).

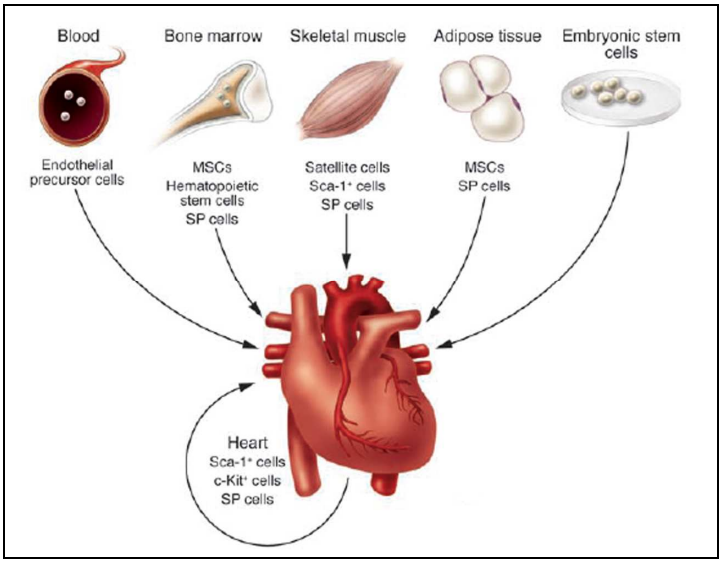


Figure 4: Sources of cells for cardiac repair. Cells in current human trials include skeletal muscle myoblasts, unfractionated bone marrow, and circulating (endothelial) progenitor cells. Cells in preclinical studies include bone marrow MSCs, multipotent cells from other sources such as adipose tissue, and novel progenitor or stem cells discovered in the adult myocardium such as cardiac resident progenitors cells. Dimmeler S et al, J.of Clinical Invest, 2005.

Although fetal and neonatal cadiomyocytes were able to form electromechanical junctions, they are currently not a feasible source of transplant cells due to the ethical and donor issues.

Embryonic stem cells can give rise to functional cardiac myocytes and could theoretically serve as reliable cell sources for cardiac tissue engineering. As previously said, they derive from the inner cell mass of a blastocyst and can differentiate into derivatives of all three germ layers including mesodermal cardiac myocytes (*Laugwitz KL et al, 2005; Badorff C et al, 2003*)**.** Yet, available ES-cell lines, be it from mice or humans, do not exhibit equal differentiation capacities, most of all are difficult to expand and form teratoma.

On the other hand the first clinically relevant cells to be proposed as a surrogate for cardiomyocytes were skeletal muscle myoblasts — undifferentiated proliferation-competent cells that serve as precursors to skeletal muscle. For clinical use, autologous human myoblasts are isolated from skeletal muscle biopsies, propagated and expanded ex vivo for a few days or weeks, then injected directly into the ventricular wall (*Hagège AA, et al, 2006*) nevertheless these cells do not form gap junction and can not contract synchronously.

Finally, bone marrow is, at present, the most frequent source of cells used for clinical cardiac repair. Adult stem cells such as bone marrow stem cells contains a complex assortment of progenitor cells, as HSCs which can develop into a wide variety of tissues, including cardiomyocytes (*Pittenger M.F et al, 2004; Malouf NN et al, 2001; Bardoff C et al, 2001***)**. Most studies support the notion that cell engraftment in animal model of myocardial infarction can improve contractile function. The mechanism behind this functional improvement remains to be elucidated, however, and to date the capacity of circulating stem cells to give rise to cardiac myocytes is controversial (*Nygren JM et al, 2004; Laflamme MA et al, 2005*)

1.2.3 Scaffolds

A critical step is the creation a suitable three-dimensional matrices composed of natural or synthetic scaffold materials that host the cells (defined as cardiac patch) to allow the maintenance of cellular viability and differentiation and favour cell integration.

A lot of effort were recently undertaken to understand the function of microenvironment cues of the stem cell niche to better control the fate of transplanted stem cells.

Several requirements have been identified as crucial for the production of tissue engineering scaffolds (*Furth ME et al, 2007*):

(1) the scaffold should possess interconnecting pores of appropriate scale to favour tissue integration and vascularisation,

(2) be made from material with controlled biodegradability or bioresorbability so that tissue will eventually replace the scaffold,

(3) have appropriate surface chemistry to favour cellular attachment, differentiation and proliferation,

(4) possess adequate mechanical properties to match the intended site of implantation and handling,

(5) should not induce any adverse response and,

(6) be easily fabricated into a variety of shapes and sizes.

Numerous studies have examined different scaffolds as well as various culture conditions for creating *in vitro* engineered myocardial tissue.

Cellular transplantation, left ventricle (LV) restraint devices, and tissue engineering approaches have emerged as possible alternatives to heart transplantation for the treatment of damaged myocardium . Initial studies focused on the injection of viable cells directly into the infarcted myocardium, a technique which has been termed cellular cardiomyoplasty. More recent approaches include the use of engineered tissue, which is

cultured *in vitro* and then implanted *in vivo* and in situ engineered tissue, which is injected directly into the myocardium (Figure 5). Polymer meshes have also been utilized to prevent LV expansion.

Table IV summarize the most used polymers in cardiac field, to date aliphatic polyesters such as polyglycolic acid (PGA), polylactic acid (PLLA), their copolymers (e.g. PLGA) and polycaprolactone (PCL) are use das solid scaffold whereas naturally derived protein (collagen, fibronectin) or carbohydrate polymers have been used as scaffolds both solid or liquid. Synthetic polymers have the advantages of having chemical and fisical properties well defined but they can induce inflammatory response, whereas natural scaffold are biocompatible and facilitate cell adhesion even though it is more difficult to obtain them.

Table IV: Synthetic and natural polymers most in use: PLA=poly(L-lactic)acid;PNIPAAM=poly(Nisopropylacrylamide);PTFE=poly(tetrafluoroethylene);PDGFBB=platelet derived growth factor BB;bFGF=basic fibroblast growth factor. Adapted from Christman KL and Lee RJ, 2006

Material	Transplantation	References
Left ventricular restraint		
Polypropylene	Alone	EnomotoY, 2005
Polyester	Alone	Olsson A, 2005
***In vitro* engineered tissue**		
Gelatin	Alone or with fetal cardiomyocytes	Leor J, 2000
Alginate	With fetal cardiomyocytes	Kellar RS, 2005
Polyglycolide/polylactide	Witt dermal fibroblasts	Zimmermann WH, 2002
Collagen type one and matrigel	With neonatal cardiomyocytes	Zimmermann WH, 2006

PTFE,PLA mesh, collagen I and matrigel	Alone or with bone marrow derived mesenchymal progenitor cells	Krupnick AS, 2005
Collagen I	Alone or with embryonic stem cells	Kofidis T, 2005
PNIPAAM	Cell sheet of neonatal caridiomyocytes or adipose derived MSC	Miyahara Y, 2006
In situ engineered tissue		
Fibrin	Alone with skeletal myoblasts, bone marrow nuclear cells or pletiotrophin plasmid	Christman KL, 2005
Collagen	Alone or with bone marrow cells	Dai W, 2005
Alginate	Alone	Leor J, 2005
Matrigel	Alone or with embryonic stem cells	Kofidis T, 2004
Collagen I and matrigel	Alone or with neonatal cardiomyocytes	Zhang P, 2006
Self assembling peptites	Alone, with neonatal cardiomyocytes or PDGFBB	Davis ME, 2005
Gelatin	With bFGF	Iwakura A, 2003

In particular, structural proteins such as collagen, laminin, elastin, and fibronectin have been used as matrices for tissue engineering (TE) and as vehicles for cell delivery.

Collagen has found widespread use as a scaffold and carrier for cells in TE and regenerative medicine, particularly in soft tissue applications such as skin (*Faraj KA et al, 2007*); not only it is a major component of the extracellular matrix that supports most

tissues and gives cells structure from the outside, but also has great tensile strength. Therefore, synthetic collagen, in particular collagen type I most abundant collagen in the human body, is mimicking normal biological extracellular matrix (ECM), which contributes to mechanical integrity and has important signalling and regulatory functions in the development, maintenance, and regeneration of tissues. ECM components, in synergy with soluble signals, provided by growth factors and hormones, participate in the tissue-specific control of gene expression through a variety of transduction mechanisms. Furthermore, the ECM is itself a 3D dynamic structure that is actively remodelled by the cells with which it interacts and is a selective key element in providing specific adhesion characteristics.

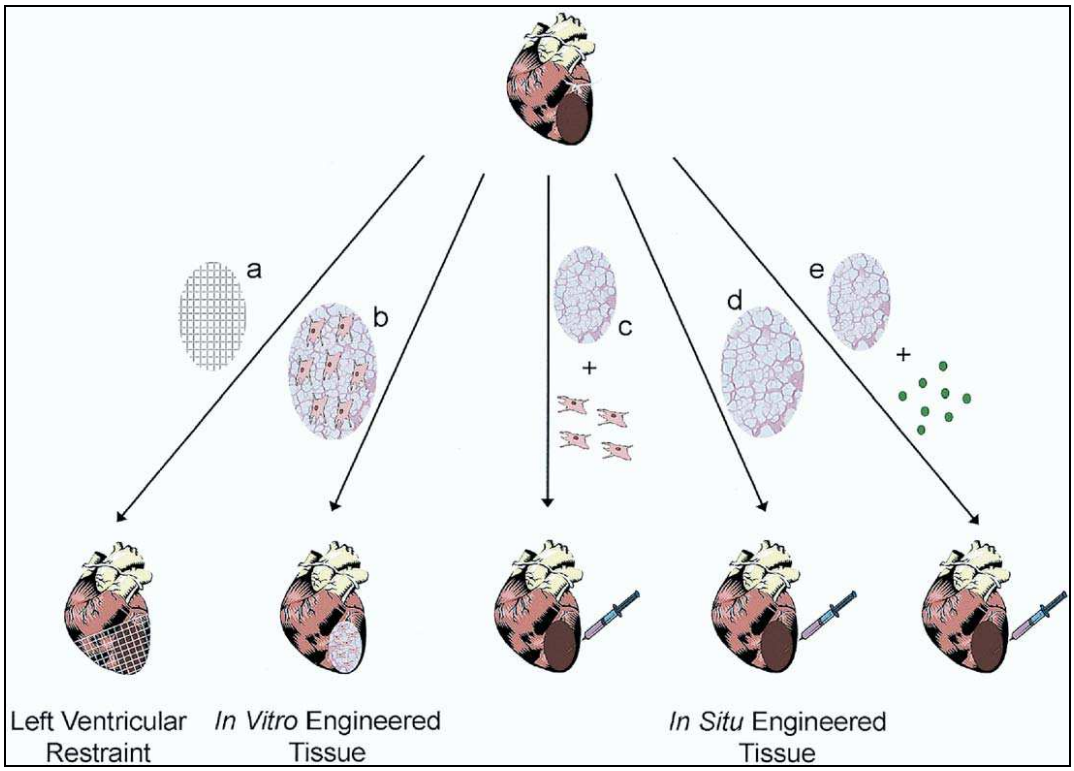


Figure 5:Strategies for treatment of myocardial infarction using biomaterials. To date, 3 different biomaterial approaches are being examined for treatment of myocardial infarction. Polymer meshes can be sutured around the heart for use as a left ventricular (LV) restraint (a) to preserve LV geometry. *In vitro* engineered tissue involves culturing cells on a biomaterial scaffold *in vitro* and then implanting the tissue onto the epicardial surface (b). In situ engineered tissue can be achieved by injecting a biomaterial alone (d), or using an injectable scaffold as a delivery vehicle for cells (c) or therapeutic agents such as genes or proteins (e). **Christman KL and Lee RJ,, 2006**

Hence, which polimer support and which stem cell are most suitable for transplantation in the injured heart?

1.3 Aim of the study

In vitro, we herein focus our study on the mesenchymal potential of $CD133^+$ isolated from the BM (*Masiero L et al, 2004*) as preliminary indicated by Trondeau T et al. and colleagues who have shown that $CD133^+$ cells from both cord blood and peripheral blood were able to give rise to mesenchymal progenitors.

About this *in vitro* part of the study, the contribution is two fold: firstly, it formulates a protocol to isolate mesenchymal stem cells from the BM using clinical-grade monoclonal antibody; secondly, it provides full characterization of the multipotency of subpopulation of $CD133^+$ cells isolated from BM of healthy donors.

Because of the mentioned characteristics, we decided to use collagen type I in our *in vivo* experiments and our goal is to ascertain capability of surviving and cell differentiation potential of human CD133+ BM cells injected on collagen patch using a nude rat model of cardiac-transmural acute necrotizing injury (ANI).

Chapter 2 Materials and Methods

2.1 In vitro experiments

2.1.1 Cell isolation

Cells were collected from remaining samples of clinical BM transplantation drawings from consenting healthy volunteer donors, according to guidelines from the Ethical Committee of the Azienda Ospedaliera di Padua, Padua, Italy and from the Groupe Hospitalier Necker-Enfants Malades, Paris, France.

Selection of $CD133^+$, $CD34^+$ and MSCs. Mononuclear cells were isolated by density gradient centrifugation over Lymphoprep™ (AXIS-SHIELD PoC AS, Norway) by manufacturer's instructions; mononuclear cells, washed three times, were resuspended in PBS 1X, incubated with anti-CD133 conjugated super paramagnetic microbeads (CD133 Isolation Kit, Miltenyi Biotech, Germany), washed and immunomagnetic-sorted with MiniMACS (Miltenyi Biotech) protocol to obtain purified $CD133^+$ cells. Selection of $CD133^+$ cells was also done automatically using CliniMACS (ClinMACS Plus superior cell selection, Miltenyi Biotech). Positive cells were plated at $1x10^3$ cells/cm^2 density in fibronectin (StemCell Technologies Inc., Canada) coated dishes (Falcon, Becton

Dickinson, CA, USA) and cultured in three different conditions: first on fibronectin-coated Petri dishes in Reyes' medium[16] used as control medium, second on fibronectin-coated Petri dishes and αMEM (Gibco) supplemented with 20% Fetal Bovine Serum (FBS), 1% penicillin/streptomycin and 1% L-glutamine (Gibco) and third on plastic Petri dishes and αMEM supplemented with 20% FBS, 1% penicillin/streptomycin and 1% L-glutamine. Cells at 60% of confluence were detached and seeded on 96-well at 0.5 cells/well to test the clonogenicity. Once adherent cells reached approximately 60-70% of confluence, they were detached with Trypsin 0.05-EDTA 0.02 w/v (Biochrom AG, Germany) sterile solution, centrifuged 5 min at 310g (1200 rpm) and re-plated at 1:3 dilution under the same culture conditions. Similarly to what previously described for CD133 selection, mononuclear cells derived from the same BM samples were resuspended in PBS 1X and labeled with anti-CD34 conjugated microbeads (MACS® Direct CD34 Isolation Kit, Miltenyi Biotech), incubated at 4°C for 30 minutes and then immunosorted by magnetic isolation to obtained purified $CD34^+$ cells. Mesenchymal cells were isolated as the adherent fraction of both the mononuclear fraction and the negative fraction of CD133 antigen as previously described[17]. Mesenchymal cells were plated at $2.5x10^3$ cells/cm^2 density and cultured as described above for $CD133^+$ cells.

2.1.2 Cell differentiation

Differentiation towards hematopoietic, adipogenic, endothelial, osteogenic, myogenic, and neuronal lineages was investigated for $CD133^+$ cells and MSCs. $CD34^+$ cells were used as control for hematopoietic differentiation; experiments were repeated with 25 different BM samples.

Hematopoietic differentiation. To induce hematopoietic differentiation 5x103 cells/cm2 were plated on tissue culture dishes (Falcon, BD) in semisolid growth media made of 1%

methylcellulose in IMDM, 30% FBS, 1% bovine serum albumin (BSA, Sigma), 10-4mol/L 2-mercaptoethanol, 2mM L-glutamine, 50ng/mL Stem Cell Factor (SCF), 10ng/mL IL-3 and 10ng/mL erythropoietin (complete media from StemCell Technologies Inc.). Pictures of colonies were taken with a Leica Camedia C-4040 camera and processed with Leica AnalySIS Software (Leica Microsystems).

Adipogenic differentiation. To induce adipogenic differentiation, 2 to 7 passages cells were plated at 4x103 cells/cm2 density on tissue culture dishes (Falcon, BD) and treated with adipogenic medium for three weeks. Medium changes were carried out 3 times a week and adipogenisis was assessed at 3, 7, 14 and 21 days. Adipogenic medium consisted of DMEM low glucose (Sigma) supplemented with 10% FBS (Gibco), 0.25mM IBMX (Sigma), 0.1μM dexamethasone (StemCell Technologies Inc.), 66nM insulin (MP Biomedicals, Germany), 1nM Triiodothyronine T3 (Sigma), 10μM rosiglitazone (Avandia®, GlaxoSmithKline, United Kingdom), 10mM hepes (Sigma), 170μM pantotenate, 33μM biotin (Ink Biomedicals, USA), 10□g/ml transferrin (MP Biomedicals), 1% penicillin/streptomycin and 1% L-glutamine (Gibco).

Endothelial differentiation. To induce endothelial differentiation, 2 to 7 passages cells were plated at 4x103 cells/cm2 density in fibronectin (StemCell Technologies Inc.) coated dishes (Falcon, BD) and treated with endothelial medium for 3 weeks with medium changes 3 times a week. Endothelial medium consisted of EBM2 supplemented with 2% FBS, 0.04% hydrocortisone, 0.4% hFGF-B, 0.1% VEGF, 0.1% R3-IGF-1, 0.1% ascorbic acid, 0.1% hEGF, 0.1% GA-1000, 0.1% heparin (complete medium from Cambrex Bio Science Walkersville Inc. USA).

Osteogenic differentiation. To induce osteogenic differentiation, 5 to 7 passages cells were plated at 4x103 cells/cm2 density on tissue culture dishes (Falcon, BD) and treated with osteogenic medium for 3 weeks with medium changes 3 times a week. Osteogenesis was assessed at 3, 7, 14 and 21 days. Osteogenic medium consisted of DMEM low glucose (Gibco) supplemented with 100nM dexamethasone, 10mM β-glycerol phosphate, 50μM

ascorbic acid 2 phosphate (StemCell Technologies Inc.), 1% penicillin/streptomycin (Gibco).

Myogenic differentiation. To induce myogenic differentiation, 2 to 7 passages cells were plated at 4x103 cells/cm2 density in Matrigel (BD) coated dishes (Falcon, BD) with 12mm round slides and treated with myogenic medium made of DMEM low glucose (Sigma) supplemented with 20% FBS (Gibco), 0.5% chicken embryo extract (MP Biomedicals, Germany), 10% horse serum (Gibco), 1% penicillin/streptomycin (Gibco) for 3 weeks. After 2 days, cells were treated with 3µM 5-azacytidin (Sigma) for 24 h. Medium changes were carried out 3 times a week and myogenesis was assessed at 10, 20 and 30 days.

Neuronal differentiation. CD133+ at 2 to 7 passages were plated at 4x103 cells/cm2 and maintained as neurospheres in the presence of DMEM F12 (Gibco), 25µg/mL Insulin, 100µg/mL Tansferrin (MP Biomedicals), 20nM Progesteron (Sigma), 60µM Putrescin (Sigma), 20ng/mL EGF (Sigma), 200µM BHA (Sigma), 40ng/mL bFGF (Sigma). Neurosphere cells were harvested, dissociated, and cultured in dishes previously coated with Poly-L-Ornithine (Sigma) and Laminin (Sigma). Neurogenic medium consisting of Neurobasal medium (Invitrogen) supplemented with B27 (Invitrogen) and 1% L-glutamine and penicillin/streptomycin. Neuronal differentiation was assessed at 14 and 21 days with PCR and immunostaining.

Cardiomyocyte differentiation. To induce cardiomyocyte differentiation, 2 to 7 passages cells were co-cultured at 2x103 cells/cm2 density in 1% gelatin coated dishes (Falcon, BD) with rat neonatal cardiomyocytes in Culture Plating Medium (CPM; 68% DMEM (Invitrogen), 17% M-199 (Sigma), 5% fetal bovine serum (Invitrogen), 10% horse serum (Invitrogen), 1% streptomycin-penicillin, and 1% L-glutamine). Neonatal rat cardiomyocyte (rCM) cultures were obtained by trypsin/EDTA digestion from newborn heart rats. Several rounds of tissue digestion were collected by centrifugation and then re-suspended in Culture Plating Medium. Co-cultures were established by admixing rCM

with hCD133+ cells in the ratio 4:1 and seeding the cell mixture on gelatine-coated glass coverslips (8x103 cells/cm2). hCD133+ had been previously labelled with the green cell tracker chloromethylfluorescein diacetate (CMFDA) following the instructions of the manufacturer (Molecular Probes, Eugene, OR). Cell viability after cell labeling was monitored by Blue Trypan exclusion test. Medium changes were carried out 3 times a week and co-cultured cells were an Immunocytochemistry: the expression of myogenic and cardiomyocyte markers in differentiated cells was assessed by immunostaining. Cells were washed twice with PBS 1X solution (Gibco), fixed with PFA 2% for 20 minutes at 4°C, permeabilized with a 0.1% Triton X-100 / PBS solution (Sigma) for 10 seconds at room temperature and then incubated for 25 minutes at 37°C with primary antibodies, diluited in a 1% PBS/BSA (Sigma) solution, and specific for MyoD (rabbit polyclonal IgG, Santa Cruz Biotechnology) and skeletal and cardiac Troponin I (mouse monoclonal IgG, Chemicon, Italy, quello per le co-colture era dato forse da schiaffino, sempre mouse monoclonal IgG). Cells were subsequentely incubated for 25 minutes at 37°C with secondary Alexa Fluorescence 594-conjugated (donkey anti-mouse IgG, Molecular Probes, Invitrogen) and Cy2-coniugated (goat anti-rabbit IgG, Chemicon) antibodies diluted in a 1% PBS/BSA and human serum solution and finally mounted with DAPI solution (Vector Laboratories, Ltd., United Kingdom). Pictures of the staining were taken using an Axioplan Zeiss fluorescent microscope.alyzed at 4, 6 and 9 days by RT-PCR and immunostaining.

Flow Cytometric Characterization. Both fresh and in culture samples after selections were analyzed. For external labeling, cells resuspended at a concentration of $5x10^5$ cells/100µl of PBS were incubated with the following monoclonal antibodies: anti-CD3 Fluorescein isothiocyanate (FITC) conjugated, anti-CD13 FITC, anti-CD29 FITC, anti-CD31 FITC, anti-CD44 FITC, anti-CD54 FITC, anti-CD25 Allophycocyanin (APC) conjugated anti-CD26 Phycoerythrin (PE), anti-CD90 FITC or PE, anti-CD73 PE , anti-CXCR4 APC,

anti-CD123 pure (all purchased from BD Pharmingen, CA, USA), anti-CD9 FITC, anti-CD124 pure (eBioscience, CA, USA), anti-CD19 FITC, anti-CD146 FITC and anti-CD184 PE (Immunotech S.A., Coulter Company, France), anti-CD34 (clone alfa-HPCA2) FITC or PE and (clone 8G12) APC, CD140b PE (BD, NJ, USA), (eBioscience), anti-CD127 PE, anti-KDR PE (R&D System, MN, USA), anti-CD133/1 PE and anti-CD133/2 (clone 293 C3) PE or APC (Miltenyi Biotech), (R&D System), (BD Pharmingen), anti-CD45 FITC (BD Pharmingen) or Peridinin-chlorophyll-protein Complex (PerCP) (clone 2D1, BD), anti-CD105 FITC (BD Pharmingen) or PE (eBioscience), anti-CD106 FITC (BD Pharmingen) or PE (eBioscience), anti-CD117 FITC (Miltenyi Biotech) or PE (Caltag, Invitrogen, Italy), HLA-ABC FITC (Human Leukocyte Antigen; Immunotech) or pure (Dako, Denmark), HLA-DR PE (Immunotech) or pure (Dako), anti-SSEA3 pure and anti-SSEA4 pure (Santa Cruz Biotechnology, Inc. USA). Pure antibodies were revealed further incubating cells for 10 min at 4°C FITC conjugated AffiniPure Rat anti-mouse IgG (Jackson Immunoresearch, United Kingdom), PE-conjugated goat anti-rat IgG 568 (Molecular Probes, Invitrogen) or FITC-conjugated goat anti-mouse 488 (Molecular Probes, Invitrogen) after rinsing the cells with PBS 1X. For internal labeling, cells were fixed at room temperature with 4% paraformaldehyde (PFA) for 10 min and permeated with 100% ethanol for 2 min after washing with PBS. Cells were incubated with 10% FBS to block non-specific binding, followed by primary antibody OCT4 (Santa Cruz Biotechnology, Inc. USA) for 1 hour; secondary FITC-conjugated antibody (Molecular Probes) was applied for 30 min. Mouse or rat non-immune immunoglobulins were always used as controls. Events were acquired and analyzed on a Coulter Epics XL-MCL (Beckman Coulter) or a FACS-Calibur (BD) cytometer with respectively EXPO^{TM} 32 ADC and BD Cell Quest Software. As control, flow cytometric analyses of MSCs were performed at passages 2 and 4 using the same methods described above for CD133^{+} cells.

Cell proliferation Assay. For cell growth assay, CD133^{+} cells and MSCs were seeded at low-density 24-well microplates in control medium; cell proliferation was monitored by

Particle Count and Size Analyzer every 2 days for 20 days. Cell proliferation was measured counting cells using a Z2 Coulter® Particle Count and Size Analyzer (Beckman Coulter, France). Cell replication ratio was obtained plotting cell number against days of culture.

Karyotype analyses. Standard chromosome preparation methods were applied to $CD133^+$ and MSCs cultures according to Back et al. Conventional $AgNO_3$ staining of the nucleolus organizing regions (NORs) was performed.

Telomerase activity. Telomerase activity of the cellular extract from $2x10^4$ cells was assayed using the TRAPeze Telomerase Detection Kit following the manufacturer's directions (Chemicon), pictures were acquired by Studio Version 9® software.

2.1.3 Cell differentiation analyses

Alkaline Phosphatase (AP) assay. AP activity was evaluated in both osteogenic induced cells and controls according to the manufacturer's instructions (Sigma-Aldrich). Briefly, cells were fixed in acetone for 15 minutes . Substrate (fast blue RR solution with naphthol AS-MX phosphate alkaline solution) was added to the cells in culture dishes that were counterstained with Mayer's Hematoxylin solution.

Oil-Red-O staining. The presence of adipose elements in induced and control cultures was determined by Oil-Red-O staining (Sigma). The slides were fixed in 10% formalin for 1 hour, washed in deionized water and air-dried. The cells were incubated with Oil-Red-O staining solution for 15 minutes and counterstained with Mayer's Hematoxylin (pH4, Sigma).

Immunofluorescence. The expression of myogenic markers in differentiated cells was assessed by immunostaining. Cells were washed twice with PBS 1X solution (Gibco), fixed with PFA 2% for 20 minutes at 4°C, permeabilized with a 0.1% Triton X-100/PBS

solution (Sigma) for 10 seconds at room temperature and then incubated for 25 minutes at 37°C with primary antibodies specific for Desmin (rabbit polyclonal IgG, Santa Cruz Biotechnology), diluted in a 1% PBS/BSA (Sigma) solution. Cells were subsequently incubated for 25 minutes at 37°C with secondary Alexa Fluorescence 488-conjugated (goat anti-mouse IgG, Molecular Probes, Invitrogen) and finally mounted with DAPI solution (Vector Laboratories, Ltd., UK). Pictures of the staining were taken using an Axioplan Zeiss fluorescent microscope. The expression of neural markers has been detected fixing the cells in 2% PFA for 10 minutes, rinsed three times with PBS, permeabilized with 0.3% Triton X-100 (Sigma) for 20 minutes, rinsed with PBS and incubated for 24 hours with primary antibody O4 (mouse monoclonal, Chemicon), MAP2 (mouse monoclonal, Chemicon) diluted in BSA/PBS. Samples were washed with PBS for 10 minutes, incubated with secondary antibody (donkey anti mouse IgG Alexa Fluorocrome 594; anti mouse Cy2 conjugated, Chemicon) for two hours at room temperature and finally washed and mounted with DAPI solution (Vector).

2.1.4 RNA extraction and RT-PCR

Total RNA was isolated from undifferentiated and differentiated cells with RNAzol™ B (Tel-Test Inc., Texas, USA) and 1μg of RNA was reverse-transcribed into first strand cDNA with Superscript II reverse transcriptase (Life Technologies, MD, USA) using Oligo-dT primer (Invitogen) following manufacturer's instructions. Both RT and PCR was done using a GeneAmp® PCR System 2700 (Applied Biosystem, CA, USA). For each PCR reaction cDNA was used in a final volume of 25μl with 200nM dNTP, 10pM of each primer, 0.3U Taq-DNA-polymerase, reaction buffer, and $MgCl_2$ (Invitrogen). Cycling conditions consisted of 94°C for 2 minutes, annealing at 63°C for 40 seconds and

elongation at 72°C for 1 minute. Cycle numbers varied between 22 and 37 cycles. Primer sequences are listed in Table 1. cDNA from human satellite cells, from human bone tissue, from human adipose tissue, from human cells from neuroblastoma, from human heart tissue and from HUVEC cells (Promo Cell Bioscience Alive) were used as positive control. cDNA from undifferentiated cells was used as negative control. PCR reactions were performed on 1% agarose gel electrophoresis and picture acquired by Studio Version 9® software. Primers sequences were build using http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi web site and were purchased from Invitrogen.

Table V. Primers details.

Primers name	**Primer sequences**	**Size**
Corebinding factor alpha 1 (Cbfa1)	**F: 5'-AGA TTT GTG GGC CGG AGT G-3'** **R: 5'-CAT CAA GCT TCT GTC TGT GCC TT-3'**	**301 bp**
GATA4	**F:5'-TCC CTC TTC CCT CCT CAA AT-3'** **R:5'-CAG ATG CCT TTA CAC GCT GA-3'**	**158 bp**
human cardiac β-MHC	**F:5'-CAA GTT TGG CCA CAC CAA-3'** **R: 5'-GTT CCA GCA GCT TTT TGT-3'**	**152 bp**
Osteocalcin	**F: 5'-TGA AGA GAC CCA GGC GCT A-3'** **R: 5'-GAT GTG GTC AGC CAA CTC GTC-3'**	**125 bp**
Myogenic Regulator Factor 4 (MRF4)	**F: 5'-GGC TCG TGA TAA CGG CTA AGG-3'** **R: 5'-CGC AAC GTT TCT TTG TGA ATG T-3'**	**151 bp**
MyoD	**F: 5'-TCC GCG ACG TAG ACC TGC C-3'** **R: 5'-GAT ATA GCG GAT GGC GTT GC-3'**	**172 bp**

Desmin	**F:5'-CCA TCG CGG CTA AGA GCA TT-3'** **R: 5'-GCC TCA TCA GGG AAT CGT TA-3'**	**440 bp**
Peroxisome Proliferator-activated Receptor gamma 2 (PPRγ2)	**F: 5'-TCA GTG GAG ACC GCC CA-3'** **R: 5'-TCT GAG GTC TGT CAT TTT CTG GAG-3'**	**152 bp**
Lipoprotein lipase (Lpl)	**F: 5'-CTG GTC GAA GCA TTG GAA T-3'** **R: 5'- TGT AGG GCA TCT GAG AAC GAG-3'**	**131 bp**
P1H12 (CD146)	**F: 5'-AGG CAT TAG CCC CGA ATC A-3'** **R: 5'-AAC CCA GTG GCC CTT TGA A-3'**	**154 bp**
Angiopoietin	**F: 5'-CCC CTT TTT GCC TTA CAA GTG ATA T-3'** **R: 5'-TCT GTA GGA ACA CAA AAG GAC AAA ATA-3'**	**159 bp**
βIII Tubulin	**F: 5'-CAT TCT GGT GGA CCT GGA AC-3'** **R: 5'-TCG CAG TTT TCA CAC TCC TTC-3'**	**195 bp**
human cardiac GATA4	**F: 5'-TCC CTC TTC CCT CCT CAA AT-3'** **R: 5'-CAG ATG CCT TTA CAC GCT GA-3'**	**158bp**
human cardiac β-Myosin HeavyChain (β-MHC)	**F: 5'-CAA GTT TGG CCA CAC CAA-3'** **R: 5'-GTT CCA GCA GCT TTT TGT-3'**	**152bp**
β_2 microglobulin	**F: 5'-GCT GTG CTC GCG CTA CTC T-3'** **R: 5'-CAA CTT CAA TGT CGG ATG GAT G-3'**	**151 bp**

2.1.5 Immunemodulatory properties assay

T limphocyte proliferation assays with phytohemoagglutinin (PHA) : to evaluate human CD133 capacity to inhibit T lymphocytes alloreaction after mitogen stimulation, co-coltures between PHA-actived lymphocytes and human CD133 at different concentrations was estabilished. Method: T lymphocytes was collected from healthy donors. Lymphocytes was seeded on 96-well dishes, at 2x105 cells/well density and in 200µl of RPMI medium added with 20%FBS, 1% L-glutamine, 1% pen/strep and 20µg/ml PHA. Cells was irradiated with 3000cGy. Different passages of irradiated (3000cGy) CD133 cells was added to PHA-stimulated lymphocytes cultures at different concentrations (2x105, 2x104, 2x103 cells/well density). Untreated PBL and MSC (at 2x105 cells/well density), other than medium, was employed as negative controls. Each experiment was carried out in triplicate. Dishes was finally incubated at 37°C and 5% CO2 atmosphere for 3 days after which lymphocyte proliferation rate was evaluated with the tritiated thymidine incorporation method.

T limphocyte proliferation assays: tritiated thymidine method: this method is based on the incorporation of radioactive nucleotides in proliferating cells during DNA replication and on the following measurement of cells radioactivity.

Method: 1 µCu of tritiated thymidine (Amersham Biosciences, UK) was added to every well of PHA-stimulated cultures after an incubation of respectively 3 and 4 days. Plates was then incubated at 37°C and 5% CO2 atmosphere for 8 hours. Cells will then be harvested using a Combi Cell Harvester (Skatron Instruments, Lier, Norway) and transferred on fibre glass filters (Skatron Instruments, Lier, Norway) that after was covered with 4ml of scintillation medium (Ultima Gold, Perkin Elmer Life and Analytical Sciences, Boston, USA). Finally, radioactivity was measured with a β-counter (Liquid

Scintillation Analyzer, Tricarb, Canberra Packard). The results was expressed as counts per minute.

2.2 In vivo experiments

2.2.1 Stem cell labelling

hCD133+ were labelled with the red fluorescent intracellular tracker (5-(and-6)-(((4-chloromethyl)benzoyl)amino)tetramethylrhodamine) CMTMR (Molecular Probes) by a 30 minutes incubation with a solution of 0,25 ul/ml CMTMR in MEMα Medium at 37°C.

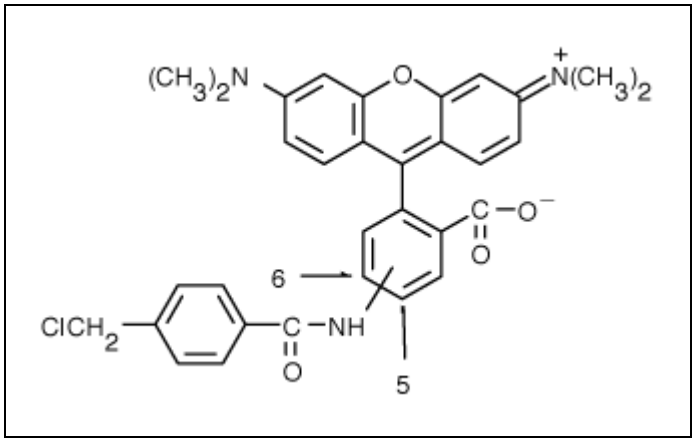


Figure 6: Chemical structure of CMTMR cell tracker.

2.2.2 GFP+ rat cardiomyocytes isolation

GFP+ rat cardiomyocytes (rCM) were obtained from 2-3 days-old GFP-positive transgenic Sprague Dawley rats by enzymatic digestion according to Radisic et al .

Animals

Male immunodeficient nude rats (rNu, Harlan, Milan, Italy) weighing about 190-200 gr and 11 weeks old were housed and maintained in a controlled environment.

Biomaterial

The 3D type I collagen scaffold was obtained as reported in Callegari A et al. 2007.

Experimental model of cardiac cryoinjury and biomaterial application.

All surgical and pharmacological procedures used in this study were performed in accordance with regulations expressed in the Guide for Care and Use of Laboratory Animals prepared by the Institute of Laboratory Animal Resources, National Research Council, published by the National Academy Press, revised 1996 (NIH Publication No. 85-23) and the Italian Health Minister Guidelines for Animal Research. The protocol was approved by the University of Padua Animal Care Committee.

The surgical procedures were carried out as previously set in Callegari et al. 2007 . rNu rats were anesthetised by i.m. injection of zoletil (4 mg/100 g body weight) along with atropin (s.c.; 5 uL/100g) and xylazin (i.p.; 0.4 mg/ 100 g).

Cells injection

Stem cells were detached from culture plastic dishes with Trypsin 0.05-EDTA 0.02 w/v (Biochrom AG) sterile solution and centrifuged 5 min at 310g (1200 rpm). 5 x 10^6

cells/animal cmtmr+ hCD133+ and gfp+ rCM were injected in 90ul of DMEM high glucose medium with hepes (Gibco) and rNU serum 1:100 solution (injection medium) in the collagen patch 15 days after its *in vivo* application on the heart cryoinjury. Animals were sacrificed at 24 hours, 15 and 30 days (this last time point only for the stem cells not for rCM) after the cells injection.

2.2.3 Ecocardiography

Transthoracic echocardiograms were performed on rats using a SONOS 5500 ultrasound unit (Philips Medical Systems, Italy) equipped with a 15-MHz linear array transducer and a 12-MHz phase array transducer. The animals were maintained lightly anesthetized with zoletil (4 mg/100 g body weight).Two dimensional and M-mode echocardiography were used to assess wall motion, chamber dimensions wall thikness and fractional shortening. Images were obtained from parasternal long axis, parasternal short axis at the mid-papillary level, apical 4-chamber, apical 2-chamber and apical 3-chamber views.Baseline echocardiograms were acquired at 2-3 days post ANI with additional echocardiograms acquired at 4 weeks post ANI. We prospectively established that ananimal must have sustained a sizable anterior ANI in order to be included in subsequent studies.

2.2.4 Histology and Immunostainig

Eight-micron thick frozen sections were cut from hearts of animals and stained with hematoxylin-eosin and Masson’s trichrome staining (Sigma).

Other cryosections were processed by immunofluorescence protocol for cardiac, immune response and inflammatory markers such as anti-cardiac troponin T (cTnT mouse IgG,

1:500, Abcam, UK), anti-α Smooth Muscle Actin (α-SM Actin, mouse IgG 1:1000 Sigma, IT), anti-von Willebrand Factor (vWf, rabbit IgG, 1:100), anti-CD31 (mouse IgG 1:100 bot from Chemicon Canada), anti-CD79a (B lymphocyte, rabbit IgG Abcam, UK), anti-macrophages (ED2 mouse IgG 1:400 Chemicon, IT), anti PH3 (mouse monoclonal, Upstate, NY).

Briefly, tissue slides were fixed in PFA 4% for 5 minute at room temperature, and incubated at 37°C for 25 minutes with the appropriate dilution of the primary antibody in PBS+1% bovine serum albumin (Gibco and Sigma). Cells were then re-incubated at 37°C for 25 minutes with the appropriate dilution of the secondary antibody (goat anti-mouse IgG coniugated with Alexa Fluorescence 564 IgG 1:150, Molecular Probes; goat anti-rabbit coniugated with Alexa Fluorescence 488 IgG 1:150, Molecular Probes) PBS+1% bovine serum albumin with human and rat serum (1:100). Cell nuclei were stained with a Hoescht solution diluited 1:5000 in PBS 1X for 5 minutes at room temperature.

Apoptosis analisys was made using apoptag detection kit (Chemicon,Canada) Observations were made using a Zeiss Axioplan epifluorescence microscope (Zeiss, Oberkochen, Germany), a Leica TCS SP5 confocal microscope and images were obtained using a Leica DC300F digital videocamera. Optical images were acquired by a Leica DMR microscope connected to a Leica DC300 videocamera.

Chapter 3 Results

3.1 In vitro

3.1.1 Cell culture

$CD133^+$, $CD34^+$ and MSCs were successfully isolated in all the BM samples examined. An average of 2% of $CD133^+$ cells were obtained among the 13 fresh samples used for FACS analyses. More than 80% of fresh $CD133^+$ cells were co-expressing CD45, CD34 and CD117 (c-kit) remarking their hematopoietic origin (Figure 8A). Cells isolated from additional samples using ClinicMACS or/and MiniMACS showed similar results. Freshly isolated $CD133^+$ cells express markers that underlined their hematopoietic and endothelial potential such as CD34 and CD31. Interestingly, once cultured, $CD133^+$ acquired markers normally expressed by MSCs such as CD146 and CD105 which were not present at the moment of isolation. Expression of CD90 (Thy-1) was similar comparing freshly-isolated and cultured $CD133^+$ cells. In parallel markers of hemato-endothelial potential where instead down-regulated. Positivity for both HLA-ABC and HLA-DR was detected but loss of the latter was observed after culture, as expected (Figure 8B). In order to expand $CD133^+$ cells, different cultured conditions were tested (Figure 2C). In all the tested conditions $CD133^+$ cells assumed a spindle-like shape reminiscent of MSCs. Interestingly,

only when cultured on fibronectin-coated dishes CD133$^+$ cells continue to express the POU-domain transcription factor POU5f1 (OCT4). In parallel, the expression of CD44, hyaluronate receptor, a stromal cell marker, CD90, HLA-ABC, CD73 and CD105 become positive highlighting an ongoing mesenchymal profile (Figure 2C). CD133$^+$ and MSCs showed significant difference growth capacities when cultured on fibronectin-coated dishes with Reyes' medium (Figure 2D) while CD34$^+$ did not survive in these conditions throughout the culture period. At passage 4, clones of CD133$^+$ cells and MSCs were harvested to analyze their morphology, karyotype as well as to study telomerase activity. Under the above-specified conditions, no significant change in morphology after expansion could be observed up to passage 4.

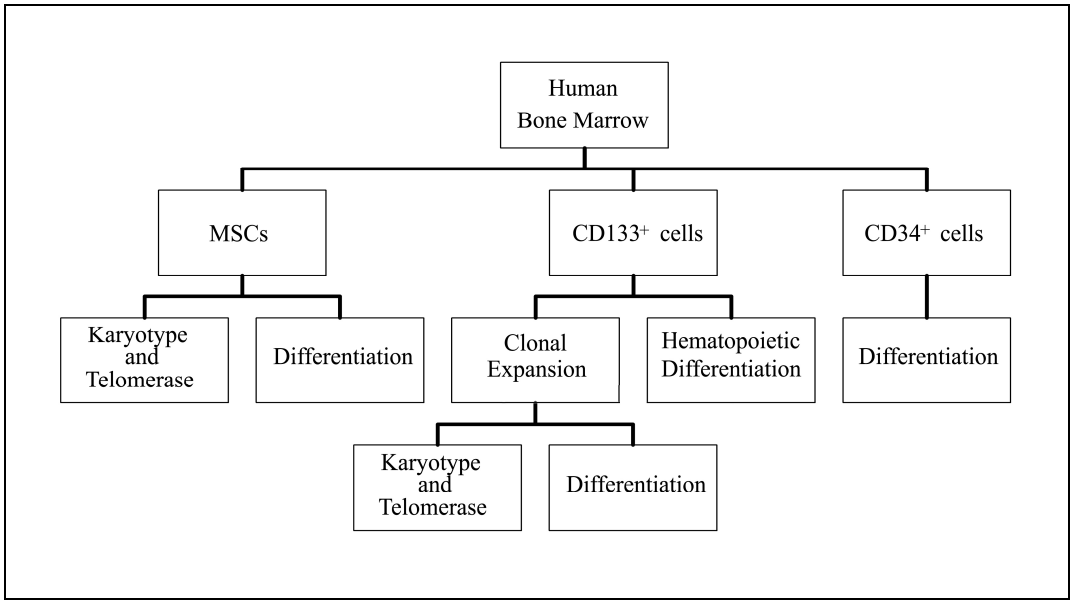


Figure 7. Working plan. From human bone marrow samples to Mesenchymal Stem Cells, CD133$^+$ and CD34$^+$ cells isolation, characterization and evaluation of their differentiation potential.

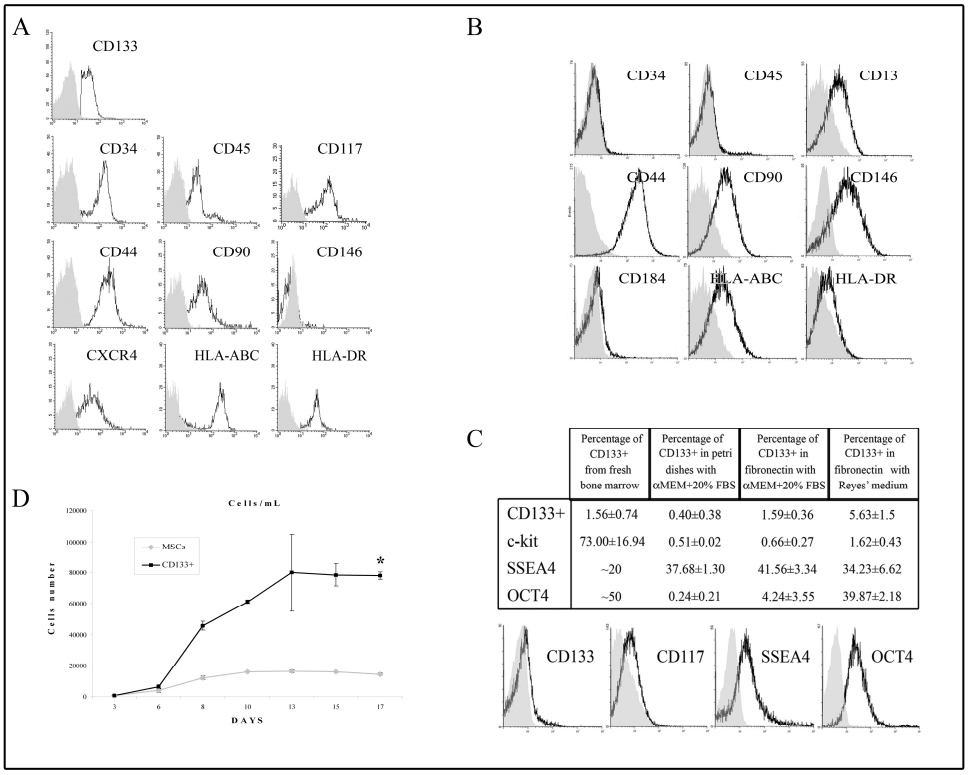
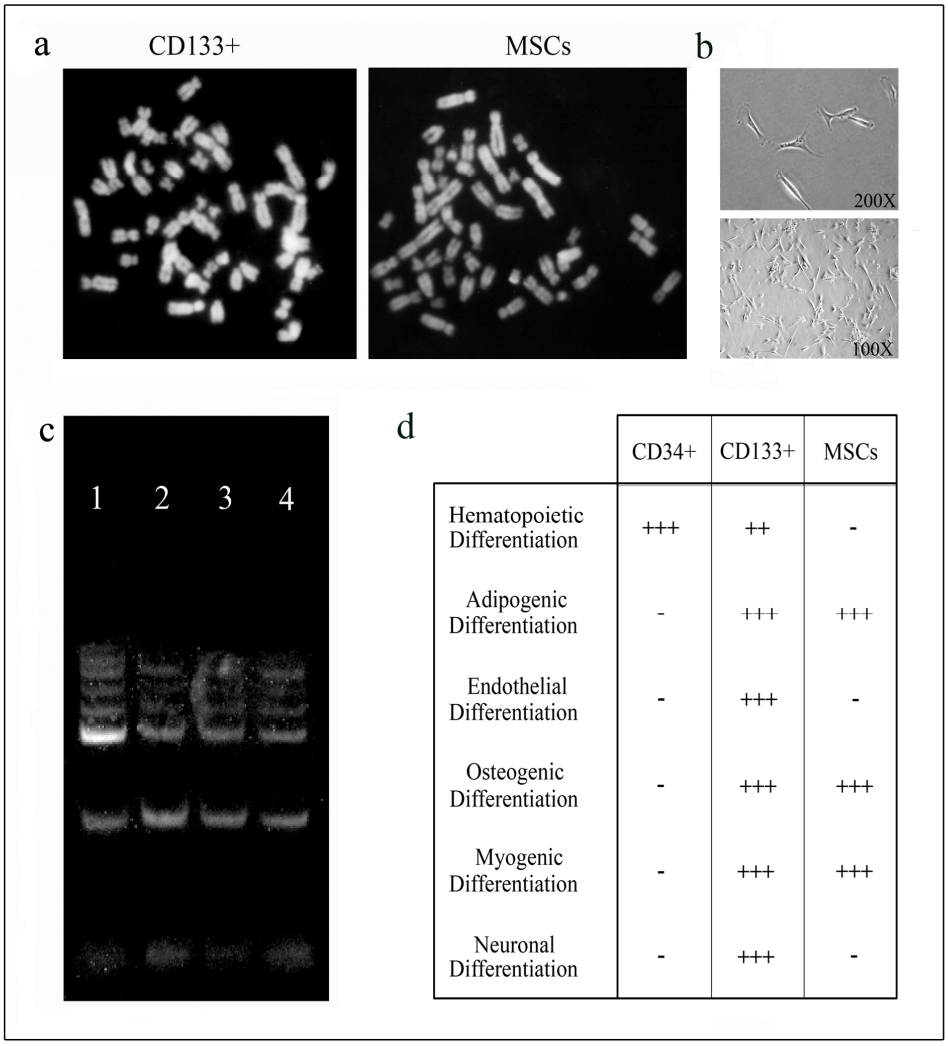


Figure 8. Immunephenotype and growth kinetics. A. Cytofluorimetric characterization of CD133$^+$ cells isolated from BM before plating; B. Cytofluorimetric characterization of CD133$^+$ cells in culture; C. Growth curve of CD133$^+$ ($p<0,05$) compared to MSCs with and without CD133 antigen (the two MSCs groups shown overlapping growth curve); D. Different CD133$^+$ cultures conditions, the histograms referred to the fourth condition (fibronectin with Reyes' medium).

The karyotype and telomerase activity showed a normal pattern (Figure 9A and 3C). However, to evaluate the best condition for their *in vitro* culture, expression of stemness markers was evaluated in the CD133$^+$ cells that underwent to three different culture

conditions as summarized in Figure 2C. In particular, when expanded in fibronectin-coated dishes and cytokines enriched medium, 5% of the selected cells retained the antigen CD133 until passage 5 but lose CD34 and CD45 while the expression of CD44 and CD90 antigens highlights an ongoing mesenchymal profile (Figure 2B-C). The negativity for CD3, CD4 and CD8 underline the undifferentiated-state of the selected population because no contamination of mature T lymphocytes and dendritic cells were found (data not shown). Interestingly, only in these conditions, $CD133^+$ cells were able to maintain the expression of POU-domain transcription factor Pou5f1 (OCT4). On the contrary, when selected cells were cultured in different conditions, CD133 expression was consistently decreased after passage 2, as well as the expression of the stemnesss markers c-kit and OCT4, whereas expression of Stage Specific Embryonic Antigen 4 (SSEA4) remain consistent on all different conditions. Expanded CD133 were also positive for HLA-ABC, but negative for HLA-DR in accordance to what has been previously reported for MSCs. While $CD133^+$ and MSCs showed good replicative capacities in these culture conditions, $CD34^+$ survived only on methylcellulose-coated dishes. In particular, when compared to MSCs derived from same BM samples, $CD133^+$ cells showed significantly higher proliferative capability (Mann-Whitney Test; $p<0,05$) as displayed in Figure 2D. Clones of $CD133^+$ and MSCs were generated and karyotype and telomerase activity were tested at the time of isolation and at passage 4 for $CD133^+$ and MSCs cells. Under the above-specified conditions, no significant changes in the morphology of the expanding cells could be observed throughout the culture period. Moreover, both $CD133^+$ and MSCs maintained a normal chromosomal length as well as the telomerase activity (Figure 9A-C). Cytofluorimetric profile of MSCs was also examined in order to evaluate their mesenchymal origin (data not shown).



	CD34+	CD133+	MSCs
Hematopoietic Differentiation	+++	++	-
Adipogenic Differentiation	-	+++	+++
Endothelial Differentiation	-	+++	-
Osteogenic Differentiation	-	+++	+++
Myogenic Differentiation	-	+++	+++
Neuronal Differentiation	-	+++	-

Figure 9. Cells characterization. A. Karyotype of CD133$^+$ and MSCs; CD133$^+$ cells at different days of culture; B. Morphological aspect of CD133$^+$ cells in culture with light microscopy; x200 original magnification (up) and x100 original magnification (down); C. Telomerase: lane 1. Positive Control, lane 2. CD133$^+$, lane 3. MSCs, lane 4. HUVEC; D.

Chart of differentiations on all cell populations. Pictures were taken with a Leica Camedia C-4040 camera and processed with Leica AnalySIS Software (Leica Microsystems).

3.2 Cell differentiation ability

3.2.1 Hematopoietic differentiation of CD133+, CD34+ and MSCs.

Freshly isolated $CD133^+$ and $CD34^+$ cells cultured on methylcellulose-based media were able to generate both CFU-GM and BFU-E colonies (Figure 10A-B). After 15 days of culture there were not statistically significant differences on the number of CFU-GM and BFU-E colonies generated by $CD133^+$ (29.75±6.61 and 17.25±10.47 respectively) or $CD34^+$ cells (25.5±6.37 and 38±1.5 respectively) derived from the same BM samples. As expected, MSCs were not able to generate neither BFU-E nor CFU-GM colonies in all the tested samples (Figure 10A). Clones of $CD133^+$ cells derived from different BM samples and expanded in mesenchymal conditions did not maintained the potential to differentiate towards the hematopoietic lineage (Figure 10A).

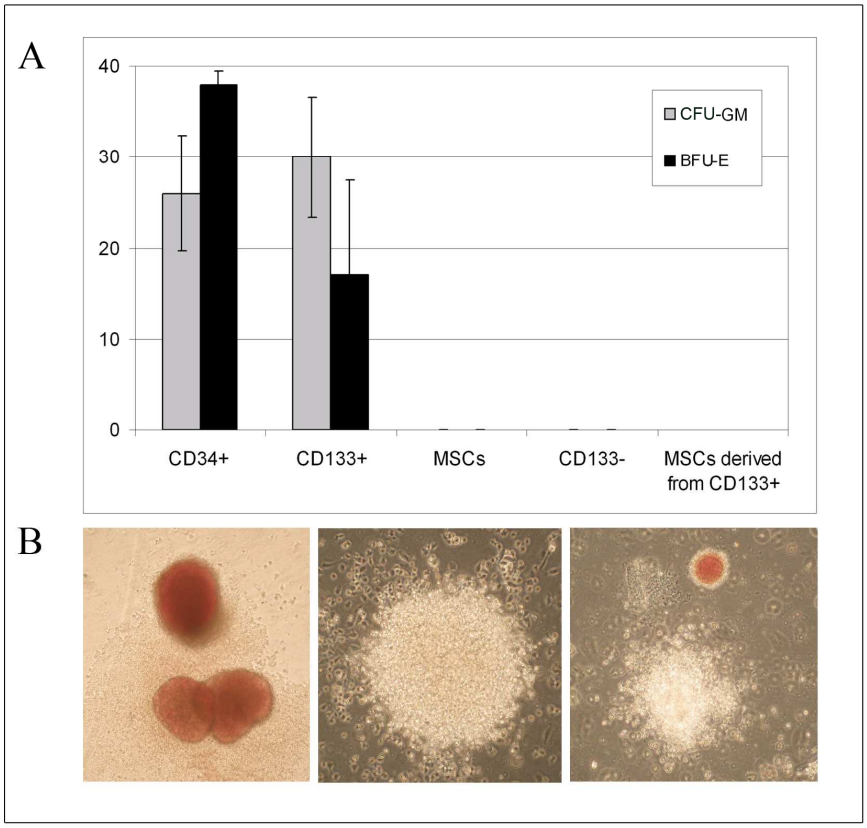


Figure 10. Hematopoietic differentiation. A. Number of hemopoietic colony (5.000 cells/ml). B. Morphological aspect of CD133^{+} hematopoietic colony: BFU-E (left), GFU-GM (centre) and; BFU-E and GFU-GM (right); x100 original magnification. Pictures of colonies were taken with a Leica Camedia C-4040 camera and processed with Leica AnalySIS Software (Leica Microsystems).

3.2.2 Mesenchymal differentiations.

In vitro differentiation of osteoblasts, adipocytes, endothelial, myocytes and neuronal cells from BM $CD133^+$ cells and MSCs. In order to test the mesenchymal potential of the clones derived by culturing $CD133^+$ cells expanded in fibronectin-coated dishes different lineages were tested. MSCs derived from same BM samples were used as control. As reported above, $CD34^+$ cells did not survive when cultured in the same conditions. To determine whether $CD133^+$ cells can differentiate into adipocytes, cells were allowed to grow to 70% confluence prior to induction. Morphological changes as well as formation of lipid vacuoles within the cells were noticeable one week post induction and visualized by Oil-Red-O staining. The expression of PPRγ and Lpl was detected after 7, 14 and 21 days of induction and increased with time (Figure 11A). $CD133^+$ cells maintained in control medium did not show any sign of adipogenic differentiation. When seeded on fibronectin-coated plates and cultured under endothelial conditions, $CD133^+$ cells changed their morphology and where able to form capillary-like structures when seeded on Matrigel-coated dishes. Moreover, expression of endothelial markers such as P1H12 (CD146) and Angiopoietin was detected at 7, 14 and 21 days after induction (Figure 11B). To investigate the osteogenic potential of $CD133^+$ selected cells, second to seventh-passage cells were cultured under appropriate condition for differentiation. When induced, the spindle shape of $CD133^+$ cells flattered and broadened with increasing time of induction, and were homogenously stained by AP. Cells maintained in control media did not show any change in their morphology and no activity of AP was detected. Moreover, only $CD133^+$ cells induced in osteogenic culture condition showed expression of Cbfa1 and Osteocalcin at RT-PCR analysis. The decrease on Cbfa1 expression in the induced cells at 21 days is compatible with the fact that this is normally expressed at the early stage of osteogenic differentiation (Figure 11C).

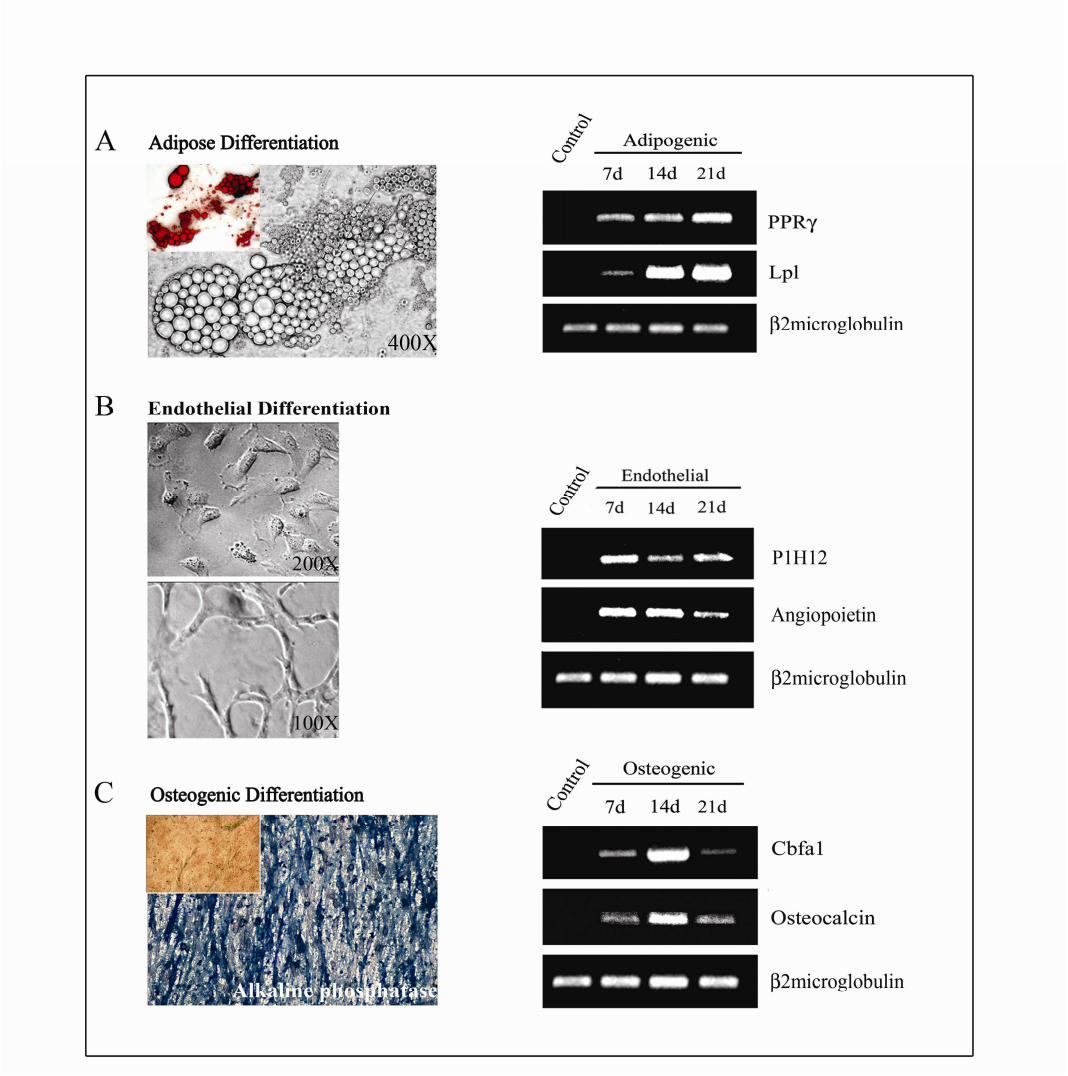


Figure 11. Differentiations - part I. A. Light microscopy showed lipidic vacuole after adipogenic stimulation; PCR results of specific adipogenic genes: transcription factor PPRgamma increase during the period of culture, whereas Lpl synthesis begins only after 14 days; x400 original magnification and x200 original magnification (inset). B. Endothelial differentiation: light microscopy showed CD133^{+} with characteristic endothelial morphology; PCR results of specific endothelial genes: the transcription factor P1H12 decrease after 14

days in culture, whereas Angiopoietin is highly express during all period of culture; x200 original magnification (up) and x100 original magnification (down). C. Alkaline phosphatase staining of CD133$^+$ cells induced in osteogenic conditions compared to CD133$^+$ in the control medium; PCR results of specific osteogenic genes: the transcription factor Cbfa1 and Osteocalcin increase during the first period and decrease at 21 days of culture; x200 original magnification and x100 original magnification (inset). All pictures were taken with a Leica Camedia C-4040 camera and processed with Leica AnalySIS Software (Leica Microsystems).

Myogenic potential was evaluated by culturing CD133$^+$ cells in Matrigel-coated plates under appropriate conditions. Myotube-like structures were noticed from 24 to 48 hours after 5-Azacytidine was added to the culture media. Increasing in the expression of MyoD and MRF4 was detected by RT-PCR after 14 and 21 days after induction (Figure 12A). Cells maintained in control medium were not able to form myotube-like structures neither to express muscle proteins. Neurospheres formation was the primary step to induce CD133$^+$ cells trough neural differentiation. After the neurospheres dissection indeed, the neuronal potential of selected cells was evaluated by culturing on pOrn and laminin coated plates under serum-free conditions. After 10 days not only morphological changes were noticeable as the formation of long extensions astrocytes-like but also the detection of O4 and MAP2 protein expression. Finally, only CD133$^+$ cells on neurogenic conditions were able to express betaIII tubulin (Figure 12C). MSCs with and without CD133$^+$ fraction were able to undergo to osteogenic, myogenic and adipogenic differentiation as previously described[18] (data not shown). No statistical differences neither in the length of time necessary for the induction of the differentiation process, nor in the gene expression was noticed between MSCs and the clones derived from the expansion of CD133$^+$. However, both MSCs fractions did not show any differentiation towards the neurogenic lineage and as expected toward endothelial lineage (data not shown).

Cardiomyocyte differentiation was assessed by co-culture method with rat neonatal cardiomyocytes. CD133+ cells expressing the cardiac marker troponin I by immunofluorescence (Figure 12B left), the early transcription factor GATA4 and the sarcomeric protein β-MHC by RT-PCR were noticed after 4 and 9 days of co-culture (Figure 12B right). CD133+ cells expressing this “cardiomyocyte-like” phenotype were about 5% of the whole co-cultured stem cell population.

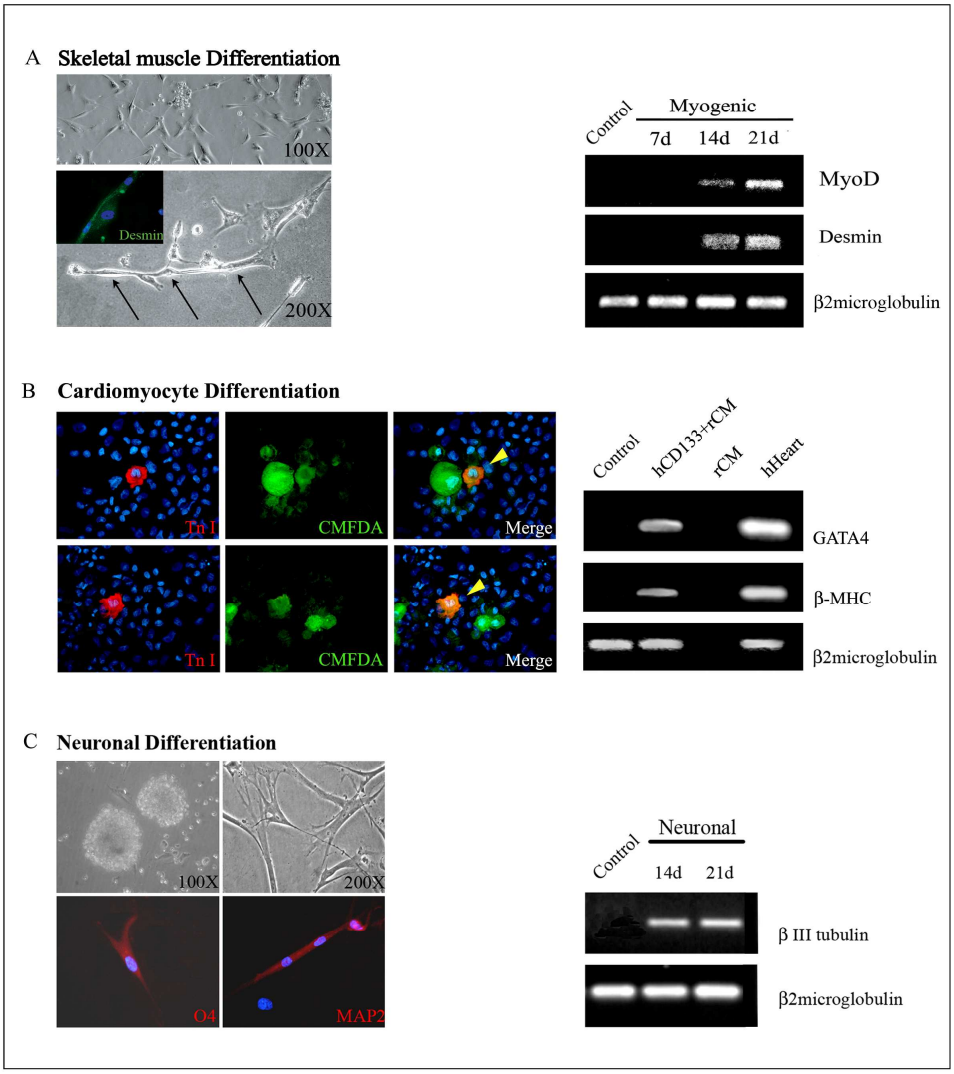


Figure 12. Differentiations – part II. A. Myogenic differentiation: more than three nuclei fused in one myotube (see black arrows). Immunostaining with Desmin in muscle differentiated CD133$^+$ cells; PCR results of specific myogenic genes: expression of MyoD and Desmin were detected only in CD133$^+$ cells cultured under myogenic conditions; x100 original

magnification (up), x200 original magnification (down) and x200 original magnification (inset).

B. Cardiomyocyte differentiation: immunofluorescence for troponin I (Tn I, red) and CMFDA (green) expression on cytospun CD133+-cmfda+ cells co-cultured with rCM, after 9 days. Yellow arrows show CD133+-cmfda+ cells expressing troponin I on merge pictures; PCR results of the specific human cardiac genes GATA4 and β-MHC: co-cultured human CD133+ cells were positive for the expression of the cardiac early transcription factor and sarcomeric protein after 4 days of co-culture with rCM (which are negative for the expression of these human genes).C. Neuronal differentiation: under these conditions $CD133^{+}$ forms neurospheres before employ typical neuronal aspect, immunostainig with O4 and MAP2 in neuronal differentiated cells; PCR results: the expression of betaIII tubulin increase during the three weeks of culture; x100 original magnification (up right), x200 original magnification (up left) and x200 original magnification (down). All pictures were taken with a Leica Camedia C-4040 camera and processed with Leica AnalySIS Software (Leica Microsystems).

3.2.3 Immunemodulatory effect of CD133+ cells

The mitogenic effect of phytohaemoagglutinin(PHA) tested in hCD133 bone marrow cells and hMSC highlight comparable profile of proliferative response (Figure 13). The already know immunemodualtory effect of hMSC appear evident also for hCD133 bone marrow cells and for both stem cells was cell-concentration dependent.

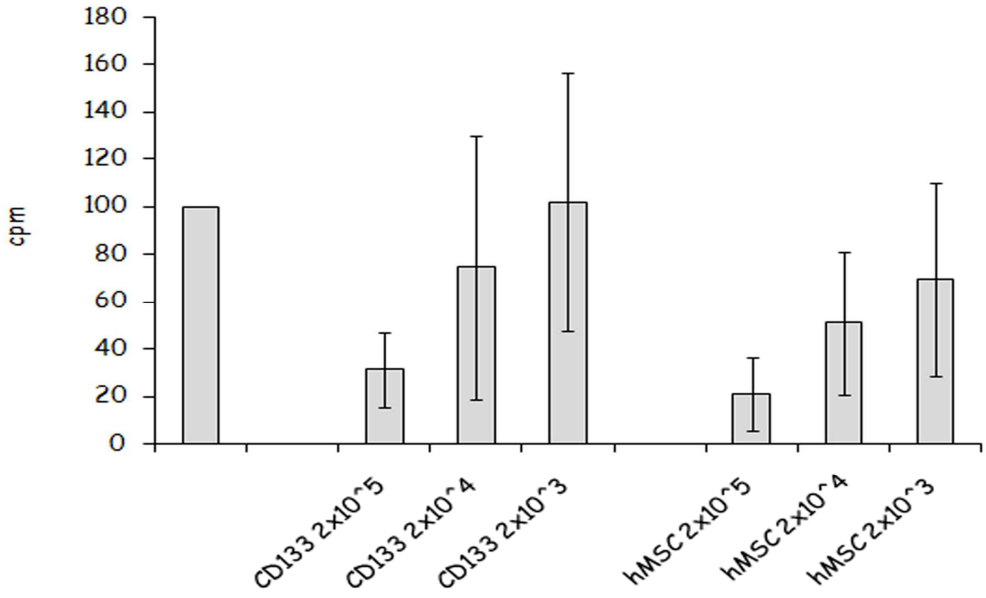


Figure 13: PHA assay for hMSC and hCD133 bone marrow cells. The immunemodulatory effect was stronger according to the used cell-concentration. Cpm: count per minute.

3.3 In vivo

3.3.1 In vivo application of CD133+ cells

In this work we analyzed CMTMR+ hCD133 bone marrow stem cells injected in collagen patch previously implanted into infracted heart of Nude Immunodeficient Rats (rNu) (Figure 14 and 15). Cryoinjured heart of rNU with patch application and injection of alternatively gfp+ rCM or cell suspension medium were used as control.

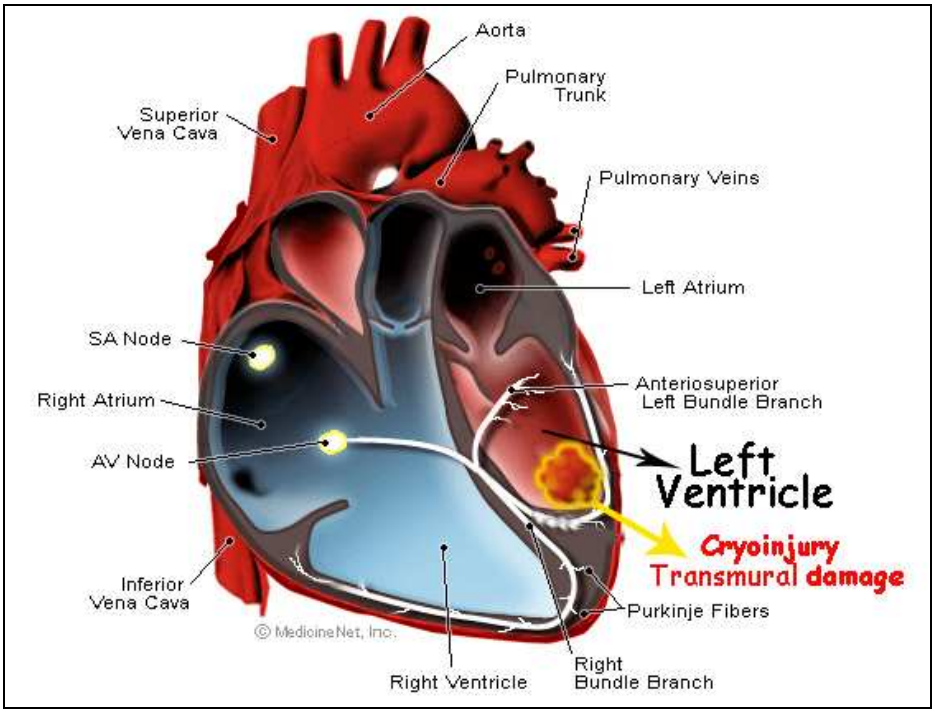


Figure 14: Cartoon of zone of infarction in left ventricle (LV) by acute necrotizing injury (ANI)

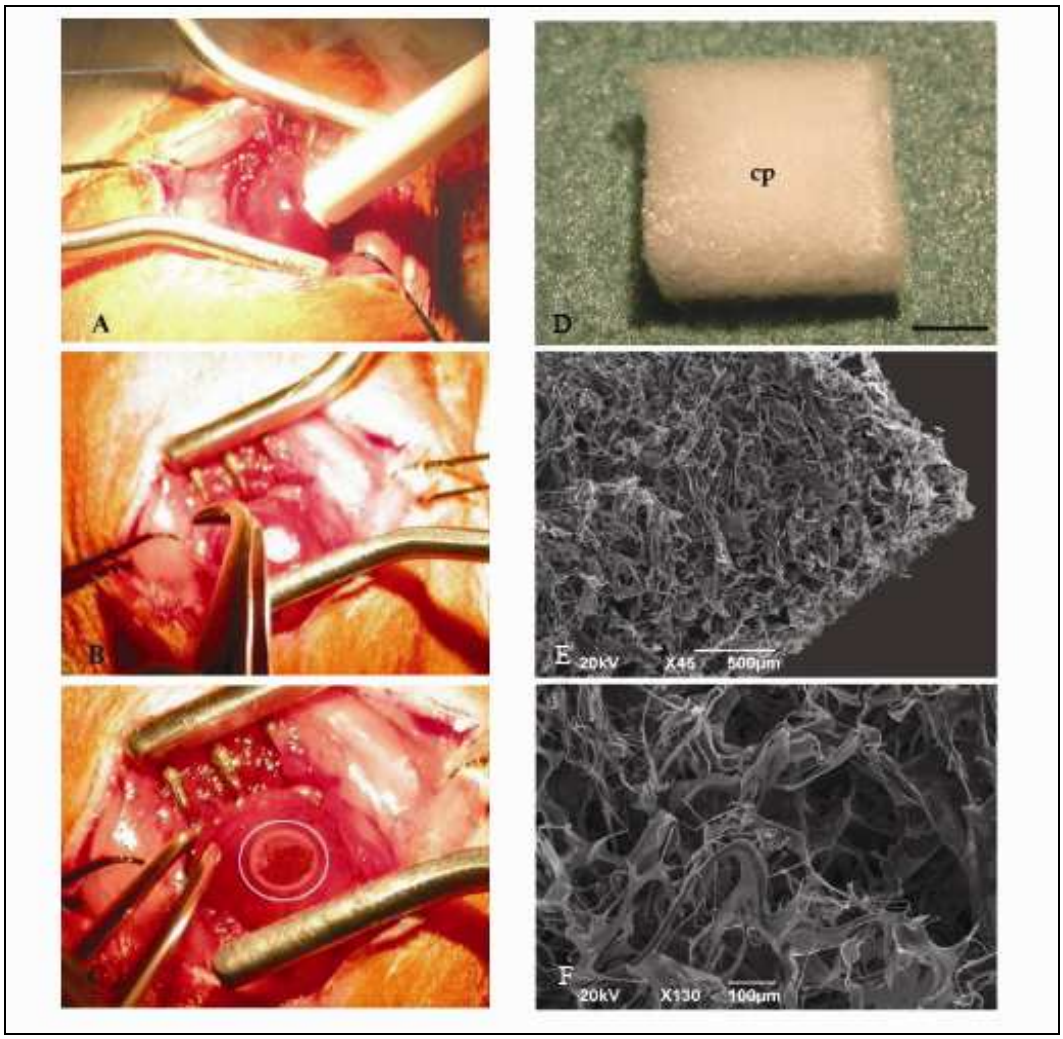


Figure 15:A, B and C showed the cryoinjury procedure in NuRat LV.C: scar after cryoinjury where the collagen patch will be applied. D:collagen patch (cp):0.8x0.8x0.5 cm. We use collagen type I because of the propensity of cardiac myocytes to form contracting aggregates by entrapment in collagen. E and F: electron scansion microscopy (SEM) images of collagen.

In particular, the animals underwent first to a cryoinjury damage of the heart performed through a left thoracotomy followed by a patch application similarly to what has been previously described (*Callegari A et al., 2007*). Two weeks after, animals were randomly divided in 2 groups. The first (model I) underwent to a second thoracotomy associated to the direct injection of the cell into the patch, the second (model II) had the cells injected systemically via the femoral vein (Figure 16).

Cells before injection were positive for the mesenchymal stem cell marker CD73, CD105, CD90, SSEA4 and in both models 4x10^ cells at passage 5 were injected. Animals were sacrificed after 24h, 15 days and 30 days after injection.

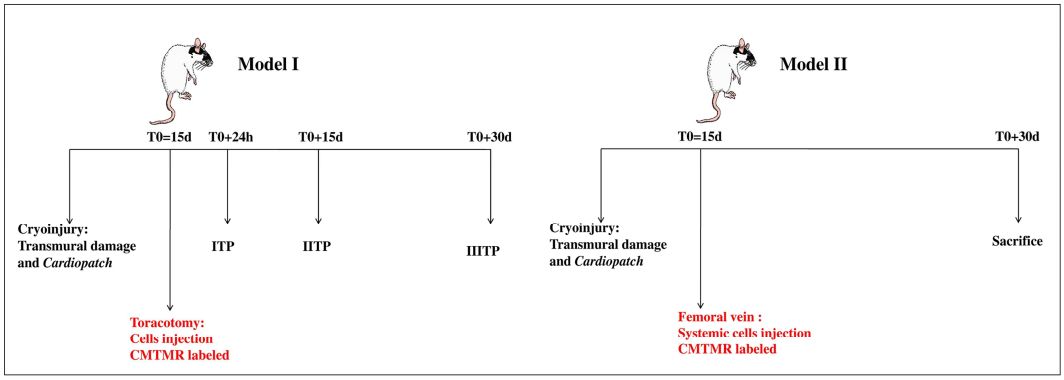


Figure 16. Cartoon of the experimental models. Model I : cryoinjury and patch application, 15 days later animals underwent new surgery and cell injection into the patch. Three different time points were chosen. Model II: cryoinjury and patch application, 15 days later cells were injected via femoral vein and one time point was chosen.

In model I after 24h about 60% of cells , both cardiomyocytes and CD133+ were found inside the patch, interestingly after 15 days cardiomyocytes were still in the patch area whereas CD133+ cells moved from the periphery of the injection site toward the cryoinjured area (Figure 17).

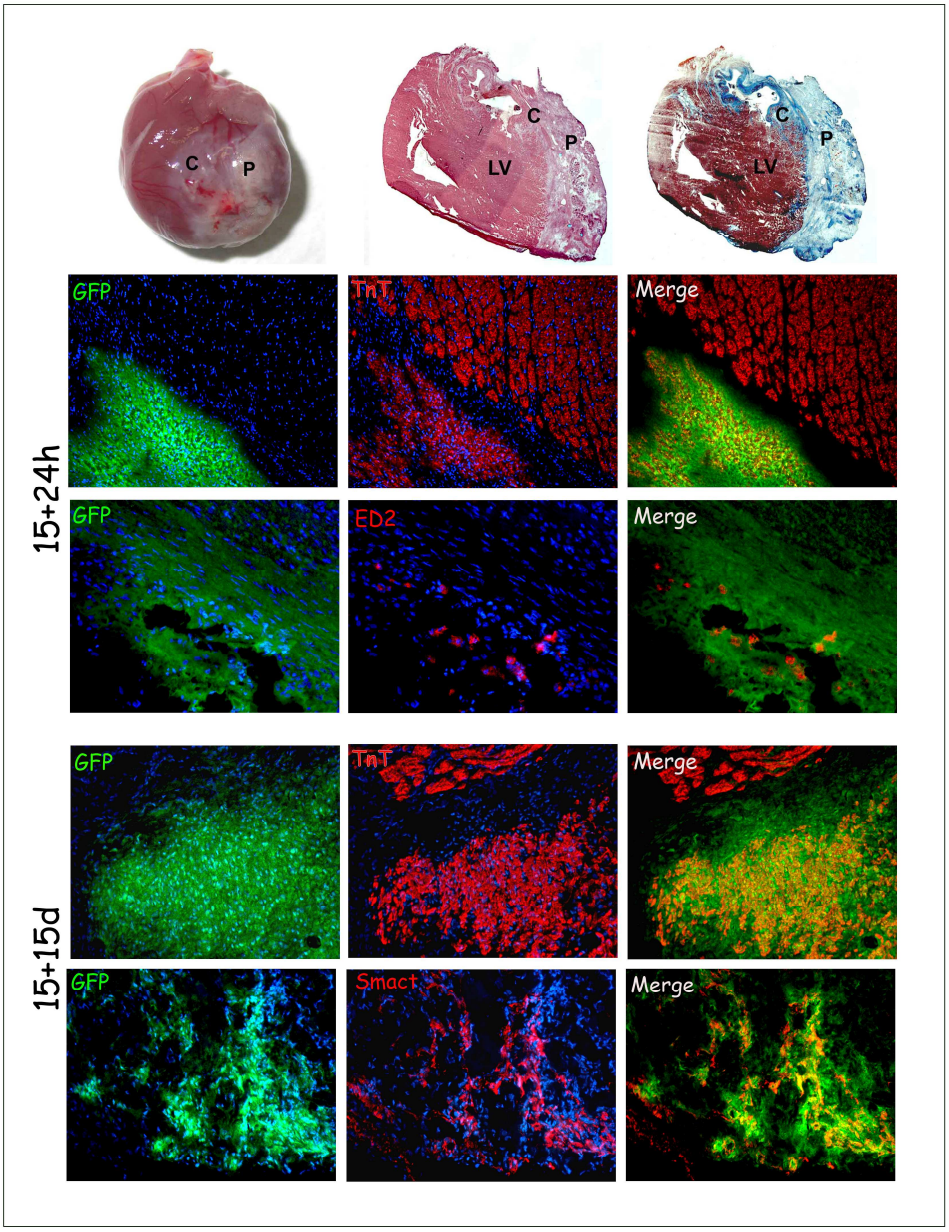


Figure 17: Model I. Gross appereance of the heart with rGFP Cardiomyocytes (high left), c=cryoinjury area, p=collagen patch; LV= left ventricles. Haematoxylin and Eosin (high

centre) and Masson's Tricrome (high right). Staining examples of TnT and ED2 antibodies after 15days post patch application and 24hour cardiomyocytes injection. TnT staining evidenced green cardiomyocytes are on the patch area; scattered macrophages expressing ED2 antibody were found at the periphery of the patch close to the cells. After 15days post patch application and 15 days cardiomyocytes injection, TnT staining highlight most of the cardiomyocytes in patch area and Smact positive cells always in collagen patch were found mixed to cardiomyocytes cells. 200x original magnification.

In Figure 18 A it is noticeable the very small amount of cells present during the different time points; after 15 days cells are dramatically decreased keeping then constant their small number probably because either they lost the cell tracker or they died since the collagen environment appear to be hostile to the growth. The very small number of cells which died by apoptosis (Figure18 B) pointed out the different mechanism of cell death chosen by the injected cells.

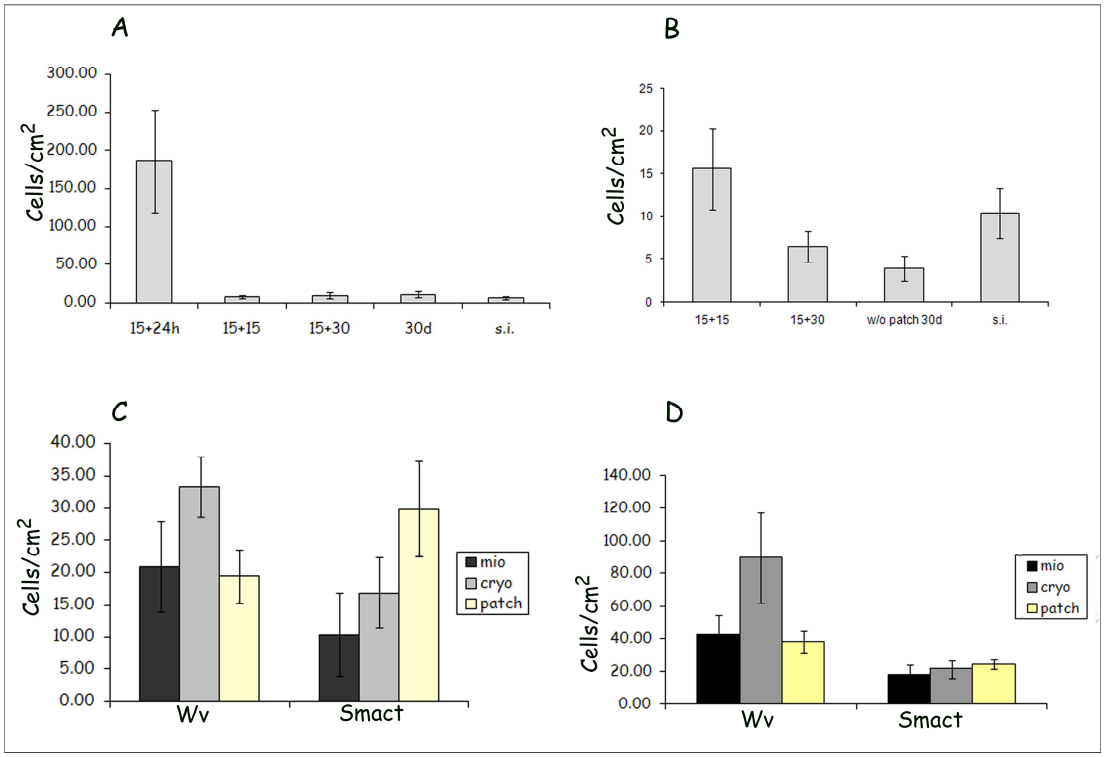


Figure 18: Model I and II. Quantitative analysis of the injected cells(A). After 24h 60% of the cells were found in the patch injection site. The cell number after 15 and 30 days post patch injection and 30 days after cell injection in cryoinjury area without patch and after systemic injection was estimated around 2%.

Very small amount of cells died by apoptosis in all different conditions (B).

Number of vases after 15 (C) and 30 (D) days of cell injection in model I. After 15 days post injection, small arterioles positive for Smact were more present in patch area whereas after 30 days new vases positive for vW factor were more abundant in cryoinjury area in respect to patch zone.

Both after 15 and 30 days serial slides of samples were stained for Von Willebrand factor and Smooth muscle actin underlining presence of new and mature vases respectively, with scattered red cells close to the vases particularly on the second time point; nevertheless immunoflurescence assays did not reveal integration of CD133 cells in capillaries or arterioles (Figure 20).

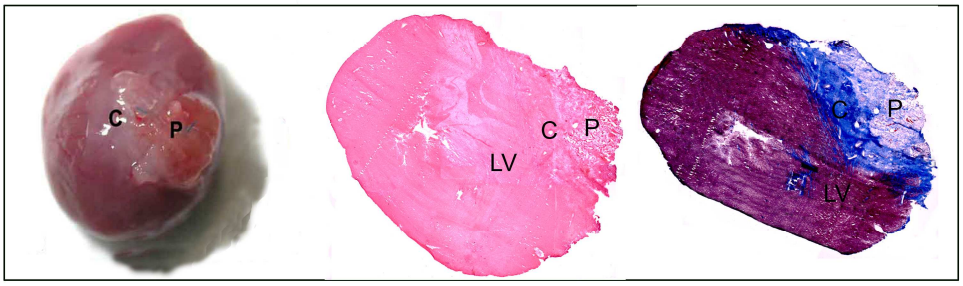


Figure 19: Model I. Gross appereance of the heart with BM CD133+cells (left), c=cryoinjury area, p=collagen patch; LV= left ventricles. Haematoxylin and Eosin (centre) and Masson's Tricrome (right).

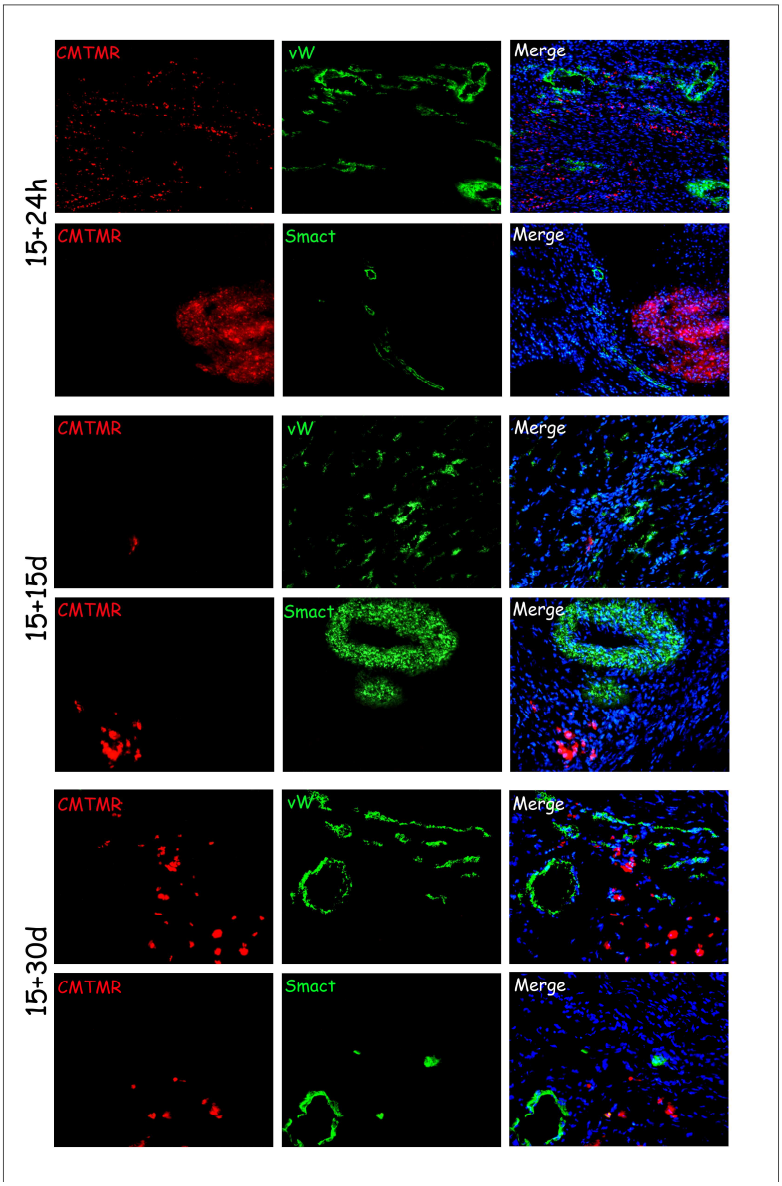


Figure 20: Model I. Staining with vW and Smact in patch area (15+24h). In 15+15days after cells injection scattered red cells are present near by vases in cryoinjury area. Similar

situation has been detected 15+30 days after cells injection where small number of cells were still present close to vW and Smact positive cells.

Quantitative analisys of the vases in patch area, cryoinjury and myocardium highlighted the strong capacity time dependent of the collagen to recruit new vases; as in Figure 18 C and D after 15 days many mature vases Smact + were present in patch area whereas after 30 days the vases number riched a plateau.

Macrophages and natural killer cells were detected, the latter were present only at the periphery of the patch while macrophages were found toward the inner part of the collagen and in cryoinjury zone. In particular, after 30 days they were still present in the patch area; moreover red spots of CMTMR+ cells were found inside macrophages, clearly showing that phagocitosys of the cell tracker took place (

Figure **21**).

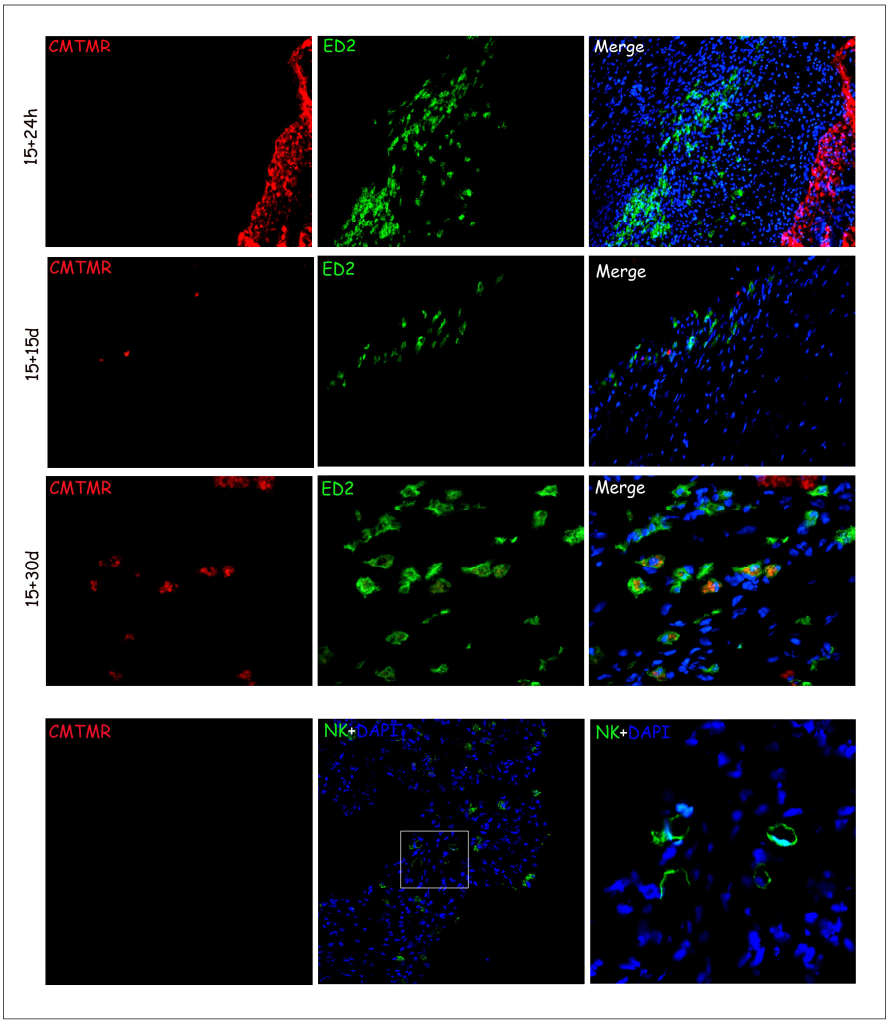


Figure 21: Model I. ED2 staining in different time points. Macrophages are present after 24h cells injection (200x magnification) mainly in the patch area, it is collagen patch applied 15 days earlier which withdraw these cells. In the other two conditions, ED2 positive cells appear also in cryoinjury zone (15+15 days. 200x magnification) often with red spot of CMTMR cell tracker inside. (15+30 days. 400x magnification). Last row: example of NK staining. Positive cells were found at the periphery of the patch unrelated to CMTMR positive cells in all three different time points (100x and 600x magnification)

In model II CMTMR+ cells were found mainly into the inner part of the patch closely related to the cryoinjured area and near by the quite large vases (Figure 22); no co-staining with ED2 macrophage marker was found even though ED positive cells with red spot of CMTMR were found mainly in the patch area.

Interestingly positive cells for the proliferation marker PH3 were abundant in the cryoinjury area of animals that underwent systemic cells injection.

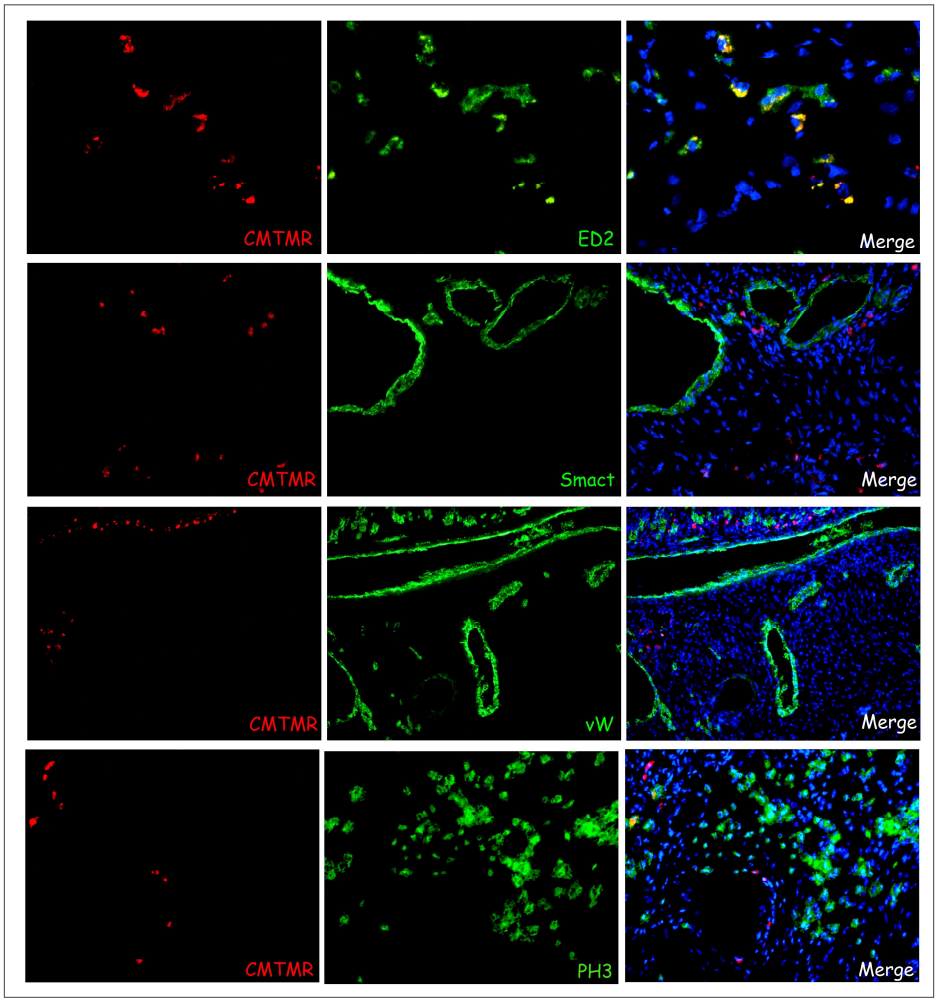


Figure 22. Model II: systemic injection. Sample were analyzed after 30 days post cells injection; in border zone (end of the path-beginning of cryoinjury) most of CMTMR positive cells were also ED2 (right above), but in cryoinjury area scattered red cells were negative for the above mentioned marker and close to the quite large vases positive for Smact and vW respectively. Last raw shows PH3 (green) staining in cryoinjury area. Interestingly PH3 positive cells were more present in cryoinjury area of model II than model I, (200x magnification).

Chapter 4 Discussion

4.1 In vitro

The present study suggests the presence in human BM of a multipotent progenitor cell with mesenchymal characteristic defined by the expression of CD133, OCT4 and SSEA4. $CD133^+$ cells were able not only to generate *in vitro* both CFU-GM and BFU-E hematopoietic colonies but also to undergo to mesenchymal and neurogenic differentiations. Indeed, this is the first study describing the potential of deriving MSCs from $CD133^+$ human BM. So far, different procedures have been described for selecting both HSCs and MSCs. For clinical application, HSCs are purified using a single isolation strategy (CD34 expression) *(Bhatia M et al., 1997;Sharkis SJ et al., 1997*). On the contrary, MSCs have been isolated from BM and other various adult specimens using different methods (*Pittenger M et al, 2002*) but a standardized method has not been described yet and as a consequence cell phenotype of MSCs has been reported to be variable among different laboratories (*Digirolamo CM et al, 1999*). Isolation of MSCs based on their phenotype is attractive for instance because the selection of a specific cell type would avoid culture contamination with already committed or differentiated cells,

which could affect the phenotype of MSCs and their differentiation process. Moreover, a selected undifferentiated population able to give rise to different lineages would possibly enhance the unsatisfactory preliminary results obtained with MSCs transplantation for tissue regeneration (*Leontiadis E et al., 2006*). In this scenario, we proved that $CD133^{+}$ cells could represent an alternative source of MSCs.

Telomerase activity and maintenance of a normal kariotype are essential characteristics for stem cell therapy (*Zimmermann S et al, 2003*). In our study telomerase activity was comparable between CD133+ and MSCs pointing out the potentially good proprieties of self renewal. As a matter of fact it is accepted that telomerase is required for replication and differentiation processes *(Hiyama Eet al., 2007*) even though hMSc express low level of telomerase activity and may elongate telomeres by a recombination based DNA replication mechanism, the alternative lengthening pathway (ALT) (*Serakinci N et al, 2008*).

An emerging body of data indicate that MSCs possess immunemodualtory properties and may play specific roles as immunemodulators in maintenance of peripheral tolerance, transplantation tolerance, autoimmunity, tumor evasion as well as fetal-maternal tolerance. MSCs are considered to be hypoimmunogenic, displaying low expression levels of human leukocyte antigen (HLA) major histocompatibility complex (MHC) class I.

Comparing CD133+ and MSC in PHA assay, same immunomodulation characteristics have been highlighted proving that our cells have same characteristics of MSC. In particular, MSCs, have been shown to suppress the activity of a broad range of immune cells, including T cells (*Bartholomew A et al, 2002; Di Nicola M et al, 2002*) (they lack indeed of surface expression of costimulatory molecules, such as CD80 -B7-1- and CD86 -B7-2-), antigen-presenting cells, natural killer (NK) cells and B cells. New studies have further shown that MSCs interact with NK cells, express NK cell receptor ligands, express Toll-like receptors (TLRs), respond to TLR ligands and act as antigen-presenting cells upon interferon-g stimulation.(*Spaggiani GM et al., 2006; Liotta F et al, 2007*).

Taken together, these data suggest that deeper studies on immunological profile of CD133 have to be arrange since it will be of paramount importance in our understanding of the biology and function of adult stem cells and in the development of therapeutic strategies aimed at exploiting or targeting BM CD133+ adult stem cells.

In order to evaluate their differentiation ability, $CD133^+$ cells were compared with $CD34^+$ and MSCs derived from same BM samples (*Hofmann WK et al, 2002; Jones EA et al., 2002*) As previously reported, we confirmed that hematopoietic colonies obtained respectively from $CD133^+$ and $CD34^+$ selected cells were comparable (*Handgretinger R et al, 2003).* As expected, MSCs derived from BM samples both before and after $CD133^+$ depletion, on hematopoietic medium did not arise colonies. These results are in keeping with previous data that remarked the hematopoietic potential of $CD133^+$ cells both *in vitro* and *in vivo*. $CD133^+$ cells have indeed the ability to generate *in vitro* a non–adherent $CD133^+CD34^-$ stem cell subset with a significantly higher frequency for SCID repopulating cells than any other $CD34^+$ or $CD34^-$ stem cell populations reported so far (*Kuci S et al., 2003; Handgretinger R et al, 2003*). Moreover it has been shown that CD133 antigen seems to appear on the surface of hematopoietic stem/progenitor cells before CD34 confirming that $CD133^+CD34^-$ cell subset appear at very early stage of human hematopoiesis (*Forraz N et al, 2004*). In addition to these data our study proved that this subset of $CD133^+$, $CD34^-$ cells, which co-express markers of stemness such as SSAE4 and OCT4 have also the ability of generating an adherent subset of cells with mesenchymal potential. In fact, while the hematopoietic potential of $CD133^+$ cells has already been extensively described, the potential of $CD133^+$ cells to differentiate towards mesenchymal lineages has been only partially explored (*Torrente Y et al, 2004*). In particular the possibility of $CD133^+$ cells derived from BM to give rise to mesenchymal lineages other than endothelial and myogenic has not been demonstrated (*Peichev M et al, 2000*). In this study we showed for the first time that MSCs derived from BM with

potentials to differentiate to various mesenchymal lineages and with some neurogenic potential, could be generated from $CD133^+$ cells. MSCs and $CD34^+$ from same BM samples were used as control. However, as previously showed $CD34^+$ fraction did not grow on mesenchymal culture conditions (*Huss R et al., 2000*) More importantly, stromal cells expanded after $CD133^+$ selections showed some differences also when compared to MSCs derived from the same BM samples. Firstly they showed a different proliferative capability. When cultured *in vitro*, $CD133^+$ cells grew quicker than MSCs underlining the beneficial characteristics for expansion and cell transplantation. Secondly some differences were also evident in terms of differentiation potential. Similarly to what has been previously described (*Pomyje J et al, 2003*), $CD133^+$ cells in endothelial condition were able to express specific endothelial markers such as P1H12 and Angiopoietin and to form capillary-like structure when seeded on Matrigel. On the contrary MSCs did not show any endothelial differentiation potential in the same conditions. Although previously reported the potential of MSCs to differentiate towards endothelial lineages is still debatable and in our experimental conditions MSCs did not form capillary-like structure neither expressed markers of endothelial differentiation (*Oswald J et al., 2004*).

Conversely, the endothelial potential of $CD133^+$ cells is so well established that at least a subpopulation of these is defined as endothelial precursors cells (*Friedrich EB et al., 2006*). It is well known that $CD133^+$-BM derived cells contributed to angiogenic process during adult life and have also shown the capacity to form new vessels (vasculogenesis) (Hilbe W et al., 2004). Remarkably, their angiogenic potential has been well established also in tumor growth and increased numbers of circulating $CD133^+$ cells occur in patient with solid tumor (*Sussman LK et al., 2003*). In this perspective, the angiogenic potential of $CD133^+$ cells would represent a further advantage on MSCs (where the angiogenic potential, if present is not comparable (*Salven P et al, 2003*) to overcome the vascularization problems usually related to cell transplantation and tissue engineering. In this perspective we could speculate in fact that $CD133^+$ cells not only differentiate into the

tissue target, but could also contribute to the vasculature architecture of that specific tissue.

Moreover, if compared to MSCs, stromal cells derived from $CD133^+$ had the advantage of being able to differentiate towards neurogenic lineages. After three weeks of neural stimulation $CD133^+$ expressed markers of neural development such as MAP2 and O4, whereas MSCs cultured in the same conditions did not (data not shown). Finally, similarly to what has been previously reported, differentiation towards myogenic lineages and myotube-like structure formation was more effective using $CD133^+$ cells that MSCs (*Torrente Y et al, 2004*). While showing some advantages to MSCs in terms of proliferation and differentiation, we showed for the first time that $CD133^+$-BM derived cells where also able to differentiate towards osteogenic and adipogenic lineages, fully proving their mesenchymal potential. In osteogenic culture conditions, $CD133^+$ cells were able to determine homogenous activity of AP similarly to what we observed for MSCs (data not shown) and osteogenic gene expression was detectable. Oil-Red-O quantification showed that $CD133^+$ induced cells were able to deposit lipid similarly to MSCs in the same conditions (data not shown) and the attained adipogenic differentiation was confirmed by the expression of specific genes. The variances in terms of proliferation and differentiation are in keeping with some difference in their phenotype during expansion. In particular stromal cells derived from $CD133^+$ co-expressed c-kit, SSAE4, OCT4 while MSCs in the same culture conditions never expressed these markers, who typically identify early progenitors.

Moreover, different methods of expansion such as fibronectin-coated or uncoated Petri dishes showed evidence of the importance of the regulation of anchorage-dependent cell growth, migration, differentiation and gene expression for the $CD133^+$ cells while MSCs cultured in the same conditions did not show any difference on growth potential and cytofluorimetric analyses (data not shown). Not only did the type of plastic indeed, but also the coating of the substrate where the cells were seeded, determine the characteristic

phenotype of a forerunner $CD133^+$ cell. Interestingly, it is conceivable that adhesion of $CD133^+$ cells to fibronectin, multi-domain adhesive glycoprotein found in blood and interstitial connective tissue (*Wang R et al, 2000*), induces formation of focal adhesions that influence cell migration and signalling (*Jackson L et al., 2007*) and do not change the immunological properties of the cells. In particular, the progenitors markers c-kit and SSEA4 were expressed by $CD133^+$ cells seeded on fibronectin, whereas they were down regulated on plastic substrate. Purified $CD133^+$ at passage 2 and 4 showed down regulation of CD133 expression in accordance to the data previously reported (Miyamoto S et al., 1998). No expression of CD4, CD8, CD34, CD38 and CD45 was found while they continue to express CD13 present on the surface of early committed progenitors of granulocytes and monocytes and by all cells of these lineages as they mature. Expression of surface proteins with a critical role for the intra- and extra-vasation process as CD13 and CD44 may indicate that $CD133^+$ cells are associated, or attached, to vessels in the BM compartment (*Suuronen EJ et al, 2006*). Expression of CXCR4 could allow them to respond to Stromal Derived Factor-1 and may confirm their ability to enter into the bloodstream if necessary. In keeping with the mesenchymal phenotype defined by In 't Anker et al (*In't Anker PS et al, 2003*) our cells were positive for the stromal cell markers CD105, CD90 and the adhesion molecule CD73 (SH2/SH3).

All together, our results *in vitro* showed that $CD133^+$ cells could represent a good source of cells for transplantation because they are able not only to reconstitute the hematopoietic compartment as previously reported but in addition they can also give rise to the stroma.

As a consequence the results of this study confirm the hypothesis that $CD133^+CD34^-$ cells identify a very early progenitor of both HSCs and MSCs.

However, more studies are necessary in order to address at least two points that are still unclear. On one hand more data are needed on the origin of $CD133^+$ cells in the BM. McGuckin et al (*Forraz N et al., 2004*) defined a cell population intracellular $CD34^+$ and $CD133^+$ that may represent a primitive cell population having yet to express external

CD34. Loges et al (*Loges S et al., 2004*) after demonstrating the hematopoietic and endothelial capacity of single $CD133^+$ precursors, speculate the identification of the human adult hemangioblast in $CD133^+$ cells since the latter are positive for the hematopoietic and endothelial markers CD31 and CD105 (*Urbich C et al., 2004*), hence VEGFR-2 are committed in Flk-1+ populations (*Cho, S.K et al, 2001*). BM or Peripheral Blood Stem Cells transplantation of $CD34^+$ cells have always faced the problem of stromal reconstitution and the regulation of mesenchymal cells has been considered important for the hematopoietic regulation and for the immunological response of the host (*Fibbe WB et al, 2003*). In particular the support of good stroma has been considered very important for the final outcome of the transplantation.

To summarize, by means of immunoselection with one anti human clinical–grade monoclonal antibody we obtained precursor cells easy to isolate and expand, with hematopoietic characteristics and mesenchymal capability. We still have to optimize the Petri dish coating to modulate the antigen profile and in this sense studies *in vitro* and *in vivo* are planned. The immune response of $CD133^+$ cells is under investigation since it is mandatory to know whether $CD133^+$ cells possess immune modulation effect as MSCs. So far, our results remark the great potential of $CD133^+$ cells from BM, and support the existence of a broadly multipotent/pluripotent cell that persists in the adult and justify the upcoming interest for possible therapeutic applications.

4.2 In vivo

Reconstituting infarcted tissue with cells capable of performing the functions of the heart or proving beneficial trophic factors for native cells are attractive solutions for myocardial repair (Figure 24*; Murry CE et al, 2006*). In most of the cellular cardiomyoplasty studies, stem cells have been administered systematically, intracoronary, or directly injected into

the myocardium. Although positive results have been observed, the accumulation of transplanted cells in other organs as the lung (*Hou D et al., 2005*), engendered cell death because of an unsuitable milieu, poor retention and the low transdifferentiation rate into cardiomyocytes are issues which need to be addressed to increase the efficacy of stem cell therapy. In this study we tested alternative approaches to deliver stem cells to the injured area; in particular CD133+ cells were delivered using a biodegradable collagen patch. Despite the fact that through this approach we were not able to appreciate cell engraftment, we could nonetheless find our cells migrated to the cryoinjury area and confirm the good mobility characteristics of BM CD133+ cells.

Collagen type I was previously used *in vitro* from Vunjak-Novakovic G.group (*Radisic M et al, 2004*) and it showed the best cardiomyocytes survival and the natural tendency of the cardiac cells to form cell junction; moreover the polymer possess neoangiogenetic properties (*Callegari A et al., 2007*).

As previously mentioned, CD133 is a primitive marker expressed in cell population with potent hematopoietic and angiogenic potential .The proangiogenic effect of BM CD133+ cells in humans has been demonstrated by improved perfusion and function of infarcted hearts after cell injection (*Bartunek J et al, 2005; Stamm C et al, 2007*); in addition, because of the importance of allowing new vases to play a crucial role on feeding the cell, we inject the cells after 15 days on scaffold implantation .

Indeed the number of new vases was enhanced on the patch area, in particular in model II where the quite large lumens were evident into the patch, close to cryoinjury zone. These vases were larger than those in cryoinjury area were they are usually big and less numerous after infarction. Interestingly, red cells were found near the large vases although we have to mention that most of the injected cells were noticed in spleen, liver and lung. The scattered red cells in patch-cryoinjury area proved that somehow CD133+ stem cells felt the biochemical signals from the damage area and possibly functional integration of non-cardiomyogenic cells might not be required to achieve a beneficial effect on cardiac

function. We performed functional analysis on one group of animal and treated animals ameliorate their heart function when compared to control rats.

In particular, most of the BM CD133+ cells home to sites of ischemia and it can be speculated a local release of factors acting in a paracrine manner on the surrounding ischemic tissue.

It has been demonstrated (*Prockop DJ et al., 2003*) that MSCs and similar cells repair tissue injury by three different mechanisms: creation of a milieu that enhances regeneration of endogenous cells, transdifferentiation, and perhaps cell fusion (Figure 23).

Although the differentiation potential of various progenitor cells was repeatedly demonstrated (*Orlic D et al, 2001*), *in vivo* cells frequently produced functional improvements without much evidence of either presence or differentiation of the cells. For instance, improvement in cardiac function after intravenous infusion of human MSC in immunedeficient mice after coronary ligation was observed but about five cells were detected in the heart after three weeks (*Iso Y, 2007*).

It has been observed that the cells release angiogenic growth factors such as vascular endothelial growth factor (VEGF), basic fibroblast growth factor (bFGF) and angiopoietin which protect cardiomyocytes against hypoxia promoting local angiogenic response (*Xu M et al, 2007*), moreover previous work suggested that both VEGF and bFGF induce the expression of the antiapoptotic gene Bcl-2 fortifying cardioprotection against myocardial cell death (*Nör JE et al, 1999; Nishida S et al., 2003*). Cytokines analyses on our samples are ongoing to detect Bcl-2 and confirm the hypotized paracrine effect of CD133+ cells.

Interestingly, immunostaining with phospho-specific antibody, PH3, revealed many cells under mitotic phosphorylation of histone 3 in cryoinjury area. These cells in proliferation, negative for the red cell tracker CMTMR, were detected in animals treated systemically: again this phenomenon may be explained by the paracrine effect of CD133+ cells, even though it is not clear why in model I proliferating cells were not so abundant.

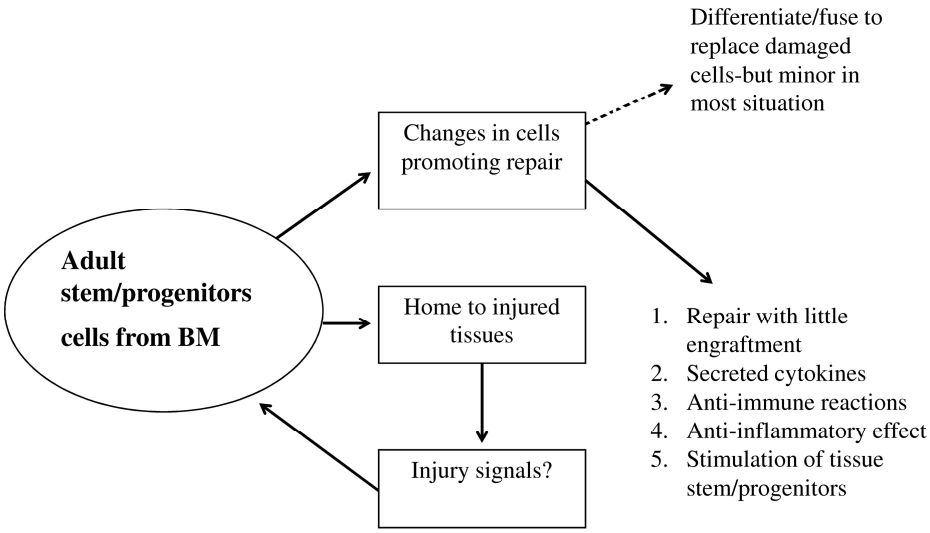


Figure 23:Schematic showing the multiple actions of mesenchymal stromal cells and perhaps other adult stem progenitor cells in repairing tissues. From Prockop DJ et al.,2007.

In both models the efficacy of cell engraftment was almost absent as more than 90% of the cell suspension injected was lost and did not engraft. Cells did not die by apoptosis and ,if they really died before secreting cytokines, other factors could have been involved to create an hostile environment, such as the constant stretching force of systolic and diastolic heart and patch movements triggered stress and hypoxia into the collagen. Cells could have died by oncosys following acute damage and swelling of the entire cell, so that it was not possible to detect the injected cells.

Therefore, the creation of engineered tissue that not only assembles cardiac cells but which also includes factors and, or cells favouring not only revascularization but also a trophic environment, will be crucial. For an optimal achievement of cardiac patches, it might be necessary to cultivate three-dimensional cell constructs in bioreactors that reproduce the normal stress and flow experienced by the tissue.

The use of CD133+ cells that possess MSC features, may be relevant for clinical application. In fact, this stem cell type showed multipotency and low immunogenicity and, hence, no rejection potential when xenotransplanted in the heart of nude rats that possess macrophages and natural killer cells. Anyway, recipient ischemic tissue may be inadeguate for donor cell retention in sufficient quantity to allow the desired effect because the survival of cells from any source implanted in the myocardium varies between 1% and 10% (*Ubrich C et al, 2004*).

Still, there lack studies on the isolation expansion and evaluation of bone marrow derived CD133+ cells for tissue regeneration; figure 24 showed as non cardiac cell sources are considered for cardiac repair.

An important point to be consider is the possibility to deliver cells by low-invasive techniques, such as the use of catheters, to avoid cell dispersion as in our systemic method.

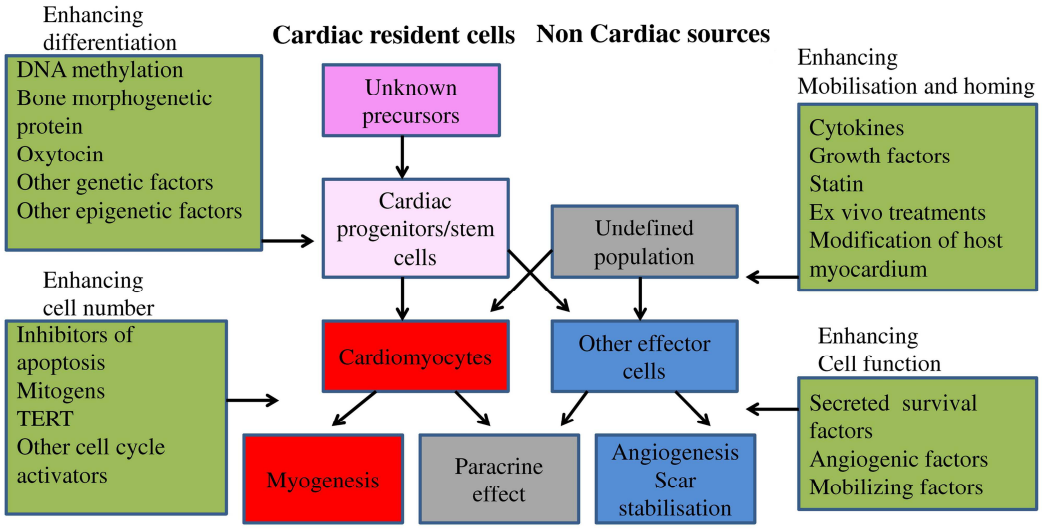


Figure 24:Current challenges for cell-based therapy in cardiac repair include identifying the origins of the novel cardiac progenitor and stem cells found within the heart, pingpointing the biological active cells from bone marrow and other mixed populations, optimizing cell mobilization and homing, augmenting grafted cells' survival, defining the cues for cardiac differentiation, promoting donor cell proliferation ex vivo (or, if safe, *in vivo*) and exploiting cell therapy as a platform for secretory signals. Adapted from Dimmeler S et al,2005.

4.3 Conclusion

While some types of stem cells are already used for clinical therapy, recent investigations raised high hopes for a broad spectrum of applications in cell-based therapies for regenerative or reparative medicine. It has been hypothesized that stem cells may at some point in the future become the basis for treatment of diseases such as Parkinson's disease, diabetes and heart disease. In this study we were able to characterize the CD133+ stem cell population from human bone marrow samples *in vitro*, highlighting the potentially good properties for clinical applications comparable to the ones owned by MSC; while *in vivo* we tested the possibility that a biodegradable and biocompatible polimer, called cardiac patch, applied on the infarction area of nude rat, could give hospitality to stem cells and deliver them to improve cardiac functionality. About 2% of cells survived and showed good mobility into the collagen patch; improved functionality was detected, however no cell engraftment was seen after four week from cell injection both locally and systemically.

Summarizing, the two different *in vivo* models aimed at define both the patch strength after cell injection in model I and the patch trophic effect in model II. Therefore to address these points it is mandatory to analyze bcl-2 expression on treated and un-treated samples with and without patch to verify whether the tissue engineering approach with this specific polimer, could have enhanced the paracrine effect of human CD133+ or changes of the polimer have to be pursue to ameliorate the cell survival.

Appendix: Why using a cryoinjury model?

Cryoinjury of the heart might be a useful model to provide therapeutic clues for interventions aimed at improving wound healing after infarction, like the identification of cytokines and growth factors involved in the accelerated wound healing.

In particular, several rat and murine models of ANI have been established over the past decades. The ligation model, in which the left anterior descending (LAD) coronary artery is permanently ligated, is the most extensively used mouse model of experimental ANI

However, the cryoinjury model, in which a frozen probe is applied directly to the left ventricular (LV epicardial surface to inflict tissue damage), has also been used in many ANI studies. In particular, it has been proposed and used as an adequate ANI model to test the effects of cardiac cell transplantation due the simplicity and reproducibility of the application of the injury (*Bittira B et al, 2002; Yau TM et al, 2001*).

In regards to our study, the decision for the use of a specific murine ANI model should be considered and, more than technical aspects, the pathophysiology of the experimental model should be further investigate for the interpretation of the study outcome. Since the ability for regeneration of myocardial tissue is limited, adequate wound healing is a critical step in the early pathophysiology after ANI. Myocardial wound healing starts by infiltration of inflammatory cells that promote wound healing by removal of necrotic cell

debris as well as by production of growth factors and cytokines that modulate neovascularization (*Francogiovanni NG et al., 2002*). Inflammatory cell infiltration is followed by scar formation by which prevents structural fragility.

Furthermore, during these early wound healing responses tightly controlled architectural changes of the heart take place, which are a major determinant of the functional outcome after ANI (*Francogiovanni NG et al., 2002; Sun Y et al, 2005*).

It is known that between ligation and cryoinjury induced ANI there are some differences,

1) that the inflammatory response is increased after cryoinjury,

2) that wound healing is accelerated after cryoinjury,

3) that the electrophysiological response differs between both models, and

4) that cryoinjury infarcted mice have only modest LV remodeling and a noticeable better functional outcome than ligation infarcted mice.

In order to create transmural injury we attempted several applications of the cryoprobe precooled in liquid nitrogen.

Although highly representative of the ischemic cell death as occurs in humans, the ligation model is inherently associated with infarcts of variable size, requiring large group numbers of mice in studies that evaluate anti-remodeling therapies. Even more importantly, LAD ligation in the mouse heart typically leads to apical infarcts with large aneurysm formation causing a particular ventricular geometry. Ligation-induced infarcts in the mouse heart appear therefore less representative for infarcts encountered in clinical practice, where acute LAD occlusion followed by aggressive reperfusion therapy often leads to moderately sized infarcts of the anterior free wall.

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