CARDIAC STEM CELL POPULATIONS FOR REPAIR OF CARDIAC TISSUE

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Abstract

Method for the isolation, expansion and preservation of cardiac stem cells from human or animal tissue biopsy samples to be employed in cell transplantation and functional repair of the myocardium or other organs. Cells may also be used in gene therapy for treating cardiomyopathies, for treating ischemic heart diseases and for setting in vitro models to study drugs.
Human cardiospheres in culture
FIGS 1B₁ - 1B₂

B₁

**Human cardiospheres**
(CGM-3.5% FCS)

[Graph showing the growth of human cardiospheres over time.]

B₂

**Mouse cardiospheres**
(CGM-3.5% FCS)

[Graph showing the growth of mouse cardiospheres over time.]
FIG. 1B

Mouse cardiospheres (serum-free CGM)

spheres (number)

0 10 20 30 40 50

0 5 10 15 20 25 30

time (days)

Δ Adult 1
◆ Adult 2
□ Newborn
△ Fetus 1
▲ Fetus 2
▲ Fetus 3
▼ Embryo 1
▼ Embryo 2
FIGS. 2C₁ - 2C₃
FIGS. 2D₁ - 2D₂

D₁

MHC I/TOUCHST

D₂

cTnI/TOUCHST

10 μm
FIGS. 2E₁ - 2E₃ and FIGS. 2F₁ - 2F₃
FIGS. 2G₃ - 2G₆
FIG. 2H
FIGS. 2J - 2J₃

CSs-derived cells (FACS analysis)

Data are presented as mean ± SD (n=3).

*Indicates a statistically significant difference from T₀.

Fluorescence intensity

Counts

Counts

day 0

day 0

CD 34

CD 31

sca-1

cKit

PE-streptavidin

Fluorescence intensity
FIGS. 3A₁ - 3A₅

MLC3F-nlacZ/B5-eGFP inoculus

a₁

GFP

200 µm

a₂

GFP

40 µm

a₃

SMA

a₄

GFP

100 µm

a₅

SMA/moebial
Myocardial repair after human CSs injection

FIGS. 3C_1-3C_8
Table 1
Myocardial Repair (Echocardiography)

<table>
<thead>
<tr>
<th></th>
<th>CAL + CSs (N=4)</th>
<th>CAL (N=5)</th>
<th>Sham (N=3)</th>
</tr>
</thead>
<tbody>
<tr>
<td>LVIDd, mm</td>
<td>4.10 (SD=0.85, SEM=0.42)</td>
<td>3.57 (SD=0.33, SEM=0.13)</td>
<td>2.13 (SD=0.06)</td>
</tr>
<tr>
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<tr>
<td>AWThd, mm</td>
<td>0.80 (SD=0.29, SEM=0.15)</td>
<td>0.60 (SD=0.20, SEM=0.08)</td>
<td>1.03 (SD=0.01)</td>
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<tr>
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<tr>
<td>FS, %</td>
<td>36.85 (SD=16.43, SEM=8.21)</td>
<td>17.87* (SD=5.95, SEM=2.43)</td>
<td>59.13* (SD=4.56, SEM=2.63)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Regional wall motion abnormality, %</td>
<td>33.82 (SD=10.05, SEM=6.53)</td>
<td>30.88 (SD=14.71, SEM=7.35)</td>
<td></td>
</tr>
</tbody>
</table>

*P<0.05 vs CAL+CSs.
†P<0.05 vs CAL.

Effect of human CSs orthotopic transplantation on echocardiographic index of myocardial performance. Data are presented as mean±SD.

LVIDd indicates left ventricular internal dimension at end diastole; AWThd, anterior wall thickness; FS, fractional shortening.
FIGS. 4A-4B

A

- pCS
- aCS
- H

- ANF (300 bp)
- Nkx2.5 (350 bp)
- Ve-cadherin (330 bp)
- GAPDH (282 bp)

B

- mCS
- H

- α-MHC (300 bp)
- TnC (490 bp)
- Cardiac α-actin (494 bp)
- GAPDH (430 bp)
CARDIAC STEM CELL POPULATIONS FOR REPAIR OF CARDIAC TISSUE

CROSS-REFERENCE TO RELATED APPLICATIONS


BACKGROUND OF THE INVENTION

[0002] 1. Field of the Invention

[0003] The invention concerns a method for the isolation and expansion of cardiac stem cells derived from postnatal cardiac tissue biopsy.

[0004] The invention deals with a method for the isolation, expansion and preservation of cardiac stem cells from human or animal tissue biopsy samples to be employed in cell transplantation and functional repair of the myocardium or other organs.

[0005] The cells may also be used in gene therapy, for treating genetic cardiomyopathies by expressing the healthy gene in cells from biopsies of subjects with genetic defects, propagating the cells in vitro and then transplanting them in the patient; for treating ischemic heart diseases by inducing the release of angiogenic growth factors by the transplanted cells; and for the setting of an in vitro models to study drugs.

[0006] 2. Prior Art

[0007] Stem cells (SC) are able to replicate and to differentiate in response to appropriate signals, thus enabling the formation or regeneration of specialized tissues.

[0008] It was thought that cardiomyocytes were terminally differentiated cells; however, emerging evidence has shown the modest potential of these cells to proliferate in animal models and in heart transplant patients (1-4).

[0009] The limited ability of adult cardiomyocytes to undergo mitosis and to regenerate the myocardium after injury leads to a permanent deficiency in the number of functioning cells, with the development and progression of cardiac insufficiency. In the end stage of the disease, the alternative treatment to transplantation is the implantation of SC in the injured myocardium (cardiomyoplasty). This method has produced promising results in animal models and has been experimented also in humans. However, the problem of having a source and an availability of SC remains (5-7).

[0010] While embryonic SC (undifferentiated cells from the embryo that can produce a wide range of specialized cells and that can be derived from the cell mass inside blastocysts which, in humans, form 4-5 days after fertilization of the ovum) have a marked capability to proliferate and differentiate, their potential immunogenicity, arrhythmogenicity, and ethical issues in particular, have limited their use. Moreover, embryonic SC are pluripotent, consequently their use carries a potential risk of generating teratomas (as occurs in animal models). Hence, before these cells can be used, they need to be differentiated in vitro in cardiomyocytes.

[0011] There exist various types of cardiomyocytes (ventricular, atrial, sinus node, Purkinje, with pacemaker functions, etc.). Embryonic SC have the potential capability to generate these cardiomyocyte phenotypes in vitro but the yield is insufficient. Furthermore, the in vivo proliferative capability of cardiomyocytes derived from embryonic SC appears to be limited by the growth of multinucleate cells.

[0012] An alternative is to use adult SC (undifferentiated cells found in differentiated tissue that are able to proliferate, reproduce and differentiate into the specialized cell types of the tissues whence they were isolated) preferably obtained from the same patient, which would afford the advantage of allowing autologous transplantation without the need for immunosuppressive therapy. For this purpose, skeletal myoblasts (satellite cells) have been employed; however, they differentiate into skeletal myocytes with morphologic and functional properties differing from those of the cardiac muscle. The inability of skeletal myoblasts to transdifferentiate into cardiomyocytes and to couple with them could give rise to arrhythmias or other anomalies.

[0013] SC derived from bone marrow offer an attractive alternative. Mesenchymal SC (MSC) of the bone marrow can differentiate into cardiomyocytes in vitro (treated with DNA-demethylating agents) and in vivo where, however, in the presence of fibrosis, they mostly generate fibroblast-like cells. Hematopoietic SC (HSC) of the bone marrow (so-called side population cells [SP cells]) are pluripotent in that they can generate vascular epithelium, smooth muscle cells and cardiomyocytes. But the functional and electrophysiologic properties of HSC- and MSC-derived cardiomyocytes are not well characterized, and the use of undifferentiated cells instead of cardiomyocytes could give rise to in vivo differentiation into fibroblasts rather than muscle cells or to the development of tumors.

[0014] Although human cardiomyocytes have been conventionally considered terminally differentiated cells (i.e. unable to re-enter the cell cycle and to divide), indirect evidence accumulating over the past two years has suggested the existence of adult SC in the heart. These cells are ideal candidates for cardioplasty in that they need no reprogramming, give rise only to cells present in the heart, i.e. cardiomyocytes and vessels (endothelial cells and smooth muscles) and may, because this is their physiologic function, survive in transplant patients, integrate into the surrounding tissues and carry out their functions for longer periods without causing any damage. Patent applications WO 03/008535 and WO 03/009550 concern methods to derive cardiomyocytes from embryonic SC. Patent applications WO 02/13740 and WO 02/09650 deal with the use of adult SC (particularly hematopoietic and/or cardiac cells, without indicating a method to isolate them, also in combination) to repair cardiac injury or to treating cardiovascular diseases in general.

[0015] Patent application WO 99/49015 deals with the isolation of pluripotent cardiac SC of the adult p53−/− mouse. In particular, the description concerns the heart-derived pluripotent SC that differentiate and proliferate to produce a variety of cell types, including cardiocytes, fibroblasts, smooth muscle cells, skeletal muscle cells, keratinocytes, osteoblasts and chondrocytes. The cells may be employed in methods to treat patients with cardiac tissue necrosis. The SC proliferate and differentiate to produce cardiocytes that replace the necrotic tissue.

[0016] However, the method differs from that of the present invention, which was based on the assumption that the cardiac muscle cells, the striate muscles and the smooth muscle cells derived from a common precursor, the myoblast. Furthermore, there is no in vivo evidence from cardiomypathic animals that supports the applicability of the method. Lastly,
the methods differ substantially. In the method described in patent WO 99/49015, adult p53-/- mouse hearts are fragmented, dissociated with DNase and collagenase. After centrifugation, the sediment myocytes are isolated on a discontinuous gradient (Percoll) and plated on a medium containing 5% FBS and then on a medium containing 15% FBS 20 days later. Between days 20 and 26, small (~5 μm) round, nonadherent, slow-growth, phase-bright cells with a high nucleus-to-cytoplasm ratio form in the suspension. These cells continue to live in the suspension for about 1.5 months in the presence of 10% horse serum. Then the cells remain suspended also without the addition of horse serum. The nonadherent SC do not form colonies in methylcellulose and proliferate in the presence of serum, SCF, aFGF, bFGF, and eFGF. The absence of horse serum, the nonadherent cells differentiate into differently appearing adherent cells the authors have identified by mainly morphologic criteria as cardiocytes, chondrocytes, fibroblasts, smooth muscle cells, skeletal muscle myoblasts, pericytes, and other cells the authors have called adherent SC. About one fourth to one fifth of these cells is positive to alkaline phosphatase (osteoblasts and endothelial cells); all cells are negative to acetylated LDL (absence of endothelial cells) and to myosin heavy chain (MF20). The cells undergo mitosis when stimulated by bFGF, aFGF and cFGF. In the absence of serum, they differentiate into cells resembling a fried egg (myocytes). After treatment with ascorbic acid/a-GP, they differentiate into chondrocyte-like cells.

Adherent cells cloned by limiting dilution give rise to mesenchymal cells, including osteoblasts, chondrocytes, adipocytes and myocytes, although they cannot be clearly identified due to often inappropriate morphologic criteria and markers. All the cells tested negative to acetylated LDL (absence of endothelial cells). None of the 11 isolated clones could be induced to differentiate toward a single mesenchymal lineage.

The isolation of the cardiac-derived SC of neonate mice (1-4 days) is also described, wherein the passage of myocytes on human fibronectin is added to eliminate the fibroblasts. However, no data are given about the characteristics of the isolated SC. Furthermore, the cells isolated with the previous method do not give rise to the formation of an essential component of the heart tissues, i.e. vessels and endothelium.

DESCRIPTION OF THE INVENTION

The method of the present invention employs heart biopsy tissue as starting material, hence an elective material that cannot be used in the method described in patent application WO 99/49015, since the material was insufficient. After fragmenting the biopsy specimen and possibly using dissociating agents (e.g. trypsin, EDTA and collagenase), the fragments are plated and added to a medium containing 10% FBS, 10-30 days later, fibroblast-like adherent cells grow from the explants over which small round, phase-bright cells migrate that tend to cluster but are either not or only weakly adherent. The cells are isolated by washing and mild dissociation (e.g. EDTA, trypsin-EDTA for 2-3 min). The cells are then plated on polylysine-treated cellular substrates in an appropriate medium until that used in the previous technique, in that it is horse-serum-free and contains other growth factors; after 2-3 days cell aggregates (cardiospheres) arise that tend to grow as floating formations. The authors have found that the cardiac-forming cells are postnatal SC that can be advantageously employed for reimplantation in the myocardium.

These cells are able to multiply, while maintaining their origin characteristics for a period (at least 60 days) that is long enough to markedly enrich the cell population. Mechanical disaggregation of the cardiospheres (CS) by repeated pipetting and changing the medium every 3 days increases the number of CS (about 100-fold every 10 days) for at least the first 20 days. Given the number of SC that can be derived from a biopsy and their ability to multiply in vitro, it is thought that they can be used to replace a greater amount of tissue than that removed.

Certain cells in the present stem-cell markers (c-kit, sca-1, CD34) that are able to differentiate toward the main components of the myocardium (cardiomyocytes and vessels). As evaluated by immunohistochemistry and/or RTPCR, certain cells spontaneously express, particularly at the border of the CS, markers for cardiomyocyte (troponin I, ANP, myosin heavy chain) and for endothelial cells (von Willebrand factor and Ve-cadherin). The human CS, in a co-culture with rat myocytes, beat spontaneously. When inoculated subcutaneously in SCID mice, the murine CS give rise to growths containing cardiac muscle tissue and vessels within several days.

The authors have thus demonstrated that the SC can be derived in a reproducible manner from biopsy tissue of the atrium, ventricle and auricula of human subjects aged from 1 month to 70 years. The CS pertaining to the invention can be cryopreserved, and they maintain their functional characteristics after thawing.

Adult cardiac SC with similar characteristics can also be isolated from the mouse. In particular, to better understand cell differentiation in CS, of several breeds of transgenic mice were studied; the findings confirmed the results obtained with human cells.

Lastly, the authors have shown in an animal model that human CS can be used for cardioplasty. When inoculated in the infarcted area (transthoracic cauterization or LAD ligation) of a SCID mouse, the cells give rise to cardiac tissue that presents good integration with the host tissue, as observed by morphology and immunohistochemistry studies.

Hence, the isolation and expansion of CS by the method of the invention is novel and advantageous compared with that described in the previous technique in terms of the origin of the sample, the methods of isolation and expansion and the morphologic and functional characteristics of the derived cells.

DETAILED DESCRIPTION OF THE INVENTION

The method comprises the following steps: biopsy sample obtained under sterile conditions and transported to the laboratory; preparation of fragments sized large enough to allow diffusion of nutrients present in the culture medium; distribution of fragments on culture plates and incubation under conditions appropriate for cell survival and growth; sampling of culture medium and cells and transfer to other culture plates under conditions adequate for cell expansion.

An object of the invention is a method to obtain stem cells able to repair damaged myocardial tissue, comprising the following steps:

a) take a biopsy specimen of cardiac tissue and keep it in an appropriate culture medium;
b) treat the specimen under appropriate conditions with mild mechanical and/or chemical and/or enzymatic techniques to obtain tissue fragments sized large enough to allow the diffusion of nutrients present in the medium;

c) leave the tissue fragments to adhere to appropriate solid supports and maintain them in a medium containing convenient serum and/or growth factors;

d) allow the cells to grow, changing the medium partially or completely, until multicellular structures form that are either weakly adherent or do not adhere to the support;

e) separate said multicellular structures from the rest of the culture;

f) treat said multicellular structures by mild dissociation until most of the small phase-bright spherical cells detach but maintain their morphologic and functional characteristics;

g) plate the cells on culture substrates treated with polylysine or other agents promoting the adhesion of the culture to the support in a medium containing at least the minimal essential constituents for the growth of mammalian cells;

h) possibly repeat steps d) to g) at least once;

i) select the cells that aggregate in phase-bright spheroid formations (cardiospheres);

j) electrically promote the formation of new cardiospheres by mild dissociation thereof and new formation;

k) eventually cryopreserve the cardiospheres for use after thawing.

l) Preferably stem cells are derived from non-embryonic cardiac tissue biopsies.

m) In one embodiment of the invention at least one of the steps follows treatment with oxygen concentrations different from that normally present in the atmosphere in order to modify the biologic characteristics of the cultures.

n) Experts in the field will understand that the CS derived with the procedure of the invention may be capable to generate continuous cell lines following spontaneous transformation or transformation induced by chemical, physical or biologic agents.

o) In another embodiment the cells giving rise to and/or constituting cardiospheres are fused with other cells.

p) In another embodiment the cells giving rise to and/or constituting cardiospheres are used for nuclear transfer to and from other cells.

q) In another embodiment the cells giving rise to and/or constituting cardiospheres are grown in at least one stage on biodegradable and/or biocompatible supports.

r) In another embodiment the cells giving rise to and/or constituting cardiospheres are cultured in bioreactors and/or fermenters.

s) It is another object of the invention the cells giving rise to and/or constituting cardiospheres able to repair myocardial tissue obtainable according to the method of previous claims. Preferably said cells are to be used in gene therapy. Preferably said cells are to be used for nuclear transfers to and from other cells. The CS derived with the method of the invention can be variously used in the repair of myocardial tissue, for nuclear transfers from and to other cells, in gene therapy for cardiopathies of genetic origin.

BRIEF DESCRIPTION OF FIGURES

FIG. 1-CS proliferation. A-1, A-2, A-3, A-4. Phase micrographs of floating CSs (cultured from 24 h to 48 h) derived from a primary culture of a human atrial bioplastic sample. B-1, B-2, B-3. Proliferation curves of human and mouse CSs (human CSs were derived from 8 different subjects (B-1, 2) and from pre- and post-natal mouse hearts (B-3, 4), respectively, in the presence (B-1, 2, 4) and in absence (B-3) of 3.5% serum. Number of spheres refers to the mean number per well from which 90% of the spheres where withdrawn at each time-point for further analysis. Note the different pattern of proliferation between the human and mouse CSs and the rapid rise of the curves, followed by an irreversible decline in the serum-free conditions. C-1, C-2. Fluorescence analysis of a single cell (C-3) obtained from a dissociated GFP-expressing CS, when plated by limiting dilution on mitomycin-treated STO-fibroblast-coated 96-wells plates in CSM, over the course of the generation of the GFP-labeling clone. This clone could be passaged and expanded on poly-D-lysine coated (IC-3, 3) 48-GFP staining of a eGFP/MLC3F clone (obtained as those human) after 48 hours exposure of growth factors-free medium: in these conditions cells in the clone become more flattened with many nuclei showing a blue color, demonstrating that a differentiation process occurred.
chronic congestive cardiomyopathy. The murine tissue came from the hearts of previously characterized homozygous MLCF3-nlacZ mice (8) homozygous troponin-I-lacZ (9) and EGFP/eGFP (10) CD1-crossed mice. The mice show localized nuclear expression (cardiac and skeletal) of the trans gene for B-galactosidase of the myosin light chain promoter, a tissue-specific nuclear expression (exclusively cardiac) of the trans gene for troponin-I and a cytoplasmic expression of the EGFP trans gene of the eukl promoter (the gene in these cell experiments), respectively. BS-EGFP mice (11), which show generalized expression of cytoplasmic GFP, were used as base strains. The crossed MLCF3-nlacZ/EGFP, MLCF3-nlacZ/EGFP-eGFP, Tn-I-lacZ-Z/EGFP-eGFP mice were bred according to experimental protocol. The human cardiac tissue biopsies were preserved in serum-free IMDM (Euroclone) at 0°C and maintained at this condition until arrival in the laboratory (within 2 h).

Processing, Isolation and Cryopreservation of Sphere-Forming Cell

After careful dissection of the macroscopically visible connective tissue, the samples were cut into 1-2 mm³ pieces, washed 3 times with Ca²⁺/Mg²⁺-free phosphate buffered solution (PBS, Invitrogen) and sequentially digested 3 times for 5 min each at 370°C with 0.2% trypsin (Gibco) and 0.1% collagenase IV (Sigma). The obtained cells, the bulk of which are elements of contaminating blood, were discarded and the remaining tissue fragments were washed with complete explant medium (CEM) IMDM supplemented with 10% fetal calf serum (FCS) (HyClone), 100 μg/ml penicillin, 100 U/ml streptomycin (Gibco), 2 mM L-glutamine (Gibco), 0.1 mM 2-mercaptoethanol (Sigma). The tissue pieces were then fixed to Petri dishes (Falcon) by light scraping with a scalpel on a plastic surface. The explants with cultured at 37°C in 5% CO₂ in complete IMDM. The murine cardiac tissues were treated similarly, except for the embryonic hearts, where enzyme digestion prior to explant digestion was omitted and the organs were partially dissociated with a 25 gauge needle. After a period of 1 to 3 weeks (depending on the origin of the sample, i.e. a shorter period for the embryonic tissue and a longer one for the adult tissue), a layer of fibroblast-like cells forms that derive from or surround the explants. The explants are then periodically treated (every 6-10 days, 4 times maximum) to isolate the sphere-forming cells. To remove the phase-bright cells, which migrate from the explants to the outer cell layer, the medium is removed, and the material is collected by washing it twice with Ca²⁺/Mg²⁺-free PBS and once with 0.53 mM EDTA (Verseco, Gibco) for 1-2 min, followed by mild trypsinization with 0.5 g/l, 53 mM Trypsin-EDTA (Gibco) at room temperature for another 2-3 min under visual microscopy control. After the cells are collected, complete medium is added to the explants, whereas the cells obtained by washing and enzymatic treatment are collected by centrifugation (1200 rpm for 7 min) and resuspended in cardiosphere-growing medium (CGM) (35% complete IMDM/65% DMEM-Ham’s F-12 mix with 2% B27 [Gibco], 0.1 mM 2-mercaptoethanol, 10 ng/ml EGF [Preprotec EC, Ltd.], 40 ng/ml bFGF [preprotek EC, Ltd.], 4 mM cardiotrophin-1 (RD), 40 nM thrombin (Sigma) (final concentrations), antibodies and L-Glu as in the complete medium. Depending on the number of cells obtained (from 10⁶ to 4x10⁶ cells/explant), the cells were resuspended by resuspending them and then plating about 2x10⁶ cells/ml on poly-D-lysine (BD) coated multi-well plates. After 12-24 h,
several cells begin to divide and after 48 h, cell groups form that are often surrounded by a thin membrane and that can grow as floating spheres and adherent spheres. The growth medium is partially changed every 2-3 days, and the spheres are mechanically triturated using a pipette or 1 ml needles. For cryopreservation, the spheres (washed in Ca²⁺-Mg²⁺-free PBS and Versene) are resuspended in the freezing medium (complete IMDM/DMEM-Ham-F-12 50: 50, 5% B27, 10% DMSO). To calculate the growth curves, all the spheres are counted during the first week of growth, and then 90% of the spheres are removed at defined times (and used for RT-PCR or immunohistochemical analysis); after adding CGM and mechanically triturating the residual spheres, they are left to proliferate until the next sampling, when they are recounted. BrdU labeling is performed for 12 h on the newly generated spheres and at defined times in the other spheres, as indicated (Roche). For clonal analysis, the human CSs are infected with a third-generation lentiviral vector, pRRL.sin.PPT-PGK.GFP expressing green fluorescent protein (GFP), as described elsewhere (12). After being washed twice, the GFP-labeled CSs are dissociated into single cells by trituration in Ca²⁺/Mg²⁺-free PBS, Versene, and 1x trypsin-EDTA solutions in sequence, resuspended in CDM, and then seeded at a predetermined concentration of 1 cell/well in 96-well plates coated with a feeder layer of mitomycin-C-treated STO fibroblasts (2 µg/mL). For differentiation on a substrate-coated surface, Ca²⁺/Mg²⁺-free PBS-washed, centrifuged and partially dissociated CSs are repeatedly pipetted and then seeded in a small volume of CEM (200-300 µl) on type I collagen (Sigma) or Matrigel-(Falcon) coated dishes and cultured for 3-6 days.

In Vivo Analysis

For heterotopic transplantation, about 60 pooled CS obtained from pre- and postnatal EGFP/MLC3F-nLacZ or EGFP/Tnl-nLacZ or MLC3F/nLacZ, Tnl-nLacZ mice were washed twice in PBS and suspended in 100 µl of Matrigel (BD) and subcutaneously injected into the dorsal region of anesthetized (ketamine, 35 mg/kg i.m.) adult NOD-SCID mice. Transplanted-cardiosphere survival and function were monitored by direct palpation of beating through the skin. After about 3 weeks, the mice were sacrificed and the isolated inoculum was embedded in OCT for immunohistochemical analysis. After thawing, 10-day cultures of cryopreserved human CS derived from ventricular and atrial cardiac explants from adult subjects were used for orthotopic transplantation. About 20 washed and partially dissociated CS were suspended in 3 µl PBS and injected in the infarcted myocardial area using a 27 gauge needle and a Hamilton syringe. Myocardial infarction was induced as described elsewhere (13) with slight modifications. Briefly, the recipient NOD-SCID mice (anesthetized with ketamine [35 mg/kg]+xylazine [5 mg/kg] i.p.) underwent transthoracic cauterization (Surgitron 140 v) with a modified electrocautery probe inserted through the internal intercostal muscle in the fourth intercostal space on the anterior surface of the heart. Electrocauterization (ca. 40 W) was applied twice for 1 sec in the cutting mode before the CS were injected (the same volume of PBS was injected in the control mice). In some mice myocardial infarction has been also induced by LAD ligation. After about 3 weeks, the mice were sacrificed and the isolated heart was embedded in OCT after extensive washing in PBS and fixing with paraformaldehyde (4%) in PBS pH 7.4.

Immunocytochemistry

[0054] Immunocytochemistry on tissue sections and on cell cultures was performed as described elsewhere (14) using the following antibodies: monoclonal anti-human-cTnl, anti-human-cardiac-MHC, anti-human nucleus and polyclonal (PAb) anti-human ANP (Chemicon); mAb anti-CD-31, CD-34 (BD Biosciences), mAb anti-human Cripto-1 (RD), monoclonal anti-Ve-cadherin, anti-sea-1, mAb anti-mouse-cKit (Pharmigen), mAb anti-human-c-Kit (DAKO); pAb anti-human-von-Willebrand-factor and mAb anti-human-KDR (Sigma); mAb MI20 and pAb anti-mouse/human MHC (14), anti-desmine and anti-Smooth-Muscle-Actin (Sigma), mAb anti-humanimouse-cTnl (15), donated by S. Schiaffino (Dept. of Pathology, Univ. of Padua), pAb anti-mouse-β-1 (Santa Cruz, USA). β-galactosidase activity was detected by light microscopy, as described elsewhere (14).

Reverse-PCR Transcription Analysis

[0055] Reverse-PCR transcription analysis was performed as described elsewhere (16). The oligonucleotides for amplifying the genes of the CS derived from the pediatric (PCS), adult subjects (aCS) and heart fragments (H) were the following:

```
bnKu3.5 (150 bp)
forw 5′-CTCCCCACTAGCCTGAGT-3′
and
rev 5′-GAGCTCACTCCGCCTGCAA-3′.

bHNF (350 bp)
forw 5′-ATTGCAATTCGCTGAGATG-3′
and
rev 5′-ATAATTCTGACCTGGGAGAC-3′.

bhe-Cad (330 bp)
forw 5′-TCTCTGTCCTGCTGACAA-3′
and
rev 5′-AAGAGAGGCTCTAGTATG-3′.

hGAPDH
forw 5′-GAAGAGGAGACCCACGACTAC-3′
and
rev 5′-CTCAACACCCACACTCTTATG-3′.
```

[0056] The oligonucleotides for amplifying the genes of the murine CS and the heart fragments (H) were the following:

```
mHSC (302 bp)
forw 5′-GAAGAGGAGGAGGTGACGCTCAAGGA-3′
and
rev 5′-TCTCTGAGAGGATTATGCTTTGAG-3′.
m cardiac actin (494 bp)
forw 5′-TGATCTGGCCCTGGAATGAG-3′
and
rev 5′-AAGAGGAGGACCACTATCAAGGAC-3′.
```
Results

Isolation and Expansion of CS

Sphere-generating cells were obtained by mild enzymatic digestion of explanted human atrial or ventricular biopsies and fetal, embryonic and postnatal mouse hearts. Soon after the generation of a layer of fibroblast-like cells from well adherent explants, small, round phase-bright cells began to migrate over this coat. These cells could be harvested periodically by treatment with EDTA and mild trypsinization, and allowed to grow on poly-D-lysine-coated culture surfaces, in a low-serum (5.5% FCS) medium supplemented with a serum substitute (B27), growth factors (EGF and bFGF), cardiotrophin-1 (CT-1) (17) and thrombin (18), which, in the first week of culture, led to a seven-fold increase in the number of spheres with respect to that obtained using the medium supplemented with the others factors either alone or in combination. Time course observations of cells derived from both human and murine explants showed that, early after their seeding (30 min), some of these cells began to divide while still in suspension; most cells became loosely adherent, others remained in suspension and some contaminating fibroblast-like cells attached firmly to the poly-D-lysine coated cell. Cellular divisions were evident also from the loosely adherent cell population and produced clusters of small, round phase-bright cells [that we termed cardiospheres (CSs)] after 10-12 hours (FIG. 1a). Within 24-36 hours from their appearance, CSs increased in size and some of them detached from the culture surface; after 48-72 hours most CSs were between 20 and 150 um in size and, when not subjected to mechanical dissociation, the largest contained dark zones within their inner mass (FIG. 1a).

Murine CSs started a spontaneous rhythmic contractile activity soon after their generation and maintained this function during their life span, while human CSs did so only when co-cultured with rat cardiomyocytes. To be sure that contraction was a new trait acquired by the cs cells, gfp-labeled human CSs (partially or totally dissociated) were co-cultured with cardiomyocytes pre-stained or not with dil. Contracting gfp-labeled cells were observed after 48 hours of co-culture; furthermore, from this time onwards, a red color stained also the green fluorescent cells, suggesting that a connection is created between the human CSs and the rat cardiac cells. In fact, labeling of human cs/rat-cardiomyocyte co-cultures (in which only human cells were pre-labeled with gfp by lentiviral infection) with cx-43, the major ventricular gap junction protein, demonstrated the typical punctuate fluorescence pattern along the cytoplasmic membrane (FIG. 2f), indicating that a functional connection is created between the two cellular populations.

CSs were found to be composed of clonally derived cells and did not simply represent cellular aggregates. In fact, when human CSs [expressing the green fluorescent protein (GFP) after infection with lentiviral vectors expressing the reporter gene] or murine CSs (derived from egfp/impl.C3F or egfp/Ctnl-mice) were dissociated and plated as single cells on mitomycin-treated STO-fibroblast-coated 96-wells or at clonal dilution on 10 cm Petri dishes, fluorescent spheres that could be sub-cloned on poly-D-lysine-coated surfaces (FIG. 1c) were generated with a 1 to 10% efficiency. These sub-clonally derived CSs show the same functional and phenotypic behavior in culture: after 3 days from their appearance, some murine clones started to, and after 48 hours of culture with CEM, the majority (6/7) of these showed expression of the lac-Z trans gene within nuclei after specific histochemical staining (FIG. 1d). Equally, human clones, derived from a single GFP-labeled cell, start a synchronous beating and express Ctnl after 48 hours of co-culture with rat cardiomyocytes.

Furthermore, when BrdU was added to the culture medium, virtually all cells in the small, and those of the inner part of the largest CSs, were labeled (FIG. 2a), indicating that these cells were newly generated.

Human CS-generating cells were capable of self-renewal. With periodical dissociation, together with partial substitution of the growth medium every 2-3 days, a log-phase expansion of spheres was obtained (FIG. 1b). Growth was slower for mouse CSs (owing, probably, to the more differentiated features assumed in culture such as beating) and, serum-dependent (FIG. 1b) as for the human ones.

As shown in FIG. 2a, confocal immunofluorescence analysis of BrdU labeled human CSS with anti-BrdU (green) and cardiac-troponin i (ctn) or atrial natriuretic peptide (amp) (red), revealed BrdU-positive cells particularly in the inner part of the spheres, while ctn- or amp-positive cells were mainly localized in the external layers. Furthermore, several CS-cells expressed cardiac differentiation markers (ctn, amp) while still dividing, as indicated by BrdU incorporation (FIG. 2a), suggesting that early cardiac differentiation already occurred during the log-phase growth; generally, within 2-3 weeks, some spheres became adherent, showing a more flattened morphology. Some small cells eventually migrated out from these “sun-like” spheres in the form of adherent (differentiated) or small, round cells that could generate new spheres. After thawing from cryopreservation, CSs proliferated again, maintaining their propensity to beat.

Phenotypic analysis of newly developing human and mouse CSs revealed expression of endothelial (Krhuman/ilk-1 (mouse), CD-31) and stem-cell (CD-34, ckit, sca-1) markers. As shown in FIG. 2b, CSs at the 2-10 cell stage, strongly reacted with antibodies against these antigens. In larger spheres, the expression pattern of some of these markers (particularly cKit) was similar to the BrdU labeling (positive staining in the center and in some peripheral zones generating satellite spheres).

A time course (0 and 6 days) of the quantitative characterization of CSs cells with these stem and endothelial markers was performed by FACS analysis (FIG. 21). As shown, at the beginning of their formation (T 0) the phenotype of these cells seems to reflect the epit-fluorescent microscopy analysis with about 10% of positive staining for all four phenotypes. However, at 6 days (T6) cKit appears as the only conserved marker, suggesting that the cKit+ cells could be the main ones contributing to the maintenance of proliferation,
while the initial positivity to the others may reflect an early activation state, as has been indicated for CD-34 in several system (19). Fluorescence microscopy analysis, performed on cryo-sectioned human CSs revealed expression of cardiac differentiation markers (cTnI, MHC) and also of endothelial markers (von Willebrand factor (vWF)) (FIG. 2c). When totally or partially dissociated into single-cells and cultured on collagen-coated dishes, in the same medium as the explants, mouse and humans CS-derived cells assumed a typical cardiomyocyte morphology, phenotype (FIG. d-d1, h) and function documented (in the mouse only) by spontaneous contraction.

As aforementioned, human CSs did not beat spontaneously; however, these began to beat within 24 h when co-cultured with postnatal rat cardiomyocytes, losing, after this time, their spherical shape and assuming a “sun-like” appearance. Markers of cardiac differentiation were co-expressed within GFP in human labeled CSs (FIG. 2e).

To follow the differentiation process of CSs during the pre- and post-natal stage, ML3CF3-rlacZ and cTnI-rlacZ mice were utilized (8,9). These mice express a form of lacZ transgene that localizes within the nucleus under the skeletal and cardiac muscle myosin light chain or cardiac troponin I promoter, respectively. CSs obtained from embryonic day 9-12, fetal day 17-18, neonatal and adult mice, showed spontaneous expression of the reporter gene in a variable percentage (10-60%) of spheres in the different culture conditions employed (FIG. 2e); moreover, as for the human ones, CSs generating cells from mouse expressed stern (CD-34, sar-L, cKit) and endothelial cell markers (flk-1 CD-31).

On this basis, we utilized transgenic mice expressing the green fluorescent protein (GFP) under the control of the c-kit promoter (10), in order to further clarify the cellular origin of these spheres and to follow the pattern of their growth process. As shown in FIG. 2i, GFP-positive cells were present from the beginning of the formation of the CSs and, albeit with reduced fluorescence intensity, also later, within the mass of the cells of the CSs and in cells migrating from old “sun-like” adherent CSs. Moreover, as suggested by the growth pattern of human CSs, when satellite secondary CSs appeared to detach from the primary ones, GFP-positive cells localized on the margins of the latter and in the inner part of the former.

We studied this process in double-heterozygous mice obtained from GFP-cKitML3CF3-rlacZ or GFP-cKit/cTnI-rlacZ crossings: as shown in FIG. 2i, beta-Gal-positivity did not co-localize with GFP in cells present within the growing areas.

In conclusion, CSs appear to be a mixture of cardiac stem cells, differentiating progenitors and even spontaneously differentiated cardiomyocytes. Vascular cells were also present, depending on the sphere’s size and time in culture. It is possible that, as for neurospheres (20), differentiating differentiated cells stop dividing and/or die while stem cells continue to proliferate in an apparent asymmetric way, giving rise to many secondary spheres and to exponential growth in vitro. Mechanical dissociation favors this process. Death, differentiation and responsiveness to growth factors of the different cells within the CS, could depend on its three-dimensional architecture and on localization within the CS (21-22). The spontaneous formation of spheres is a known prerogative of neural stem cells, some tumor cell lines (LM) (22), endothelial cells (23) and fetal chicken cardiomyocytes (24). All these models (ours included), that mimic the true three-dimensional architecture of tissues, consist of spheroids of aggregated cells which develop a two-compartment system composed of a surface layer of differentiated cells and a core of unorganized cells that first proliferate and thereafter disappear over time (perhaps through apoptotic cell death). As well documented in fetal chick cardiomyocytes and endothelial cell spheroid culture, three-dimensional structure affects the sensitivity of cells to survival and growth factors (22,23). In particular, central spheroid cells do not differentiate and are dependent on survival factors to prevent apoptosis, while the cells of the surface layer seem to differentiate beyond the degree that can be obtained in two dimensional culture and become independent of the activity of survival factors. Furthermore, cell-cell contact and membrane-associated factors are known to be important for the division of neural precursor cells (25), in accordance with the notion that stem cells will only retain their pluripotency within an appropriate environment, as suggested by the “niche” hypothesis (26).

To investigate the survival and morpho-functional potential of the CSs in vivo, two sets of experiments were performed: in the first, CSs were injected in the dorsal subcutaneous region of SCID mice; in the second, they were injected into the hearts of SCID-beige mice, acutely after myocardial infarction. The intention of ectopic transplantation experiments was to study the pattern and the behavior of growth of CSs in a neutral milieu (i.e. one without specific cardiac induction), in order to verify their unique potential of generation of the main cardiac cell types and to exclude the possibility of neoplastic transformation. For these experiments about 60 pooled spheres/inoculum/mouse from pre- and post-natal ML3CF3-rlacZ/B5-eGFP Tnl-rlacZ/B5-eGFP mice or ML3CF3-rlacZ/CD-1 and cTnI-rlacZ/CD-1 mice, were employed. During the first 10 days, beating was appreciable through the skin over the injection site, distant from large blood vessels. On day 17, animals were sacrificed and the inoculum recognized as a translucent formation, grain-like in size, wrapped in ramified vessel-like structures. Observation of unfixed cryosections by fluorescence microscopy revealed the presence of open spheres from which cells appeared to have migrated; clusters of “black holes”, particularly in the periphery of the structure, were evident. The tissue contained tubular formations, surrounded by nuclei (Hoechst-positive), identified as cardiac sarcomeres because they were positive for cTnI and sarcomeric myosin (FIG. 3a). Alpha-SMA-positive structures (known to be transiently expressed during cardiomyogenesis (27), were present in the remainder of the spheres and associated with the vasculature (the clusters of “black-holes”); this exhibited well-differentiated structures with a thin endothelium expressing Ve-cadherin (FIG. 3a) and a relative large lumen containing erythrocytes, indicating the establishment of successful perfusion by the host. Light microscopic observation of the inoculum, after X-Gal staining, showed strong nuclear expression of striated muscle-specific lacZ in the remainder of the spheres and in some cells close to them. No multi-differentiated structures suggesting the presence of tumor formation were observed.

To test the acquisition of functional competence and the cardiac regenerative potential of the CSs when challenged into an infarcted myocardium, orthotopic transplantation experiments with human CSs were performed. To do this, thawed (cryo-preserved) adult human CSs, coming from three atrial (one male and two female) and one ventricular
(one female) biopsies were injected into the viable myocardium bordering a freshly produced infarct. Each mouse received CSs from a single passage of an explant (derived from a single subject). Four control infected animals were injected with an equal volume of PBS. After eighteen days from the intervention, the animals were sacrificed and infarct size was determined. Infarct size was 34.9±7.1 (36.6) and 31.9±6.9 (3.5) in the CS-treated group and PBS-injected group, respectively (p=n.s.). However, echocardiography showed better preservation of the infarcted anterior wall thickness (0.80±0.29 (0.15) versus 0.60±0.20 (0.08) p=n.s.) and particularly of FS% (36.85±16.43 (8.21) versus 17.87±5.95 (2.43) p<0.05) in the CS-treated group compared to the PBS-injected group (FIG. 3-table 1).

[0072] At the time of evaluation, bands of regenerating myocardium were present (with different degrees of organization and thickness) throughout most of the infarcted areas, as evaluated with hematoxilin-eosin histochemistry and MHC immunofluorescence (FIG. 3c). In the regenerating myocardium, cells expressing lamin A/C (a specific human nuclear marker) co-localize also with cardiomyocytes stained positive for MHC, newly generated capillaries stained for smooth α-actin and PECAM (FIG. 3c), and with connexin-43 expressing cells (which, as in the co-culture experiments, defines a connection between the human cells and the regenerating myocardium).

[0073] Thus CSs can be considered as clones of adult stem cells, maintaining their functional properties in vitro and in vivo also after cryo-preservation.

[0074] While this manuscript was in preparation, two papers have been published concerning the isolation of cardiac stem or progenitor cells from adult mammalian heart (28, 29). Isolation of these cells was based exclusively on the expression of a stem cell-related surface antigen: c-kit in the first paper and Sca-1 in the second one. In the first study (28) freshly isolated c-kit<sup>+</sup>Lin<sup>-</sup> cells from rat heart were found to be self-renewing, clonogenic and multipotent, exhibiting biochemical differentiation into the myogenic, smooth muscle cell, or endothelial cell lineage but, differently from cells grown under the conditions described here, failed to contract spontaneously. When injected into an ischemic heart these cells regenerated functional myocardium. In the second study (29), Sca-1<sup>+</sup> c-kit<sup>+</sup> Lin<sup>-</sup> cells from mice heart were induced in vitro to differentiate toward the cardiac myogenic lineage in response to 5′-azacytidine. When given intravenously after ischemia/reperfusion, these cells homed to injured myocardium and differentiated into cardiomyocytes with and without fusion to host cells. Our data obtained on GFP-cKit transgenic mice also suggest that the adult cardiac stem cell is cKit<sup>+</sup>. It is possible that CSs enucleate a mixed population of cells that, as a niche, could promote the viability of cKit progenitors and contribute to their proliferation. The data obtained in the present paper confirm the existence of an adult cardiac stem cell. More importantly, they demonstrate for the first time that it is possible to isolate cells from very small fragments of human myocardium and expand these cells in vitro many fold (reaching numbers that would be appropriate for in vivo transplantation in patients) without loosing their differentiation potential, thus opening previously unforeseen opportunities for myocardial repair.

**Transgenic Mice**

[0075] To follow the differentiation process of CSs during the pre- and post-natal age, ML3F-nlacZ and Tnl-nlacZ mice were utilized. These mice express a form of the lacZ transgene that localizes within the nucleus under the skeletal and cardiac muscle myosin light chain or cardiac troponin-I promoter, respectively (8, 9). CSs derived from embryonic day 9-12, fetal day 17-18, neonatal and adult mice, showed spontaneous expression of the reporter gene in a variable percentage (10-60%) of spheres at the different culture conditions employed (FIG. 4a). Moreover, the mouse CS start to beat at the start of formation (particularly in the embryo) and continue to beat over the course of their life span. The human CS expressed stem (CD-34, CD-4, cKit<sup>+</sup>) and endothelial cell markers (βK<sub>1</sub>, CD-31).

[0076] In order to further clarify the cellular origin of these spheres and to follow the pattern of their growth process, we utilized transgenic mice expressing the green fluorescent protein (GFP) under the control of the c-kit promoter (10). GFP-positive cells were present from the beginning of the formation of the CSs and, albeit with reduced fluorescence intensity, also later. Moreover, as suggested by the growth pattern of human CSs, when satellite secondary CSs appeared to detach from the primary ones, GFP-positive cells localized on the marginal part of the latter and in the inner part of the former. We studied this process in double-heterozygous mice obtained from EGFPC-Kit<sup>+</sup>/ML3F-nlacZ or Tnl-nlacZ crossings. As shown in FIG. 4b, beta-Gal-positivity did not co-localize with EGFP in cells present within the growing areas.

**Genetic Phenotype**

[0077] The RT-PCR panel created on murine or human CS RNA extracts is shown in FIG. 5. A more typical profile of cardiac progenitors seems to be that of the human samples (in log-growth phase) compared with the murine samples, in which it is easier to have proliferation and differentiation occurring together.

**REFERENCES**


SEQUENCE LISTING

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What is claimed is:

1. A population of cardiospheres for administration to a subject for the repair of damaged cardiac tissue, comprising: cardiospheres isolated from said subject and expanded in culture,
   wherein the cardiospheres are spherical multicellular aggregates about 20 microns to about 150 microns in size,
   wherein the cardiospheres comprise a mixed population of cells that generates only tissue present in the heart, thereby reducing the risk of tumor formation,
   wherein the mixed population of cells generates cardiomyocytes, endothelial cells and smooth muscle and is therefore able to repair both damaged muscle and vessels of the heart,
   wherein the cardiospheres express a stem cell marker,
   wherein the cardiospheres express at least one endothelial marker selected from the group consisting of KDR, flk-1, and CD31

2. The cardiospheres of claim 1, wherein the cardiospheres further express one or more of cardiac troponin, cardiac myosin heavy chain, and von Willebrand factor.

3. The cardiospheres of claim 1, wherein said cardiospheres express said stem cell marker within 12 hours of said cardiospheres being generated.

4. The cardiospheres of claim 3, said stem cell marker is CD34.

5. The cardiospheres of claim 1, wherein said cardiospheres express at least one of said KDR, flk-1 and CD31 within 12 hours of said cardiospheres being generated.

6. The cardiospheres of claim 1, wherein said cardiospheres are clonally derived.

7. The cardiospheres of claim 1, wherein said cardiospheres are dissociated into a plurality of single cells prior to administration.

8. The cardiospheres of claim 7, wherein said cardiospheres or single cells are able to generate continuous cell lines following spontaneous transformation or transformation induced by chemical, physical or biologic agents.

9. The cardiospheres of claim 7, wherein said cardiospheres or the single cells are fused with other cells prior to administration.

10. The cardiospheres of claim 7, wherein said cardiospheres or the single cells are used as a source or as a recipient for nuclear transfer to or from other cells prior to administration.

11. The cardiospheres of claim 1, wherein said cardiospheres are expanded on a solid support.

12. The cardiospheres of claim 11, wherein said solid support comprises a biodegradable support or a biocompatible support.

13. The cardiospheres of claim 11, wherein said solid support comprises a surface treated with one or more of collagen and matrigel.

14. The cardiospheres of claim 1, wherein no reprogramming of said cardiospheres is required to achieve said repair of damaged cardiac tissue.

15. The cardiospheres of claim 1, wherein said expansion allows replacement of a greater amount of cardiac tissue than the amount of cardiac tissue from which the cardiospheres were derived.

16. The cardiospheres of claim 1, wherein said cardiospheres retain their functional properties after cryopreservation.

17. A population of cardiospheres for the repair of damaged cardiac tissue, comprising:
   cardiospheres isolated from non-embryonic cardiac tissue and expanded in culture,
   wherein the cardiospheres are spherical multicellular aggregates,
   wherein the cardiospheres are clonally derived,
   wherein the cardiospheres comprise a mixed population of cells that generates cardiomyocytes, endothelial cells and smooth muscle and is therefore able to repair both damaged muscle and vessels of the heart,
wherein the cardiospheres express at least one endothelial marker selected from the group consisting of MDR, flk-1, and CD31, and wherein the cardiospheres express one or more of cardiac troponin, cardiac myosin heavy chain, and von Willebrand factor.

18. The cardiospheres of claim 17, wherein the cardiospheres are isolated by: fragmenting said non-embryonic cardiac tissue in vitro to obtain tissue fragments; adhering at least some of the tissue fragments to a solid support and culturing the tissue fragments in a first culture media having nutrients, the tissue fragments sized so that the nutrients in the media can diffuse to a substantial portion of the tissue fragments; culturing the tissue fragments until phase-bright cells form; isolating the phase-bright cells;
culturing the phase-bright cells on a treated substrate to obtain one or more cardiospheres.

19. The cardiospheres of claim 17, wherein said non-embryonic cardiac tissue is obtained from a site selected from the group consisting of the ventricle, atrium, and auricle of the heart.

20. The cardiospheres of claim 17, wherein said damaged cardiac tissue is a result of a myocardial infarction and wherein administration of said cardiospheres reduces infarct size.

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