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(54) Title: CUSTOMIZING STEM CELL-DERIVED CARDIOMYOCYTES FOR TRANSPLANTATION

(57) Abstract: The present invention relates to methods of customizing the biological activity (e.g. rhythmic firing rate) of cardiomyocytes derived from pluripotent or multipotent stem cells, followed by transplantation to modify cardiac functions *in vivo* (e.g. to augment or attenuate the heart rate by modifying the cellular excitability of recipient cells).

CUSTOMIZING STEM CELL-DERIVED CARDIOMYOCYTES FOR TRANSPLANTATION

Cross Reference To Related Applications

This application claims priority to U.S. Provisional Patent Application Serial No. 60/ 598,171 filed August 2, 2004, the entire disclosure of which are incorporated herein by reference in its entirety.

STATEMENT OF FEDERALLY SPONSORED RESEARCH

Funding for the present invention was provided in part by the Government of the United States by virtue of Grant No. HL72857 by the National Institutes of Health. Thus, the Government of the United States has certain rights in and to the invention claimed herein.

FIELD OF THE INVENTION

The present invention relates to the customization of cells derived from pluripotent or multipotent stem cells and the use of such cells in transplantation to modify organ function (examples include, but are not limited to, cardiac impulse generation).

BACKGROUND OF THE INVENTION

The present invention relates to a stem cell-derived biological heart pacemaker, and more importantly to an implantation in the heart of a biological pacemaker that is molecularly determined.

Throughout this application, various publications are referenced to by numbers. Full citations for these publications may be found at the end of the specification immediately

following the Abstract. The disclosures of these publications in their entireties are hereby incorporated by reference into this application in order to more fully describe the state of the art to those skilled therein as of the date of the invention described and claimed herein.

The heart beats 2-3 billion times during the lifespan of an average person with a regular rhythm to pump blood throughout the body. These pumping actions require the highly coordinated efforts of specialized chamber-specific cardiomyocytes (CMs) such as atrial, ventricular and pacemaker cells. Autonomous rhythmic heart beats are modulated by sympathetic and parasympathetic means according to everyday needs; such normal rhythms originate in the sino-atrial (SA) node of the heart, consisting of pacemaker cells that generate spontaneous rhythmic action potentials (i.e. pacing) which subsequently propagate (i.e. conduction) to induce coordinated muscle contractions of the atria and ventricles for effective blood pumping. Since terminally-differentiated adult CMs lack the ability to regenerate, malfunctions or significant loss of specialized CMs due to disease or aging can lead to consequences such as lethal arrhythmias. Cell replacement therapy is a promising option but is limited by the availability of transplantable human CMs. Self-renewable embryonic stem cells (ESCs), derived from the inner cell mass of blastocysts, can propagate indefinitely in culture while maintaining their normal karyotypes and pluripotency to differentiate into all cell types. Therefore, ESCs may provide an unlimited supply of CMs for transplantation and cell-based therapies.

Although it is presently known that human CMs can be derived from hESCs *ex vivo*, and that electrical coupling does exist within hESC-derived beating outgrowths[6], their ability to functionally integrate with recipient cells after engraftment remains to be tested.

New methods to genetically custom-tailor and to successfully engraft hESC derivatives in vivo so as to enable modification of an organ function (e.g. correction of a cardiac electrical defects, such as the sick sinus syndrome, by stem cell-derived heart cells) would be highly desirable.

SUMMARY OF THE INVENTION

The present invention demonstrates that by using a combination of electrophysiological, imaging and gene transfer techniques, donor hESC-derived CMs can form a functional syncytium with and therefore modify the excitability of recipient CMs, *in vitro*, and the intact ventricular myocardium, *in vivo*.

A preferred embodiment of the present invention relates to a method of inducing or modifying rhythmic electrical and contractile activities of in vivo cardiac tissue comprising the integration of electrically active donor cardiomyocytes derived from genetically engineered human embryonic stem cells (that exhibit a particular phenotype) into recipient cardiomyoctes of in vivo cardiac tissue. These integrated in vitro donor and in vivo recipient cardiomyocytes can form a functional syncytium.

In other embodiments of the present invention the recipient cardiomyocytes of the in vivo cardiac tissue can be ventricular myocytes or atrial nodal myocytes.

In still another embodiment of the present invention, the integration of electrically active donor cardiomyocytes into recipient cardiomyocytes of in vivo cardiac tissue is by transplantation into the in vivo cardiac tissue. Such transplantation can be achieved by direct injection of the donor cardiomyocytes into the in vivo recipient cardiac tissues or cardiomyocytes.

In one other embodiment of the present invention, the genetically engineered human embryonic stem cells are altered by a transgene delivery vehicle (e.g. lentivirus, adeno-associated virus, extra-chromosomal vectors or other relevant appropriate vectors), for instance, by genetically targeting the activity of particular ion channels via *ex vivo* gene transfer into pluripotent stem cells of specific normal and/or engineered ion channel proteins, whose expression can be induced or repressed by the addition or removal of specific ligands. The same approaches can be applied to engineer other multipotent stem cells (e.g. adult progenitor and resident stem cells).

The present invention also relates to a method of inducing cardiac differentiation of human embryonic stem cells in vitro comprising treatment of the stem cells with a recombinant transgene delivery vehicle to exhibit a particular phenotype. This vector can contain nucleotide sequences for genes (such as Green Fluorescent Protein and/or other recombinant genes that encode for specific functions e.g. an ion channel) and a specific promoter such as an internal composite constitutive promoter CAG containing the CMV enhancer and the β-actin promoter or a tissue-specific promoter.

Thus the present invention relates to "custom-tailored" CMs that can provide an extremely flexible approach to modify cardiac functions such as cardiac excitability. Spontaneously electrically-active donor hESC-derived CMs that have been genetically engineered can act as a surrogate pacemaker to induce rhythmic electrical and contractile activities in otherwise quiescent recipient ventricular CMs after *in vitro* and *in vivo* transplantation. Furthermore, the beating rate of hESC-derived CMs can be modified by genetically targeting the activity of hyperpolarization-activated cyclic-nucleotide-modulated (HCN) pacemaker current to achieve maximum therapeutic flexibility. Other proteins (e.g. Na-Ca exchanger, ryanodine receptors, SERCA pumps, and such ionic channels as the inward rectifier potassium channels, etc) can be similarly targeted. Overall, the present invention relates to novel methods for correcting cardiac excitability defects (e.g. sick sinus syndromes) and for regenerating the damaged myocardium by constructing a self-renewable *ex vivo* library of customized stem cell-derived CMs that exhibit a range of tailored phenotypes (e.g. firing rates).

BRIEF DESCRIPTION OF THE FIGURES

Figure 1 represents an illustration of synchronized beating of hESC-derived CMs and neonatal rat ventricular myocytes (NRVMs) wherein A) Representative confocal images (top, phase contrast; bottom, green fluorescence) of control and LV-CAG-GFP-transduced undifferentiated hESC colonies, differentiating hESCs, and spontaneously-beating hEB outgrowths as indicated; B) LV-CAG-GFP-transduced

hESC line displayed a normal karyotype; C) Tabulated distribution of individual hEB (open symbols) and averaged (bars) data summarizing the percentage of hEBs containing contracting outgrowths on Day 10 after attachment. Each data point represents an individual differentiation reaction; D) Spontaneous beating rate measured in beats per minute (bpm) was identical for control and LV-CAG-GFP-transduced groups of contracting hEBs.

Figure 2 represents an illustration of A) Left: Spontaneously beating hEB outgrowth, which stably expresses GFP, was micro-dissected and transplanted onto a quiescent monolayer of NRVMs; Right: The beating rate of the spontaneously contracting hEB exhibited similar beating rates before (47±5 bpm, "alone") and after (49±4 bpm, "co-cultured") transplantation onto a monolayer of NRVMs. B) Ca^{2+}-transient recording from NRVMs located 1cm away from the transplanted beating hEB using rhod 2-AM as an indicator before (Left panel) and during (Middle panel) a spontaneous contraction; Normalized fluorescence intensity was measured over 10 sec in a co-culture (Right); C) The immuno-fluorescence image with red fluorescence indicating the presence of Cx43 at the border between NRVMs and GFP-expressing hESC-derived CMs. Although this image containing a 3-dimensional hEB was focused on the contact surface between hEB and NRVMs, Cx43 was indeed expressed throughout the two cell types (data not shown).

Figure 3 represents an illustration of spontaneous and rhythmic electrical signals generated and propagated from the engrafted hESC-derived graft wherein A) Multi-electrode array (MEA) recording of extracellular field potentials (Left); The field potential as a function of time contour map (Right) located the origin of potential wave front as the site of implantation of the beating hEB outgrowth (black area); B) MEA recording from another co-culture preparation before (Left) and after (Right) 200 μM lidocaine; The time contour map demonstrates the delay of conduction from a hEB (black area) upon lidocaine application; The pacing origin also corresponded to the site of implanted hEB.

Figure 4 represents an illustration of A) Optical action potentials were mapped with a voltage sensitive dye using the photodiode array focusing on a region containing a spontaneously beating hEB transplanted on quiescent NRVM monolayer; Right: A conduction contour map generated from the left image, demonstrating centrifugal

propagation of action potential wavefront from the site of transplanted hEB (red) to the periphery; B) A gap junction uncoupler, heptanol, reversibly eliminated the action potential propagation to the neighboring NRVM sites but did not affect the action potentials in the pacing origin of the hEB. NRVM1 and NRVM2 represent two distinct sites at 3.2 and 3.6 mm, respectively, away from the pacing origin; C) Superimposed optical action potential profiles demonstrate the delay of activation and slower rate of depolarization of NRVMs. D) β-adrenergic stimulation with 1 μM isoproterenol (Iso) significantly accelerated the spontaneous beating rate of hEB (P = 0.01) while ZD7288 attenuated the beating activity. n = 9 for each group.

Figure 5 represents an illustration of the effect of AV nodal cryoablation on control (A-F) and hESC-CM-injected (G) adult guinea pig heart to eliminate the intrinsic heart rhythm so as to create a sick sinus syndrome model to test the efficacy of electrically active hESC-derived cardiac cells to act as an in vivo surrogate pacemaker to pace the otherwise quisecent myocardium.

Figure 6 represents an illustration of isopotential contour maps of a guinea pig heart pre-injected with hESC-CMs before (left) and after (right) cryoablation. Note the rapid epicardial wavefront breakthrough of pre-ablation vs. planar spread of depolarization of post-ablation. The right atrium had been removed in these recordings.

Figure 7 represents illustrations of the functional consequences of lentivirus-mediated gene transfer of HCN1-encoded pacemaker channels wherein (A) is a representative family of hyperpolarization-activated currents recorded from HEK293T cells transduced with LV-CAG-HCN1-GFP; control non-transduced cells did not induce any measurable currents; (B) Steady-state activation curves of WT and HCN1-GFP fusion channels; (C) Confocal images of LV-CAG-HCN1-GFP-transduced HEK293T cells (left, transparent; middle green fluorescence; right, overlay); (D) LV-CAG-HCN1-GFP transduction of hESC-derived beating cardiomyocytes (circles significantly increased the beating activity of hEB's, a change which was not observed with control non-transduced contracting hEBs (squares) recorded under identical conditions (open symbols, individual data; solid, averaged).

DETAILED DESCRIPTION OF THE INVENTION

As used herein, the use of the word "a" or "an" when used in conjunction with the term "comprising" in the claims and/or the specification may mean "one," but it is also consistent with the meaning of "one or more," "at least one," and "one or more than one."

Although various lines of evidence suggest that adult stem cells possess a remarkable degree of plasticity by having the capacity to transdifferentiate into other lineages *in vivo*, this concept has been challenged by experiments showing that some results might have been misinterpreted by their fusion with existing cell types rather than by direct conversion[28,29]. Two recent studies further indicate that hematopoietic stem cells adopt only hematopoietic fates and do not transdifferentiate into cardiac cells for myocardial regeneration[30,31]. In contrast, hESCs are proven to possess the potential to differentiate into multiple cell types including CMs[1-6]. Here we have further demonstrated that hESCs retain their ability to differentiate into CMs even after stable genetic modification (by a recombinant lentivirus, but other appropriate means of genetic modification such as those described in the paragraphs that follow can also be employed). Furthermore, differentiation of hESCs into the cardiac lineage does not result in transgene silencing (when the appropriate promoter is chosen). Given that mouse and human ESCs differ in a number of important aspects (e.g. conventional methods of genetic manipulations such as electroporation and transfection that are highly effective for mESC prove to be highly inefficient or even ineffective for hESCs; please see [32] for review), this straightforward finding, however, is critical if genetic methods such as the use of a cardiac-specific promoter for driving the expression of a fluorescent reporter or an antibiotic resistance gene are to be combined with gene transfer techniques for deriving and selecting hESC-derived (chamber-specific) CMs[32-34].

Normal heartbeats originate in the sino-atrial (SA) node, a specialized cardiac tissue consisting of only a few thousand electrically-active pacemaker cells that generate spontaneous rhythmic action potentials which subsequently propagate to induce coordinated muscle contractions of the atria and ventricles. Not surprisingly,

malfunction or loss of pacemaker cells due to disease or aging necessitates the implantation of electronic pacemakers. While effective, the use of such devices is also associated with significant risks (e.g. infection or death), expense and other disadvantages such as the need for periodic battery replacements. Using adenovirus-mediated somatic gene transfer techniques, Nuss and colleagues have demonstrated that "latent" pacemaker activity of normally-silent ventricular myocytes can be unleashed to produce spontaneous firing activity by genetic inhibition of the inwardly rectifying K^+ current I_{K1} encoded by the Kir2 gene family[35]. However, the induced automaticity was ~3-fold slower than normal, and genetic suppression of I_{K1} does not provide a direct means to modulate the induced rhythm. It is better to target a genuine pacemaker gene such as the HCN channel. Although transient overexpression of HCN2 in the left atrium similarly induced an ectopic pacemaking activity, however, vagal suppression was required to unleash the effect limiting any potential therapeutic applications[36]. More importantly, transgene expression in the heart in these experiments was sporadic. Clearly, the distribution of the transgene(s) in the heart needs to be carefully targeted in order to achieve therapeutic goals because uncontrolled creation of ectopic pacemaker sites in the heart would lead to chaotic heartbeats. Localized transduction of the atrium, even if feasible, could be technically challenging. Furthermore, the creation of pacemakers by gene therapy relies upon the conversion of pre-existing heart tissue, rather than the implantation of a new "surrogate node". Recently, undifferentiated human mesenchymal stem cells (hMSCs) have been tested as an alternative vehicle for delivering the HCN2 gene into the heart[37]. However, these modified undifferentiated hMSCs are incapable of pacing quiescent cells since they are neither electrically-active nor genuine cardiac cells. Since hESCs have been proven to possess the potential to differentiate into CMs, damaged SA nodal cells, at least conceptually, can be better replaced or supplemented with electrically-active hESC-derived CMs. Indeed, we have demonstrated in the present study the ability of electrically-active hESC-derived CMs to functionally integrate with, and actively stimulate recipient ventricular CMs, *in vitro* and the ventricular myocardium, *in vivo*. Maximal therapeutic flexibility could even be achieved by *ex vivo* genetic manipulation of hESC-derived CMs[8] (e.g. the HCN gene[38]) to exhibit a desired firing frequency (cf. Figure 7D). Furthermore, such an *ex vivo* approach is potentially advantageous over the in situ gene transfer approach by being able to first isolate clonal genetically-modified cell lines whose transgene location, and other properties,

have been thoroughly characterized before transplantation to minimize the risk of inappropriate gene insertion (and thus, the associated oncogenesis). Our data also suggest that, unlike electronic devices, hESC-derived pacemakers retain their responsiveness to β-adrenergic stimulation as well as other ion channel modulators such as lidocaine and ZD7288. Sensitivity to drugs and cAMP, etc, can also be engineered by modifying stem cells (or their derivatives) with recombinant genes encoding specific proteins that perform particular functions.

The present invention leads to electrically-active genetically-modified hESC-derived CMs that are capable of actively pacing recipient ventricular CMs, *in vitro* and *in vivo*. It provides a platform for modifying cardiac excitability with genetically-engineered hESC-derived cardiac derivatives. Our approach can also be applied to other multipotent stem cells (e.g. cardiac resident stem cells). The present invention can provide a safer and cheaper alternative, or supplemental method, to implantable electronic devices for correcting defects in cardiac impulse generation.

I. Definitions

As used herein, the term "cardiomyocyte" refers to any cell in the cardiac myocyte lineage that shows at least one phenotypic characteristic of a cardiac cell. Such phenotypic characteristics can include expression of cardiac proteins, such as cardiac sarcomeric or myofibrillar proteins or atrial natriuretic factor, or electrophysiological characteristics. As used herein, the term "cardiomyocyte" and "myocyte" are interchangeable.

As used herein, the term "stem cell" refers to an "undifferentiated", multipotent/pluripotent cell capable of proliferation, self-maintenance, production of a differentiated cell or regeneration of a stem cell may be tissue. In preferred embodiments of the present invention, a stem cell is capable of differentiating into a differentiated myocardial cell, such as a cardiomyocyte.

II. In Vitro Modification of Human Embyonic Stem Cells

A nucleic acid molecule introduced into a stem cell is in a form suitable for expression in the cell of the gene product encoded by the nucleic acid. Accordingly, the nucleic acid molecule includes coding and regulatory sequences required for transcription of a gene (or portion thereof) and, when the gene product is a protein or peptide, translation of the gene product encoded by the gene. Regulatory sequences which can be included in the nucleic acid molecule include promoters, enhancers and polyadenylation signals, as well as sequences necessary for transport of an encoded protein or peptide, for example N-terminal signal sequences for transport of proteins or peptides to the surface of the cell or for secretion.

Nucleotide sequences which regulate expression of a gene product (e.g., promoter and enhancer sequences) are selected based upon the type of cell in which the gene product is to be expressed and the desired level of expression of the gene product. For example, a promoter known to confer cell-type specific expression of a gene linked to the promoter can be used. A promoter specific for cardiac gene expression can be linked to a gene of interest to confer muscle-specific expression of that gene product. Cardiomyocyte-specific regulatory elements which are known in the art include upstream regions from the myosin heavy chain gene, the myosin heavy chain gene, the sodium-calcium exchanger 1 gene, atrial natriuretic factor, and the troponin gene (Mar and Ordahl, (1988) Proc. Natl. Acad. Sci. USA. 85:6404), etc. Regulatory elements specific for other cell types are known in the art (e.g., the insulin regulatory elements for pancreatic islet cell-specific expression; various neural cell-specific regulatory elements, including neural dystrophin, neural enolase and A4 amyloid promoters). Alternatively, a regulatory element which can direct constitutive expression of a gene in a variety of different cell types, such as a viral regulatory element, can be used. Examples of viral promoters commonly used to drive gene expression include those derived from polyoma virus, Adenovirus 2, cytomegalovirus and Simian Virus 40, and retroviral LTRs. Alternatively, a regulatory element which provides inducible expression of a gene linked thereto can be used. The use of an inducible regulatory element (e.g., an inducible promoter) allows for modulation of the production of the gene product in the cell. Examples of potentially useful inducible regulatory systems for use in eukaryotic cells include hormone-regulated

elements (e.g., see Mader, S. and White, J. H. (1993) Proc. Natl. Acad Sci. USA 90:5603-5607), synthetic ligand-regulated elements (see, e.g. Spencer, D. M. et al. (1993) Science 262:1019-1024) and ionizing radiation-regulated elements (e.g., see Manome, Y. et al. (1993) Biochemistry 32:10607-10613; Datta, R. et al. (1992) Proc. Natl. Acad. Sci. USA 89:10149-10153). Additional tissue-specific or inducible regulatory systems which may be developed can also be used in accordance with the invention.

There are a number of techniques known in the art for introducing genetic material into a cell that can be applied to modify a cell of the invention. In one embodiment, the nucleic acid is in the form of a naked nucleic acid molecule. In this situation, the nucleic acid molecule introduced into a cell to be modified consists only of the nucleic acid encoding the gene product and the necessary regulatory elements. Alternatively, the nucleic acid encoding the gene product (including the necessary regulatory elements) is contained within a plasmid vector. Examples of plasmid expression vectors include CDM8 (Seed, B. (1987) Nature 329:840) and pMT2PC (Kaufman, et al. (1987) EMBO J. 6:187-195). In another embodiment, the nucleic acid molecule to be introduced into a cell is contained within a viral vector. In this situation, the nucleic acid encoding the gene product is inserted into the viral genome (or a partial viral genome). The regulatory elements directing the expression of the gene product can be included with the nucleic acid inserted into the viral genome (i.e., linked to the gene inserted into the viral genome) or can be provided by the viral genome itself.

Naked DNA can be introduced into cells by forming a precipitate containing the DNA and calcium phosphate. Alternatively, naked DNA can also be introduced into cells by forming a mixture of the DNA and DEAE-dextran and incubating the mixture with the cells. or by incubating the cells and the DNA together in an appropriate buffer and subjecting the cells to a high-voltage electric pulse (i.e., by electroporation). A further method for introducing naked DNA cells is by mixing the DNA with a liposome suspension containing cationic lipids. The DNA/liposome complex is then incubated with cells. Naked DNA can also be directly injected into cells by, for example, microinjection. For an in vitro culture of cells, DNA can be introduced by microinjection in vitro or by a gene gun in vivo.

Alternatively, naked DNA can also be introduced into cells by complexing the DNA to a cation, such as polylysine, which is coupled to a ligand for a cell-surface receptor (see for example Wu, G. and Wu, C. H. (1988) J. Biol. Chem. 263:14621; Wilson et al. (1992) J. Biol. Chem. 267:963-967; and U.S. Pat. No. 5,166,320). Binding of the DNA-ligand complex to the receptor facilitates uptake of the DNA by receptor-mediated endocytosis. An alternative method for generating a cell that is modified to express a gene product involving introducing naked DNA into cells is to create a transgenic animal which contains cells modified to express the gene product of interest.

Use of viral vectors containing nucleic acid, e.g., a cDNA encoding a gene product, is a preferred approach for introducing nucleic acid into a cell. Infection of cells with a viral vector has the advantage that a large proportion of cells receive the nucleic acid, which can obviate the need for selection of cells which have received the nucleic acid. Additionally, molecules encoded within the viral vector, e.g., by a cDNA contained in the viral vector, are expressed efficiently in cells which have taken up viral vector nucleic acid and viral vector systems can be used either in vitro or in vivo.

Defective retroviruses are well characterized for use in gene transfer for gene therapy purposes (for a review see Miller, A. D. (1990) Blood 76:271). A recombinant retrovirus can be constructed having a nucleic acid encoding a gene product of interest inserted into the retroviral genome. Additionally, portions of the retroviral genome can be removed to render the retrovirus replication defective. The replication defective retrovirus is then packaged into virions which can be used to infect a target cell through the use of a helper virus by standard techniques.

The genome of an adenovirus can be manipulated such that it encodes and expresses a gene product of interest but is inactivated in terms of its ability to replicate in a normal lytic viral life cycle. See for example Berkner et al. (1988) BioTechniques 6:616; Rosenfeld et al. (1991) Science 252:431-434; and Rosenfeld et al. (1992) Cell 68:143-155. Suitable adenoviral vectors derived from the adenovirus strain Ad type 5 dl324 or other strains of adenovirus (e.g., Ad2, Ad3, Ad7 etc.) are well known to those skilled in the art. Recombinant adenoviruses are advantageous in

that they do not require dividing cells to be effective gene delivery vehicles and can be used to infect a wide variety of cell types, including airway epithelium (Rosenfeld et al. (1992) cited supra), endothelial cells (Lemarchand et al. (1992) Proc. Natl. Acad Sci. USA 89:6482-6486), hepatocytes (Herz and Gerard (1993) Proc. Natl. Acad. Sci. USA 90:2812-2816) and muscle cells (Quantin et al. (1992) Proc. Natl. Acad Sci. USA 89:2581-2584). Additionally, introduced adenoviral DNA (and foreign DNA contained therein) is not integrated into the genome of a host cell but remains episomal, thereby avoiding potential problems that can occur as a result of insertional mutagenesis in situations where introduced DNA becomes integrated into the host genome (e.g., retroviral DNA). Moreover, the carrying capacity of the adenoviral genome for foreign DNA is large (up to 8 kilobases) relative to other gene delivery vectors (Berkner et al. cited supra; Haj-Ahmand and Graham (1986) J. Virol. 57:267). Most replication-defective adenoviral vectors currently in use are deleted for all or parts of the viral E1 and E3 genes but retain as much as 80% of the adenoviral genetic material.

Adeno-associated virus (AAV) is a naturally occurring defective virus that requires another virus, such as an adenovirus or a herpes virus, as a helper virus for efficient replication and a productive life cycle. (For a review see Muzyczka et al. Curr. Topics in Micro. and Immunol. (1992) 158:97-129). It is also one of the few viruses that may integrate its DNA into non-dividing cells, and exhibits a high frequency of stable integration (see for example Flotte et al. (1992) Am. J. Respir. Cell. Mol. Biol. 7:349-356; Samulski et al. (1989) J. Virol. 63:3822-3828; and McLaughlin et al. (1989) J. Virol. 62:1963-1973). Vectors containing as little as 300 base pairs of AAV can be packaged and can integrate. Space for exogenous DNA is limited to about 4.5 kb. An AAV vector such as that described in Tratschin et al. (1985) Mol. Cell. Biol. 5:3251-3260 can be used to introduce DNA into cells. A variety of nucleic acids have been introduced into different cell types using AAV vectors (see for example Hermonat et al. (1984) Proc. Natl. Acad. Sci. USA 81:6466-6470; Tratschin et al. (1985) Mol. Cell. Biol. 4:2072-2081; Wondisford et al. (1988) Mol. Endocrinol. 2:3239; Tratschin et al. (1984) J. Virol. 51:611-619; and Flotte et al. (1993) J. Biol. Chem. 268:3781-3790).

When the method used to introduce nucleic acid into a population of cells results in modification of a large proportion of the cells and efficient expression of the gene product by the cells (e.g., as is often the case when using a viral expression vector), the modified population of cells may be used without further isolation or subcloning of individual cells within the population. That is, there may be sufficient production of the gene product by the population of cells such that no further cell isolation is needed. Alternatively, it may be desirable to grow a homogenous population of identically modified cells from a single modified cell to isolate cells which efficiently express the gene product. Such a population of uniform cells can be prepared by isolating a single modified cell by limiting dilution cloning followed by expanding the single cell in culture into a clonal population of cells by standard techniques.

Alternative to introducing a nucleic acid molecule into a cell to modify the cell to express a gene product, a cell can be modified by inducing or increasing the level of expression of the gene product by a cell. For example, a cell may be capable of expressing a particular gene product but fails to do so without additional treatment of the cell. Similarly, the cell may express insufficient amounts of the gene product for the desired purpose. Thus, an agent which stimulates expression of a gene product can be used to induce or increase expression of a gene product by the cell. For example, cells can be contacted with an agent in vitro in a culture medium. The agent which stimulates expression of a gene product may function, for instance, by increasing transcription of the gene encoding the product, by increasing the rate of translation or stability (e.g., a post transcriptional modification such as a poly A tail) of an mRNA encoding the product or by increasing stability, transport or localization of the gene product. Examples of agents which can be used to induce expression of a gene product include cytokines and growth factors.

III. Methods for Treating Disorders Characterized by Insufficient Cardiac Function Using Electrically Active Cardiomyocytes of the Present Invention

Another aspect of the invention pertains to methods for treating disorders characterized by insufficient cardiac function in a subject. These methods include administering to a subject having such a disorder cardiomyocytes of the invention.

Such cardiomyocytes are described in detail above. The term "treating" as used herein includes reducing or alleviating at least one adverse effect or symptom of a disorder characterized by insufficient cardiac function. Adverse effects or symptoms of cardiac disorders are numerous and well characterized. Non-limiting examples of adverse effects or symptoms of cardiac disorders include: arrhythmia, dyspnea and palpitations. For additional examples of adverse effects or symptoms of a wide variety of cardiac disorders, see Robbins, S. L. et al. (1984) Pathological Basis of Disease (W. B. Saunders Company, Philadelphia) 547-609; Schroeder, S. A. et al. eds. (1992) Current Medical Diagnosis & Treatment (Appleton & Lange, Connecticut) 257-356. Transplantation of cardiomyocytes of the invention into the heart of a human or animal subject with a cardiac disorder results in replacement of lost cardiomyocytes and restoration of autonomous rhythmic heat beats. Cardiomyocytes are introduced into a subject with a cardiac disorder in an amount suitable to replace lost cardiomyocytes such that there is an at least partial reduction or alleviation of at least one adverse effect or symptom of the cardiac disorder.

As used herein the terms "administering", "introducing", and "transplanting" are used interchangeably and refer to the placement of the cardiomyocytes of the invention into a subject, e.g., a subject, by a method or route which results in localization of the cardiomyocytes at a desired site. The cardiomyocytes can be administered to a subject by any appropriate route which results in delivery of the cells to a desired location in the subject where at least a portion of the cells remain viable. It is preferred that at least about 5%, preferably at least about 10%, more preferably at least about 20%, yet more preferably at least about 30%, still more preferably at least about 40%, and most preferably at least about 50% or more of the cells remain viable after administration into a subject. The period of viability of the cells after administration to a subject can be as short as a few hours, e.g., twenty-four hours, to a few days, to as long as a few weeks to months. One method that can be used to deliver the cardiomyocytes of the invention to a subject is direct injection of the cardiomyocytes into the ventricular myocardium or the atrium of the subject as needed. See e.g., Soonpaa, M. H. et al. (1994) Science 264:98-101; Koh, G. Y. et al. (1993) Am. J. Physiol. 33:H1727-1733. Cardiomyocyte can be administered in a physiologically compatible carrier, such as a buffered saline solution. Additional

delivery methods which may be developed can also be used in accordance with the invention.

To accomplish these methods of administration, the cardiomyocytes of the invention can be inserted into a delivery device which facilitates introduction by, injection or implantation, of the cardiomyocytes into the subject. Such delivery devices include tubes, e.g., catheters, for injecting cells and fluids into the body of a recipient subject. In a preferred embodiment, the tubes additionally have a needle, e.g., a syringe, through which the cells of the invention can be introduced into the subject at a desired location. The cardiomyocytes of the invention can be inserted into such a delivery device, e.g., a syringe, in different forms. For example, the cells can be suspended in a solution or embedded in a support matrix when contained in such a delivery device. As used herein, the term "solution" includes a pharmaceutically acceptable carrier or diluent in which the cells of the invention remain viable. Pharmaceutically acceptable carriers and diluents include saline, aqueous buffer solutions, solvents and/or dispersion media. The use of such carriers and diluents is well known in the art. The solution is preferably sterile and fluid to the extent that easy syringability exists. Preferably, the solution is stable under the conditions of manufacture and storage and preserved against the contaminating action of microorganisms such as bacteria and fungi through the use of, for example, parabens, chlorobutanol, phenol, ascorbic acid, thimerosal, and the like. Solutions of the invention can be prepared by incorporating cardiomyocytes as described herein in a pharmaceutically acceptable carrier or diluent and, as required, other ingredients enumerated above, followed by filtered sterilization.

Support matrices in which the cardiomyocytes can be incorporated or embedded include matrices which are recipient-compatible and which degrade into products which are not harmful to the recipient. Natural and/or synthetic biodegradable matrices are examples of such matrices. Natural biodegradable matrices include, for example, collagen matrices. Synthetic biodegradable matrices include synthetic polymers such as polyanhydrides, polyorthoesters, and polylactic acid. These matrices provide support and protection for the cardiomyocytes in vivo.

The term "subject" is intended to include mammals, particularly humans, susceptible to diseases characterized by insufficient cardiac function.

As used herein, the phrase "disorder characterized by insufficient cardiac function" includes an impairment or absence of a normal cardiac function or presence of an abnormal cardiac function, such as cardiac arrhythmias. Abnormal cardiac function can be the result of disease, injury, and/or aging. As used herein, abnormal cardiac function includes morphological and/or functional abnormality of a cardiomyocyte or a population of cardiomyocytes. Non-limiting examples of morphological and functional abnormalities include physical deterioration and/or death of cardiomyocytes, abnormal growth patterns of cardiomyocytes, abnormalities in the physical connection between cardiomyocytes, under- or over-production of a substance or substances by cardiomyocytes, failure of cardiomyocytes to produce a substance or substances which they normally produce, and transmission of electrical impulses in abnormal patterns or at abnormal times. Abnormal cardiac function is seen with many disorders including, for example, ischemic heart disease, e.g., angina pectoris, myocardial infarction, chronic ischemic heart disease, hypertensive heart disease, pulmonary heart disease (cor pulmonale), valvular heart disease, e.g., rheumatic fever, mitral valve prolapse, calcification of mitral annulus, carcinoid heart disease, infective endocarditis, congenital heart disease, myocardial disease, e.g., myocarditis, cardiomyopathy, cardiac disorders which result in congestive heart failure, and tumors of the heart, e.g., primary sarcomas and secondary tumors.

IV. General Cell Culture Methods:

The present invention relies on routine techniques in the field of cell culture, and suitable conditions can be easily determined by those of skill in the art (see, e.g., Freshney et al., Culture of Animal Cells, 3rd ed. (1994)). In general, the cell culture environment includes consideration of such factors as the substrate for cell growth, cell density and cell contract, the gas phase, the medium, the temperature, and the presence of growth factors.

Exemplary cell culture conditions for stem cells are described in, e.g., U.S. Pat. Nos. 6,017,527 and 5,851,756; Inaba et al., J. Exp. Med. 176:1693 (1992); Inaba

et al., J. Exp. Med. 175:1157 (1992); Inaba et al., Current Protocols Immunol., Unit 3.7 (Coico et al., eds. 1998); Schneider et al., J. Immunol. Meth. 154:253 (1992); and Lutz et al., supra.

The cells of the invention can be grown under conditions that provide for cell to cell contact. In a preferred embodiment, the cells are grown in suspension as three dimensional aggregates. Suspension cultures can be achieved by using, e.g., a flask with a magnetic stirrer or a large surface area paddle, or on a plate that has been coated to prevent the cells from adhering to the bottom of the dish. For example, the cells may be grown in Costar dishes that have been coated with a hydrogel to prevent them from adhering to the bottom of the dish.

For cells that grow in a monolayer attached to a substrate, plastic dishes, flasks, roller bottles, or microcarriers are typically used. Other artificial substrates can be used such as glass and metals. The substrate is often treated by etching, or by coating with substances such as collagen, chondronectin, fibronectin, laminin or poly-D-lysine. The type of culture vessel depends on the culture conditions, e.g., multi-well plates, petri dishes, tissue culture tubes, flasks, roller bottles, microcarriers, and the like. Cells are grown at optimal densities that are determined empirically based on the cell type.

Important constituents of the gas phase are oxygen and carbon dioxide. Typically, atmospheric oxygen tensions are used for dendritic cell cultures. Culture vessels are usually vented into the incubator atmosphere to allow gas exchange by using gas permeable caps or by preventing sealing of the culture vessels. Carbon dioxide plays a role in pH stabilization, along with buffer in the cell media, and is typically present at a concentration of 1-10% in the incubator. The preferred CO.sub.2 concentration for dendritic cell cultures is 5%.

Cultured cells are normally grown in an incubator that provides a suitable temperature, e.g., the body temperature of the animal from which is the cells were obtained, accounting for regional variations in temperature. Generally, 37.degrees. C. is the preferred temperature for dendritic cell culture. Most incubators are humidified to approximately atmospheric conditions.

Defined cell media are available as packaged, premixed powders or presterilized solutions. Examples of commonly used media include Iscove's media, RPMI 1640, DMEM, and McCoy's Medium (see, e.g., GibcoBRL/Life Technologies Catalogue and Reference Guide; Sigma Catalogue). Defined cell culture media are often supplemented with 5-20% serum, e.g., human, horse, calf, or fetal bovine serum. The culture medium is usually buffered to maintain the cells at a pH preferably from about 7.2 to about 7.4. Other supplements to the media include, e.g., antibiotics, amino acids, sugars, and growth factors (see, e.g., Lutz et al., supra).

V. Kits

Therapeutic kits of the present invention are kits comprising the stem cells. Such kits will generally contain, in suitable container means, a pharmaceutically acceptable formulation of the stem cells. The kit may have a single container means, and/or it may have distinct container means for each compound.

When the components of the kit are provided in one and/or more liquid solutions, the liquid solution is an aqueous solution, with a sterile aqueous solution being particularly preferred. The stem cell compositions may also be formulated into a syringeable composition. In which case, the container means may itself be a syringe, pipette, and/or other such like apparatus, from which the formulation may be applied to an infected area of the body, injected into an animal, and/or even applied to and/or mixed with the other components of the kit.

However, the components of the kit may be provided as dried powder(s). When reagents and/or components are provided as a dry powder, the powder can be reconstituted by the addition of a suitable solvent. It is envisioned that the solvent may also be provided in another container means.

The container means will generally include at least one vial, test tube, flask, bottle, syringe and/or other container means, into which the stem cells are placed, preferably, suitably allocated. The kits may also comprise a second container means for containing a sterile, pharmaceutically acceptable buffer and/or other diluent.

The kits of the present invention will also typically include a means for containing the vials in close confinement for commercial sale, such as, i.e., injection and/or blow-molded plastic containers into which the desired vials are retained

Irrespective of the number and/or type of containers, the kits of the invention may also comprise, and/or be packaged with, an instrument for assisting with the injection/administration and/or placement of the ultimate the stem cell composition within the body of an animal. Such an instrument may be a syringe, pipette, forceps, and/or any such medically approved delivery vehicle.

All publications and patent applications cited in this specification are herein incorporated by reference as if each individual publication or patent application were specifically and individually indicated to be incorporated by reference.

Although the foregoing invention has been described in some detail by way of illustration and example for purposes of clarity of understanding, it will be readily apparent to one of ordinary skill in the art in light of the teachings of this invention that certain changes and modifications may be made thereto without departing from the spirit or scope of the appended claims.

EXEMPLIFICATION

Materials and Methods

Maintenance and differentiation of hESC

The hESC line H1 (Wicells, Madison, WI) was maintained on irradiated mouse embryonic fibroblast (MEF) feeder layer and propagated as previously described [1]. Briefly, the culture media consisted of Dulbecco's modified Eagle's medium (Invitrogen Corp., Carlsbad, California, USA) supplemented with 20% fetal bovine

serum (HyClone; Logan, Utah, USA), 2 mM L-glutamine, 0.1 mM ß-mercaptoethanol, and 1% nonessential amino acids. MEF cells were obtained from 13.5 day embryos of CF-1 mice. For cardiac differentiation, the method of forming embryoid bodies (hEB) from enzymatically dispersed hES cells in suspension in Petri dishes in the absence of LIF and bFGF was employed[2-4]. On day 6, suspended hEBs were plated onto gelatin-coated 6-well plates. The media, which contained 15% FBS and 0.1mM non-essential amino acids in DMEM, was replenished daily during differentiation. Spontaneously beating outgrowths, which typically appeared around 7 days after plating of hEBs, were identified by visual inspection. Both continuously and episodically beating hEBs were observed. While both groups were considered when assessing the percentage of spontaneously-contracting hEBs present in the population, only the former was measured for beating frequency (in duplicate at 35±2°C) and transplantation experiments to minimize the variability in activity. The variability in beating frequency is presumably the result of electrical inhomogeneities [4].

Lentivirus-mediated stable genetic modification of hESCs.

For stable genetic modification, we employed the self-inactivating HIV1-based lentiviral vector (LV)[9] for transgene delivery. LV was chosen as the vehicle because, unlike adenoviruses, it allows persistent transgene expression without eliciting host immune responses. With six of the nine genes removed, including all the crucial virulent factors of wild-type HIV1, LV is non-pathogenic. Furthermore, LV is effective even in mitotically-inactive terminally-differentiated cells such as CMs[10], in contrast to other oncoretroviruses that also integrate transgene into the host genome. These unique properties make LV ideal for modifying hESCs and their cardiac derivatives. The plasmid pLV-CAG-GFP was created from pRRL-hPGK-GFP SIN-18 (generously provided by Dr. Didier Trono, University of Geneva, Switzerland) by replacing the human phosphoglycerate kinase 1 (hPGK) promoter with the composite CAG promoter. Unlike CMV, CAG does not lead to transgene silencing in hESCs. Recombinant lentiviruses were generated using the 3-plasmid system[11] by co-transfecting HEK293T cells with pLV-CAG-GFP, pMD.G and pCMVΔR8.91. The latter plasmids encode the vesicular stomatitis virus G envelope protein and the HIV-1 *gag/pol, tat,* and *rev* genes required for efficient virus production, respectively. For

each 100-mm dish of HEK293T cells plated at 80-85% confluency, 5, 2.5, and 10 μg of pLenti-CAG-GFP, pMD.G and pCMVΔR8.91 DNA were used for transfection, respectively. Lentiviral particles were harvested by collecting the culture medium at 48 hours post-transfection. The titer, measured in transducing units (TU) per milliliter, was determined by examining the percentage of green HEK293T cells obtained 48 hours after transduction with the corresponding serially diluted lentivirus-containing supernatants using fluorescence-activated cell sorter (FACS) analysis (FACScan or FACSort, Becton Dickinson, Franklin Lakes, NJ). Lentiviruses generated using this protocol typically had titres in the range of 1×10^6 to 6×10^6 TU/mL. Lentiviruses were stored at -80°C before use.

hESCs were transduced by adding purified lentiviruses to cells at a final concentration of 10,000 TU ml^{-1} with 8 μg/mL polybrene to facilitate transduction. The multiplicity of infection (MOI) was ~5 for each round of transduction. After 4 to 6 hours of incubation with LV-CAG-GFP, 2 mL of fresh medium per 60 mm dish was added. Transduction was allowed to proceed for at least 12-16 hours. Cells were washed with PBS twice to remove residual viral particles. For obtaining a homogeneous population of LV-CAG-GFP, green portions of hESC colonies were microsurgically segregated from the non-green cells, followed by culturing under undifferentiating conditions for expansion. This process was repeated until a homogenous population of green hESC, as confirmed by FACS, was obtained.

Assessment of functional coupling after in vitro transplantation.

Monolayers of neonatal rat ventricular myocytes (NRVMs) were prepared as previously described [12], and plated at a density of ~1.5 x 10^3 cells/mm^2. Typically, some preparations of NRVMs became electrically quiescent without any spontaneous contractions after 10-14 days in culture. Only absolutely quiescent NRVMs were chosen for experiments. Ca^{2+} transients were recorded by incubating cells at 37°C and 5% CO_2 with rhod-2 AM (Molecular Probes, Eugene, Oregon, USA) using laser scanning confocal microscopy. GFP was excited with the 488 nm line of the krypton-argon laser and visualized using the 525 nm band-pass emission filter. The rhod-2 signal was generated by 568 nm excitation coupled with a 600 nm band-pass emission filter. *In vitro* multi-electrode array (MEA) recordings were performed at 37°C by simultaneously recording from 60 microelectrodes arranged in an 8x8 layout grid with

an inter-electrode distance of 200μm. The raw signals were collected at 25kHz, band-pass filtered, and amplified (Multi-channel Systems, Reutlingen, Germany), followed by analysis using MC Data Tool V1.3.0 to generate a conduction map based on the time differences at which signals were detected at each of the microelectrodes. For high-resolution optical mapping of monolayers, cells were incubated in 10 μM RH237 (Molecular Probes, Eugene, Oregon, USA) for 15 min at 37°C in a DMEM media without phenol red in a 5% CO_2 environment, followed by recording using a custom-built system after washing out the dye.

Immunostaining.

A spontaneously beating hEB co-cultured with a monolayer of NRVMs was fixed in 4% paraformaldehyde for 5 min at 21°C, washed four times with PBS and blocked with 10% BSA with 0.075% saponin in PBS for 2 hours at 21°C. Fixed cells were incubated with primary antibody against connexin 43 (x 800 dilution, Chemicon, Temecula, California, USA) overnight at 4°C. Cells were visualized by incubation with anti-mouse Alexa-Fluor 595 (x 500 dilution, Molecular Probes Inc., Eugene, Oregon, USA) and observed under laser-scanning confocal microscopy.

In vivo Transplantation and Optical Action Potential Mapping

For *in vivo* transplantation, 5 microdissected beating hEB outgrowths prepared as described above were injected subepicardially into the left ventricular (LV) anterior wall (~1-2 mm deep) of adult breeder guinea pigs using a 21-gauge needle after thoracotomy. The area of injection (anterior epicardium, midway between apex and base) was chosen such that it is most suitable for mapping due to minimal heart curvature and thus motion artifact can be suppressed by gentle stabilization. A small suture in the immediate vicinity of the injected area was typically introduced to further assist our identification of the region of interest during the mapping experiments. Furthermore, a parallel optical port was designed to enable visualization of the exact mapped area, which was centered upon the injected region. The optical port also allowed visualization of the suture as well as GFP signal upon excitation with light at 400nm.

Injected animals were allowed to recover from the surgical procedure for 48-72 hours before performing further experiments. No tumor, inflammation or immune rejection was observed during the time course of our experiments. To determine if electrically active hESC-derived CMs could indeed capture the myocardium, we implemented a novel experimental model that enables us to investigate in detail, the functional electrophysiological interaction between the transplanted human cells and the recipient ventricular myocardium. This model was designed to specifically test the ability of hESC-derived CMs to modulate the intrinsic rhythm of the intact organ by direct impulse propagation from the injected exogenous cells to the surrounding myocardium. In preliminary experiments we found that the average RR interval of Langendorf perfused guinea pig hearts under our experimental conditions (T=36°C) is 435ms, corresponding to a heart rate of 138bpm, which is significantly greater than the *in vitro* beating rate of hESC-derived CMs in culture (cf. Figure 1C). Therefore, we needed to suppress the heart rate of these preparations in order to avoid over-drive suppression of the electrical activity of hESC-derived CMs by the intrinsic guinea pig heart rhythm. In addition to having a relatively fast heart rate, isolated, perfused, and stained guinea pig hearts consistently exhibit complete heart block as evidenced by a clear dissociation of the P-waves and the QRS complexes on the volume conducted ECG (data not shown). Since the junctional escape rhythm was too fast relative to the rate of the transplanted hES derived CMs, an alternative approach, based on performing AV nodal cryoablation in the isolated guinea pig heart, was developed. Briefly, following surgical dissection of the right atrium, a custom designed cryo-probe was inserted into the right ventricular (RV) cavity and placed in contact with the high septum, 1 mm below the base of the heart. Liquid nitrogen was then rapidly and continuously passed through the probe using a commercially available cryo-gun (Brymill Inc) for 2 minutes resulting in ablation of the RV facing septum, and the endocardial surface of basal RV free wall but not to the LV, as assessed by histological examination of the preparation, and a lack of change in electrophysiological measurements including epicardial action potential duration and morphology, LV epicardial and endocardial diastolic pacing thresholds and effective refractory periods (cf. Table 1). Cryoablated hearts of pre-injected (n=6) and non-injected (n=6) animals were stained with the voltage-sensitive dye, di-4-ANEPPS, and

placed in a custom designed, temperature-controlled, imaging chamber for optical action potential measurements, as previously described [13].

able 1. Summary of electrophysiological parameters recorded from control guinea pig hearts before and after atrial removal and cryoablation.

	Mean APD	Conduction velocity (longitudinal)	Diastolic pacing threshold	Diastolic pacing threshold
			EPICARDIAL	ENDOCARDIAL
Pre-cryoablation	179 ±8	56.2 ±5.3	0.3 ±0.1	0.5 ±0.2
Post-cryoablation	181 ±9	55.4 ±2.4	0.4 ±0.2	0.4 ±0.2
	p = 0.7	p = 0.8	p = 0.2	p = 0.7

Statistics

All data reported are means ± S.E.M. Statistical significance was determined for all individual data points and fitting parameters using one-way ANOVA and Tukey's HSD *post hoc* test at the 5 % level.

EXAMPLES

Example 1. ***Generation of a stably transduced GFP-expressing hESC line***

To achieve stable genetic modification of hESCs, we first constructed LV-CAG-GFP, which directs GFP expression under the control of CAG, an internal composite constitutive promoter containing the CMV enhancer and the β-actin promoter. Transduction of hESCs with LV-CAG-GFP enabled the generation of an hESC line that stably expresses GFP (Figure 1A) for facilitating the identification of hESC-derived CMs after engraftment. Undifferentiated LV-CAG-GFP-transduced hESCs were positively stained for molecular markers of pluripotency such as Oct4, SSEA4, TRA-60 and TRA-80 (data not shown), remained green for >2 years, maintained a normal karyotype without detectable insertion, deletion or rearrangements (Figure

1B), and propagated normally with a split cycle not different from that of non-transduced hESCs (i.e. ~8 days).

Example 2. ***Stably genetically-modified cardiac tissues from pluripotent hESCs***

Although a modified hESC line that stably expresses GFP has the ability to differentiate into hematopoietic cells [14], it is not known whether hESCs that have been subjected to similar sustained genetic manipulation also retain their ability to differentiate into the cardiac lineage. Given that transgene silencing has been observed in mammalian ESCs as well as several primordial cell types derived from them [15,16], we verified that genetic modification persists during and after cardiac differentiation (see Discussion for applications). To test whether the pluripotency of hESCs to differentiate into CMs was altered by LV-mediated genetic modification, we induced and compared *in vitro* cardiac differentiation of control and LV-CAG-GFP-transduced hESCs. Spontaneously-beating outgrowths of human embryoid bodies (hEBs), which contain CMs [2-6], could be readily obtained from control and LV-CAG-GFP-transduced hESCs. Figure 1A also shows that GFP expression remained robust during and after cardiac differentiation. Both groups behaved similarly with regard to the time course of the appearance of visibly-beating hEB outgrowths (a plateau of ~13% in the entire differentiated hEB population was reached on day 10 under our experimental conditions) and the associated beating frequency (~50 bpm, Figures 1C-D; $P>0.05$). These results demonstrate that persistent genetic modification of hESCs can be achieved without altering their cardiogenic potential.

Example 3. ***Electrically-active donor hESC cardiac derivatives form a functional syncytium with quiescent recipient ventricular cells in vitro.***

To investigate whether donor GFP-expressing hESC-derived CMs can functionally integrate with recipient CMs, we adopted an *in vitro* transplantation model. For each experiment, a single CM-containing beating outgrowth (~500μm in diameter) was micro-surgically dissected from an hEB differentiated from LV-CAG-GFP-transduced hESCs and transplanted onto a quiescent monolayer of neonatal rat ventricular myocytes (NRVMs) (see Methods). After co-culturing for 2-3 days,

synchronous rhythmic contractions of GFP-expressing hEB and NRVMs was observed with a rate of 49±4 bpm (n=14) (Figure 2A; see video 1 provided in Supplemental Information). Note that this rate was similar to that of spontaneously-contracting hEB alone (cf. Figure 1D) but much lower than that typically observed with beating NRVMs (about 200-300bpm before they became quiescent). Furthermore, co-culturing hESC-derived CMs and NRVMs without direct contact did not lead to spontaneous contractions (data not shown), excluding the so-called field effect transmission[17], and the possibility that paracrine factors of hESC-derived CMs influence the excitability and/or contractions of NRVMs. Spontaneous Ca^{2+} transients, not seen in otherwise quiescent NRVMs, could be recorded from co-cultured NRVMs more than 1 cm away from the human cells (Figure 2B). Collectively, these observations suggest that the transplanted cells were electrically driving the recipient cells. Consistent with this notion, immunostaining the co-culture with a primary antibody against the gap junction protein connexin-43 (Cx43) showed expression of Cx43 throughout hEB and NRVMs, and along their contact surface, suggesting a functional Cx-mediated coupling between the two cell types (Figure 2C; note that the 3-dimensional preparation shown was focused on the contact surface between hEB and NRVMs but Cx43 was also expressed throughout the two cell types, see supplemental video 2). Indeed, multi-electrode array (MEA) recording of extracellular field potentials revealed a coordinated pattern of electrical conduction: rhythmic signals generated from an early-activated region corresponding to the transplantation site (green) spread to the rest of the monolayer (Figure 3A). As anticipated[18,19], application of lidocaine (100 μM), a Na^+ channel blocker, significantly slowed electrical conduction of another preparation by approximately 3-fold (Figure 3B; note the crowding of the isochrones in the right panel).

High-resolution optical mapping further displayed a consistent time delay between action potentials (AP) recorded from hEB and NRVMs remote from the transplanted site (Figure 4A-C; note that the AP morphologies were also different). The patterns of activation were comparable to those obtained from MEA recordings. Heptanol is known to block gap junction proteins and interfere with electrical coupling[20]. Application of 0.4 mM heptanol uncoupled the hESC-derived CMs from the NRVMs without suppressing the pacemaker activity of the human cells *per se*

(Figure 4B, right panel). Such an uncoupling effect was readily reversible upon washout of heptanol (data not shown).

β-adrenergic stimulation is a potent physiological mechanism to accelerate cardiac pacing[21]. We next sought to determine if the functional syncytium formed between hEB and NRVMs could display a change in beating rates in response to the β-adrenergic agonist isopreterenol (Iso). Indeed, the beating frequencies of the co-culture increased significantly from 48±5 bpm to 63±8 bpm upon washing in of 1 μM Iso ($p<0.05$). These results were consistent with a previous finding that β-adrenergic receptors are already expressed in hESC-derived CMs [3] and further indicate that stimulation of these receptors produce a positive chronotropic response that can be transmitted to the recipient NRVMs. Recently, the cardiac current I_f, encoded by the hyperpolarization-activated cyclic-nucleotide-modulated (HCN) channel gene family which is known to play an important role in pacing[22], has been identified in hESC-derived CMs[23]. Unlike Iso, the addition of the bradycardic agent ZD7288, a HCN-specific blocker[24], significantly reduced the beating activity of our co-culture (Figure 4D; $p<0.05$). Addition of neither Iso nor ZD7288 affected quiescent NRVMs without engrafted hESC-derived CMs (data not shown).

At the end of each experiment, synchronous beating could be terminated by crushing or surgically excising the transplanted human cells (n=17; analogous to ablation), bolstering the notion that hESC-derived CMs were indeed the origin of pacing. To further confirm that synchronous beating was not an epiphenomenon due to stretch-activated automaticity of NRVMs from the overlying contracting hESC-derived CMs, we studied the effect of 2,3-butanedione monoxime (BDM) to uncouple excitation-contraction coupling[25]. As anticipated from an electrical integration of donor with recipient cells, the co-culture ceased to contract upon 1mM BDM application although optically-mapped electrical conduction was unaffected (data not shown).

Example 4. ***In vivo transplantation.***

Although the data presented thus far indicate that electrically-active donor hESC-derived CMs can engraft to, and form an electrical syncytium with, quiescent recipient ventricular CMs after *in vitro* transplantation, it is still necessary to

demonstrate the functional efficacy of our approach, *in vivo*. Therefore, optical action potential mapping was performed in hearts of control (sham or non-injected) versus hESC-derived CM-injected guinea pigs that were subjected to a cryoablation technique (see Materials and Methods) designed to suppress the intrinsic heart rate (as a model for the sick sinus syndrome). The functional consequences of the cryoablation protocol are shown in Figure 5, which demonstrates complete suppression of the intrinsic heart rhythm by the ablation procedure in non-injected heart (Figure 5, C&F). Importantly, tissue damage incurred by this procedure was limited to the RV endocardium and RV septum, without affecting the LV. In fact, successful LV pacing could be achieved with no change ($p>0.05$) in the diastolic pacing threshold via unipolar extracellular wires placed on the LV epicardium and LV endocardium (Figure 5, D&E) suggesting that the procedure did not produce tissue damage in either region. To further discount the potential for tissue damage induced by the cryoablation procedure, LV epicardial action potential durations, morphologies, and longitudinal epicardial conduction velocity were also measured and compared. These parameters, as summarized in Table 1, were identical before and after the procedure, further indicating the absence of changes in intrinsic electrophysiological properties in the myocardial region of interest where hESC-derived CMs were transplanted.

Whereas *ex vivo* optical mapping of control adult cryoablated guinea pig hearts exhibited complete electrical silence throughout the entire LV (n=6; Figure 6A), relatively slow spontaneous action potentials could be readily recorded from the LV of animals that were pre-transplanted with spontaneously-beating hESC-derived CMs, *in vivo* (n=4; Figure 6A). Shown in Figure 6B are isopotential contour maps recorded every 0.6 ms that depict the sequential spread of membrane depolarization across the anterior epicardial surface of a representative guinea pig heart injected with hESC-derived CMs before and after the cryoablation procedure. Before the procedure, the heart rate was relatively high and the spread of membrane depolarization was very rapid across the epicardial surface (Figure 6B, left panel; supplemental video 3) and consistent with epicardial wavefront breakthrough from deeper intramyocardial layers, suggesting a normal ventricular activation sequence via the His-Purkinje conduction system and that the transplanted hESC-derived CMs have been overridden. Interestingly, following the cryoablation procedure, a slow heart rate persisted *exclusively* in hearts pre-injected with hESC-derived CMs but not in controls

(cf. Figure 6A). Importantly, the spread of membrane depolarization in these hearts was consistent with epicardial surface wavefront propagation, always proceeding from the site of transplantation as identified by the GFP epifluorescence of hESC-derived CMs (Figure 6B, right panel; supplemental video 4). This finding is consistent with that recently reported by Field and colleagues (2003) which demonstrates that mouse embryonic CMs transplanted into the left ventricle of syngeneic adult mice fully integrate into the host tissue matrix and form gap junction proteins [26], and further demonstrates that exogenous transplanted cells can even electrically drive the surrounding myocardium. By contrast, injection of saline without hESC-CMs did not induce automaticity. Same as control non-injected hearts, sham injected hearts also exhibited complete electrical silence after cryoablation. Similar to the injection of mESC-CMs into mouse hearts[27], hESC-CM injection into guinea pig heart did not lead to tumor or arrhythmias (presumably because hESC-CMs pacemaking activity was suppressed by the 2-3 times faster guinea pig heart rate) over the time course of our experiments.

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We claim:

1. A method of inducing or modifying rhythmic electrical and contractile activities of in vivo cardiac tissue comprising the integration of electrically active donor cardiomyocytes derived from genetically engineered human embryonic stem cells into recipient cardiomyoctes of in vivo cardiac tissue to achieve a preferred therapeutic outcome.

2. A method of claim1 wherein the recipient cardiomyocytes of the in vivo cardiac tissue are ventricular myocytes.

3. A method of claim1 wherein the recipient cardiomyocytes of the in vivo cardiac tissue are atrial myocytes.

4. A method of claim 1 wherein the integration of electrically active donor cardiomyocytes into recipient cardiomyocytes of in vivo cariac tissue is by transplantation into the in vivo cardiac tissue.

5. A method of claim 4 wherein transplantation is by direct injection of the donor cardiomyocytes and the recipient in vivo cardiomyocytes.

6. A method of claim 1 wherein the electrically active donor cardiomyocytes and recipient in vivo cardiomyocytes form a functional syncytium.

7. A method of claim1 wherein the genetically engineered human embryonic stem cells and their cardiac derivatives are altered by a transgene delivery vehicle.

8. A method of inducing cardiac differentiation of human embryonic stem cells in vitro comprising treatment of the stem cells with a transgene delivery vehicle.

9. A method of claim 8 wherein the transgene delivery vehicle contains a nucleotide sequence encoding a normal or genetically-modified recombinant ion channel protein such as the HCN gene, or other functional proteins.

10. A method of claim 8 wherein the transgene delivery vehicle contains a nucleotide sequence for a cardiac tissue specific promoter.

11. A method of claim 10 wherein the promoter is an internal composite constitutive promoter containing the CMV enhancer and the β-actin promoter.

12. A method for treating a disorder characterized by insufficient cardiac function in a human subject comprising: administering to the subject a composition comprising an electrically active donor cardiomyocytes derived from genetically engineered human embryonic stem cells.

13. The method of claim 12, wherein the subject has congestive heart failure.

14. The method of claim 1, wherein the subject has had a myocardial infarction.

15. The method of claim 1, wherein the subject has had a cardiac arrhythmia (e.g. bradycardia, tachycardia, abnormal sinus node function, atrioventricular block, etc).

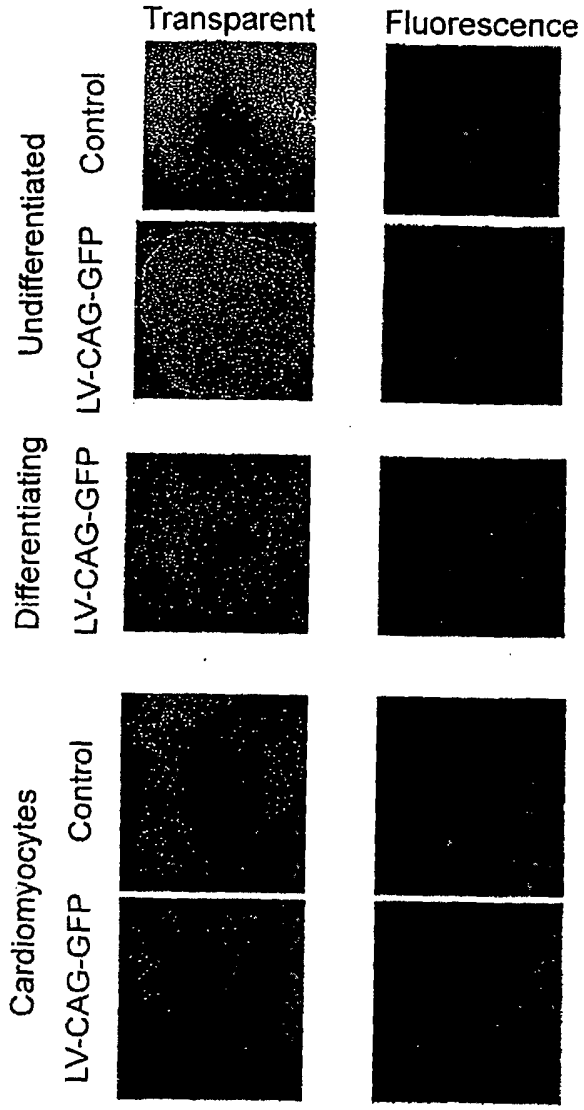


FIG. 1A

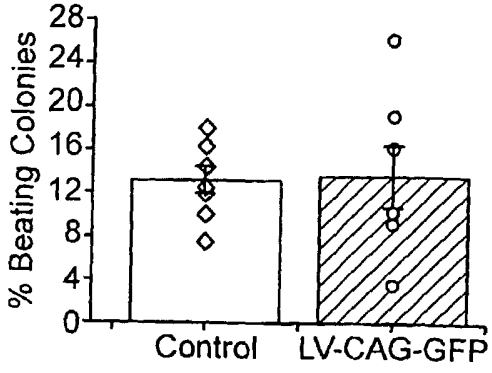


FIG. 1B

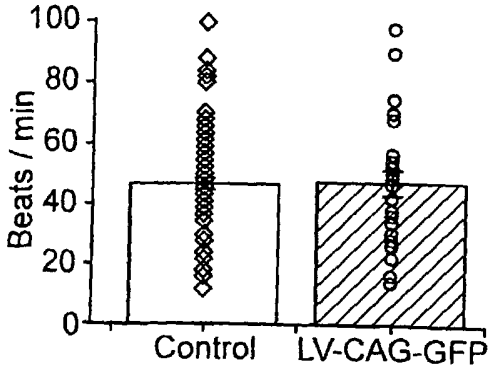


FIG. 1C

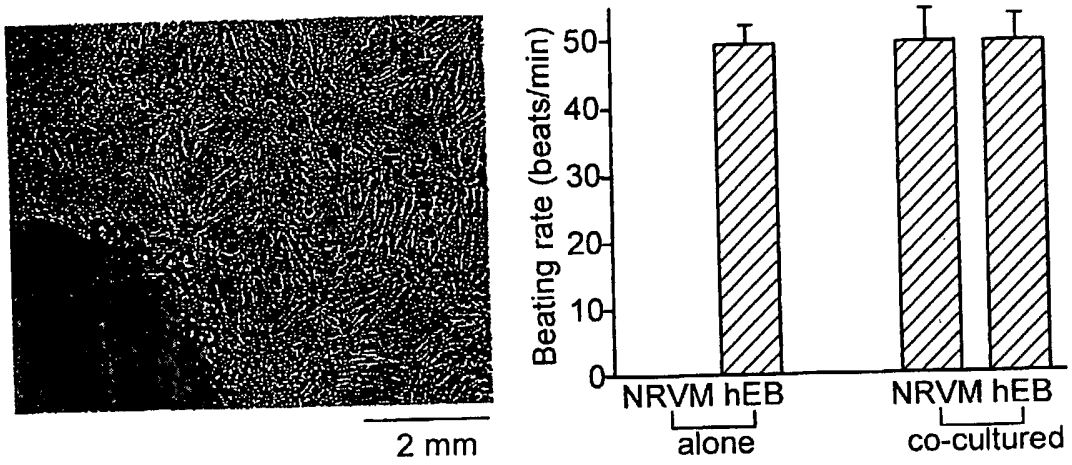


FIG. 2A

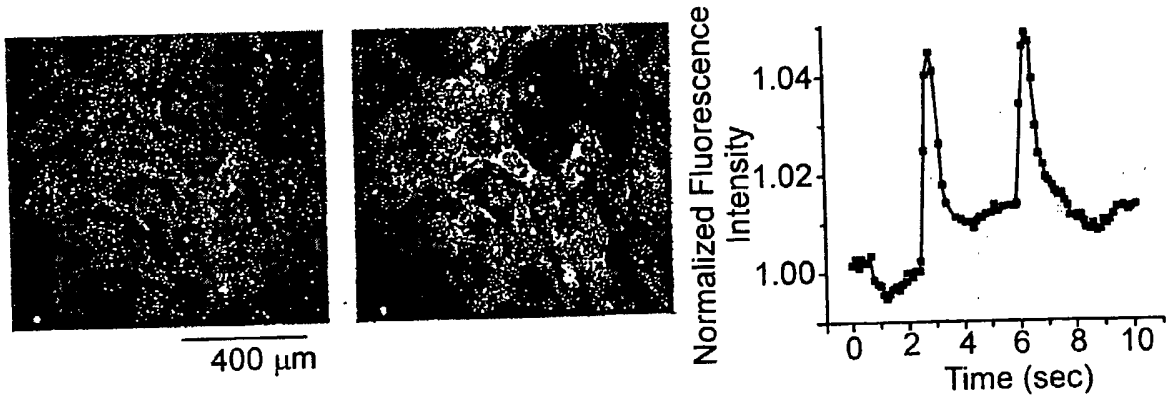
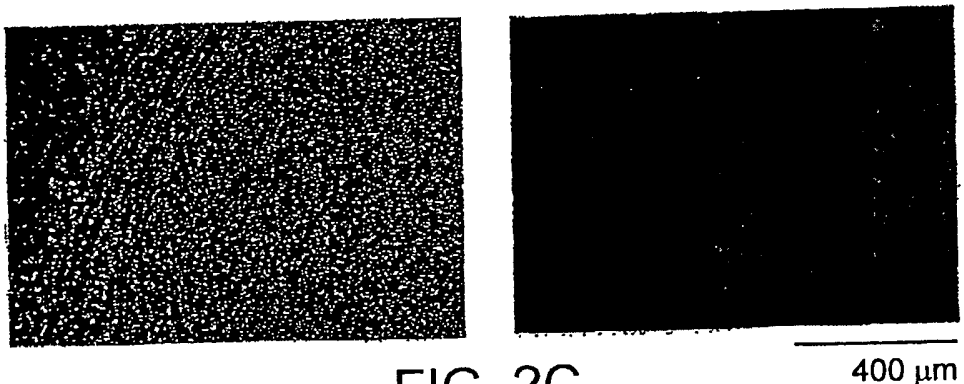


FIG. 2B



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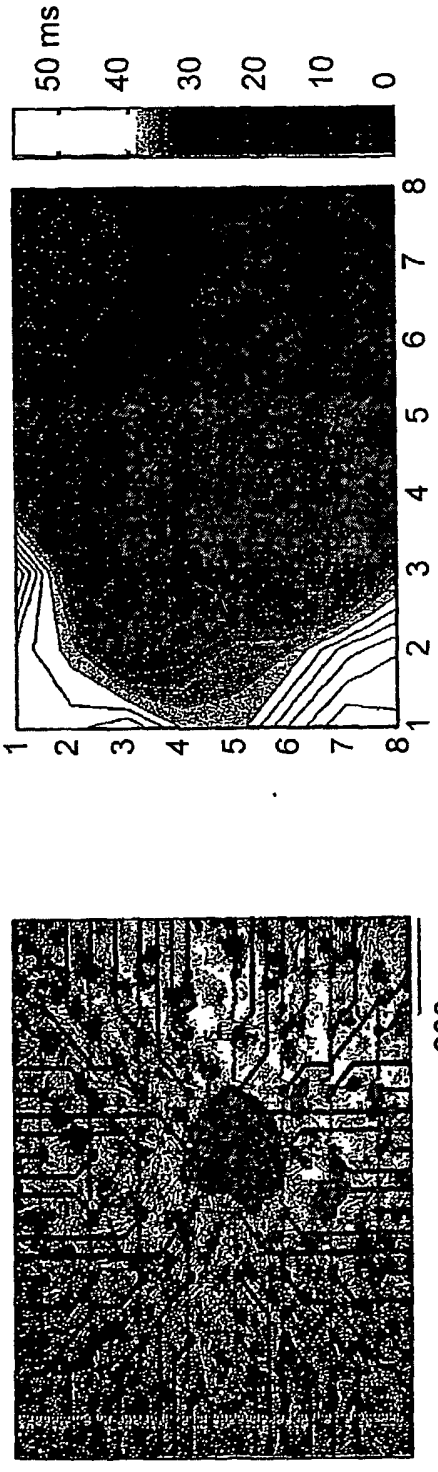
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200 µm

FIG. 3A

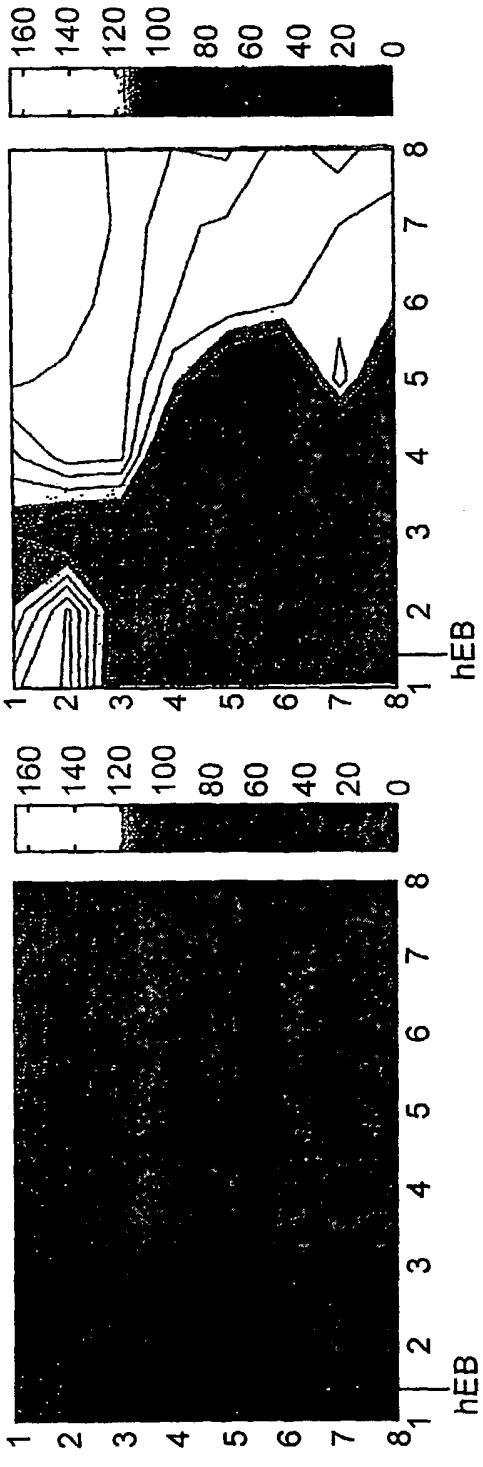
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FIG. 3B

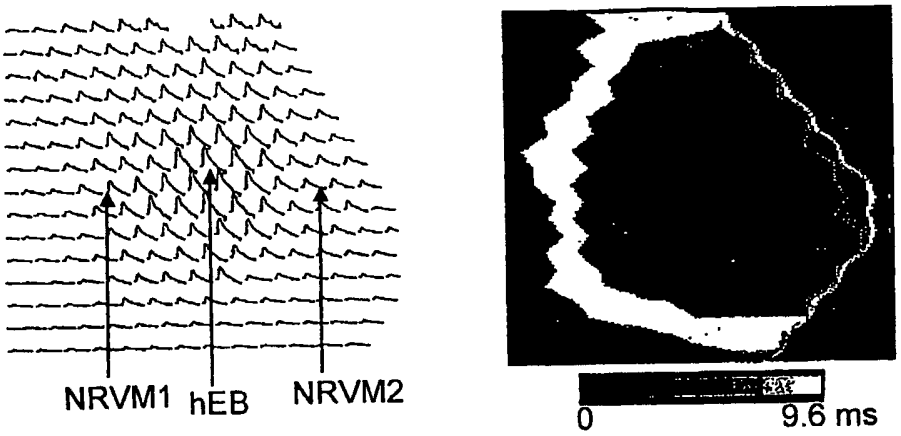
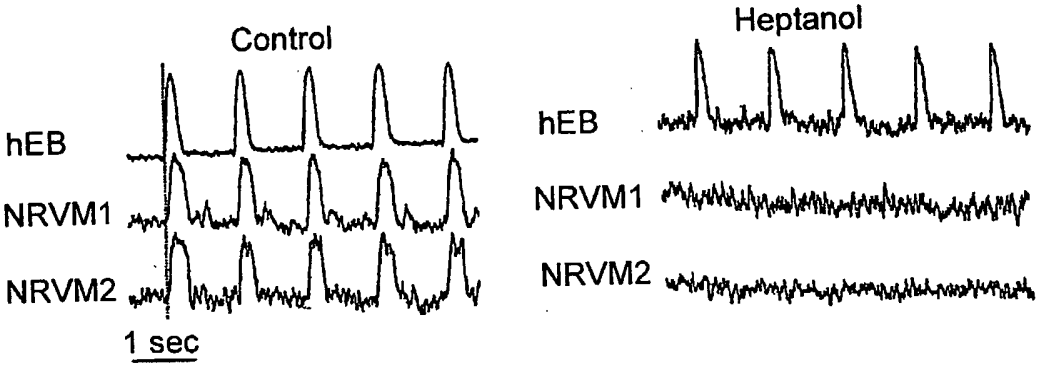


FIG. 4A



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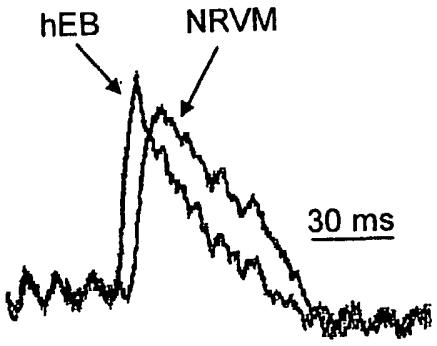


FIG. 4C

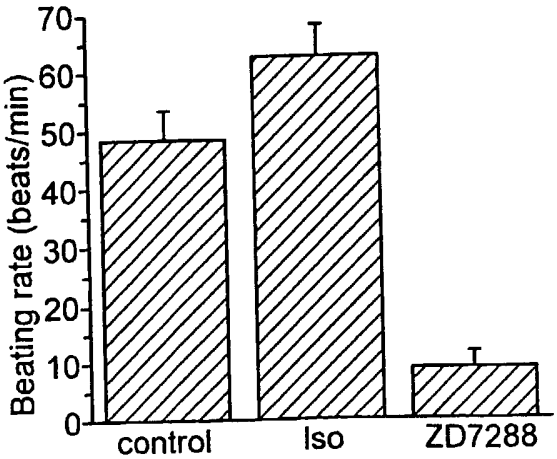


FIG. 4D

FIG. 5A

FIG. 5B

FIG. 5C

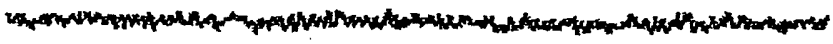


FIG. 5D

FIG. 5E

FIG. 5F

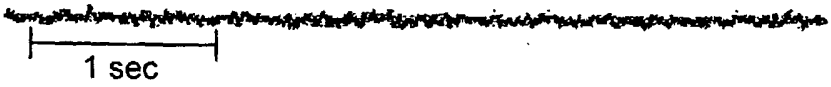
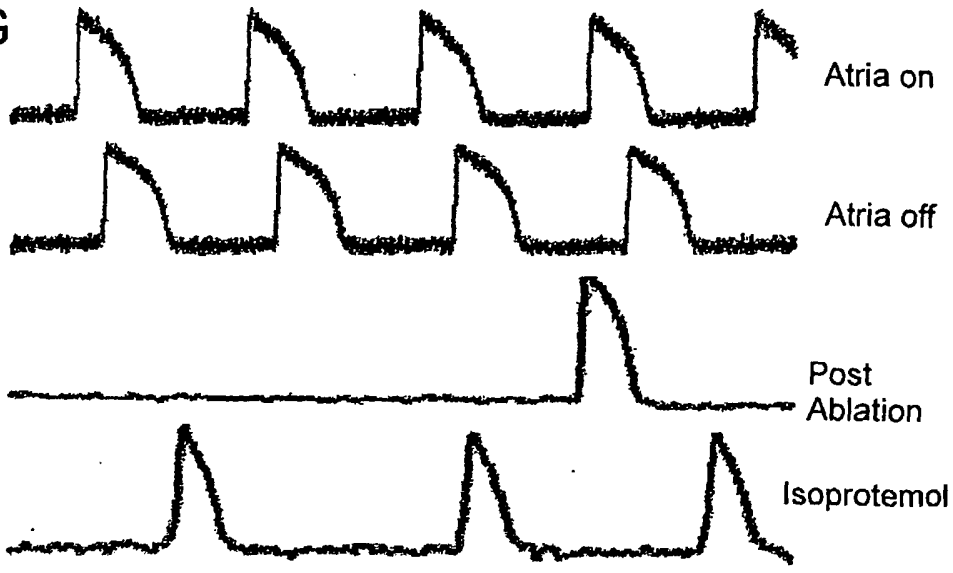


FIG. 5G



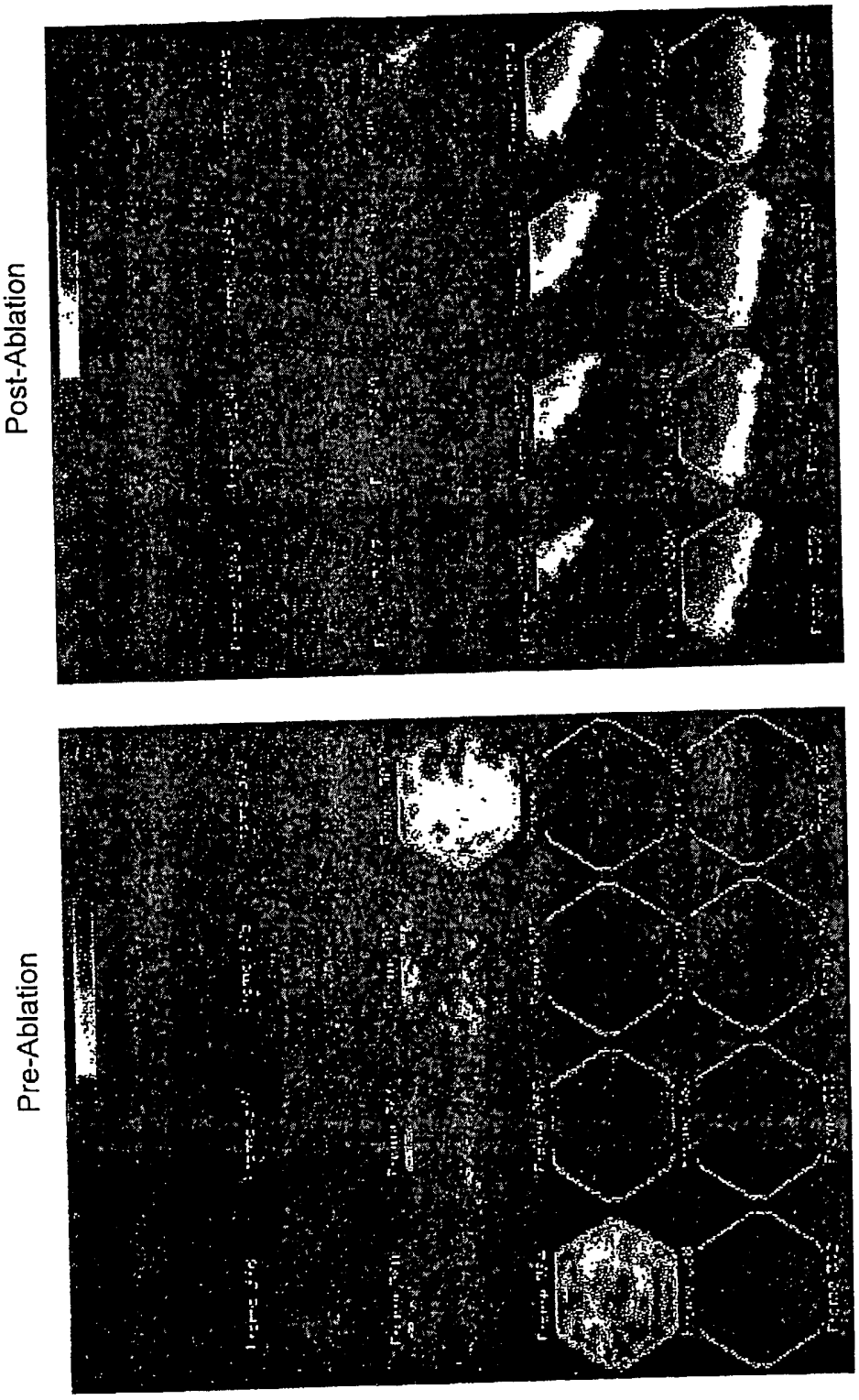


FIG. 6

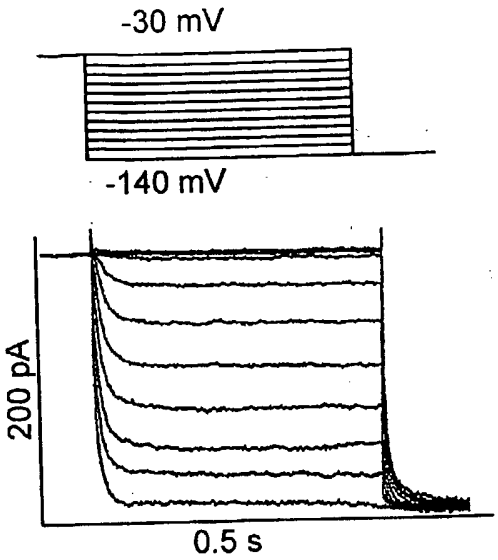


FIG. 7A

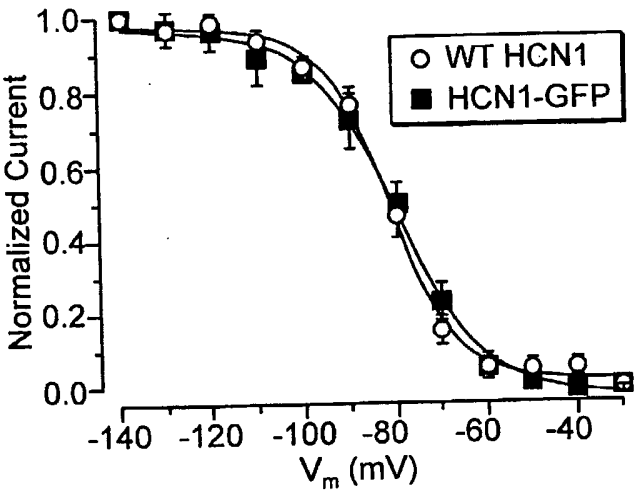
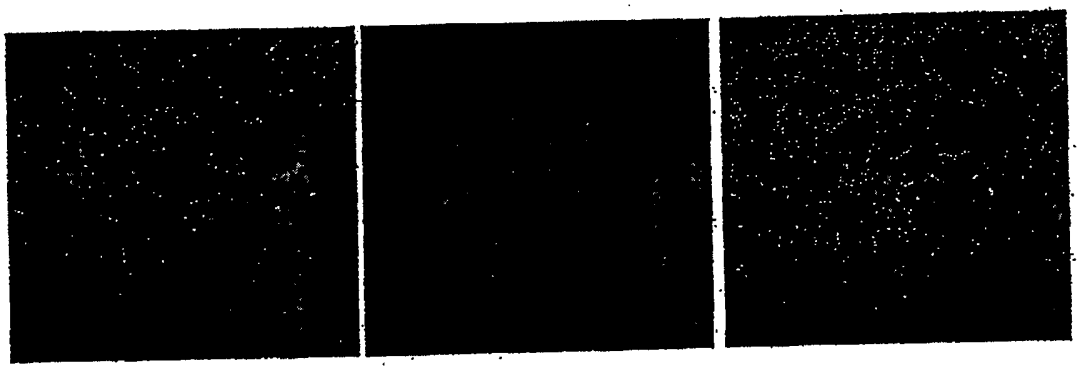


FIG. 7B



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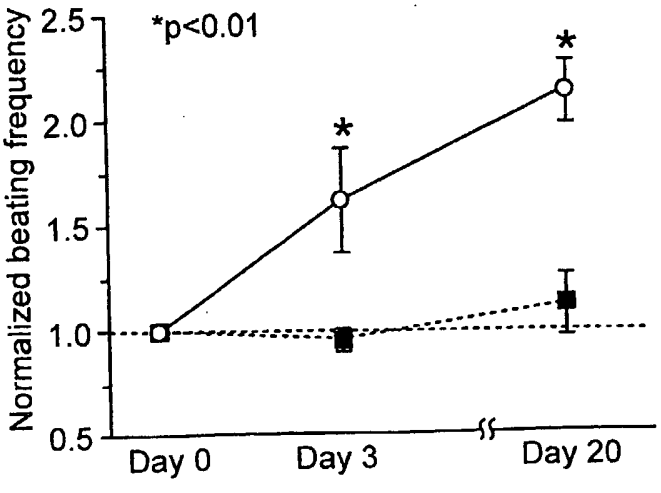

FIG. 7D