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Cardiomyocytes Differentiated from Human Pluripotent Stem Cells as an Experimental Model for Anesthetic-induced Preconditioning

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Kardiomiociti diferencirani iz ljudskih pluripotentnih stanica kao eksperimentalni model za prekondicioniranje potaknuto anesteticima

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LIST OF ABBREVIATIONS

5-HD	5-hydroxydecanoate
APC	Anesthetic preconditioning
BMP4	Bone morphogenetic protein 4
CACNA1C	Calcium channel, voltage-dependent
c-MYC	c-avian myelocytomatosis viral oncogene homolog
CsA	Cyclosporine A
cTnT	Cardiac troponin T
Ctrl	Control
EB	Embryoid body
EGFP	Enhanced green fluorescent protein
ETC	Electron transport chain
Eth	Ethidium
FGF	Fibroblast growth factor
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GATA4	GATA binding protein 4
HCN4	Hyperpolarization-activated cyclic nucleotide-gated channel 4
hESC	Human embryonic stem cells
hiPSC	Human induced pluripotent stem cells
I/R	Ischemia and reperfusion
ISL1	ISL LIM homeobox 1
Iso	Isoflurane

KCND3 Potassium voltage-gated channel, Shal-related subfamily, member 3

KCNH2 Potassium voltage-gated channel, subfamily H (eag-related), member 2

KCNJ2 Potassium inwardly-rectifying channel, subfamily J, member 2

KLF4 Kruppel-like factor 4

LIN28 Lin-28 homolog

MEF Mouse embryonic fibroblast

MEF2C Myocyte enhancer factor 2A

MESP1 Mesoderm posterior basic helix-loop-helix transcription factor 1

MLC 2a Myosine light chain 2a

MLC 2v Myosin light chain 2v

mPTP Mitochondrial permeability transition pore

mtioKATP Mitochondrial ATP-sensitive potassium channel

MYH6 Myosin, heavy chain 6, cardiac muscle, alpha

MYL7 Myosin light chain 7

Nanog Nanog homeobox

Nkx2.5 NK2 homeobox 5

OCT4 Octamer-binding transcription factor 4; also known as POU5F1 (POU class 5 homeobox 1)

PAX6 Paired box 6

PC Preconditioning

PI Propidium iodide

PKC Protein kinase C

qPCR Real time quantitative PCR

ROS	Reactive oxygen species
RPL13A	Ribosomal protein L13a
SCN5A	Sodium channel, voltage-gated, type V, alpha subunit
SOX17	Sex determining region Y-box 17
SOX2	Sex determining region Y-box 2
SSEA3 and 4	Stage-specific embryonic antigen 3 and 4
Tbx20	T-box 20
TGFB	Transforming growth factor β
TMRE	Tetramethylrhodamine ethyl ester
TNNT2	Troponin T type 2, cardiac
Wnt	Wingless-type MMTV integration site family
$\Delta\Psi m$	Mitochondrial membrane potential

1. INTRODUCTION AND THE PURPOSE OF THE DISSERTATION

Pluripotent stem cells

Stem cell pluripotency. By definition, stem cells have the capacity for self renewal and differentiation toward one or more mature cell types.(1-3) Thus, virtually any cell type in the body can be derived from pluripotent stem cells. Therefore, pluripotent stem cells have immense potential for various applications.

In general, the pluripotency of stem cell lines is tested by analyzing the expression of pluripotency markers and their ability to differentiate into more mature cell types. Pluripotent stem cells can differentiate into derivatives of all three embryonic germ layers.(4) As such, they have an immense potential for various applications in clinical practice and industry like generation of human cardiomyocytes for transplantation therapy of infarcted myocardium or for in vitro pharmacological testing.(5, 6) Human embryonic stem cells (hESCs) have been the most widely studied pluripotent stem cells, but their use is hampered by ethical issues and immune rejection of allogenic graft.(2, 7) However, they are considered as gold standard in stem cell biology due to their natural pluripotency. Recently, generation of human induced pluripotent stem cells (hiPSCs) by introduction of pluripotency factors into skin fibroblasts resolved these two issues.(8) Similar to hESCs, hiPSCs exhibit indefinite self-renewal and the ability to differentiate into virtually any cell type in human body.(3) Hence, hiPSCs provide the possibility of performing cell replacement therapy with patient-matched cells, circumventing severe complications of life-long immunosupression therapy that inevitably follows allogenic grafts.(9)

Two hESC, H1 and H9, and two hiPSC lines, C2a and C6a, used here were previously validated for their pluripotency.(10-12) H1 and H9 lines are morphologically similar to rhesus monkey ESC and have normal karyotypes. They also express high telomerase activity which indicates their immortality. H1 and H9 cells also express characteristic markers specific for undifferentiated cells, i.e. alkaline phosphatase, SSEA3, SSEA4, TRA-1-60 and TRA-1-81.(11) Teratomas are formed when these cells are transplanted to immunocompromised, SCID-beige mice. Teratomas are tumors formed from more than one germ layer indicating pluripotency of these cells. As pluripotent stem cells, H1 and H9 can be differentiated into all three germs layers and their derivatives, such as cardiomyocytes, neurons, hepatocytes and other cell types.(11, 12)

C2a and C6a hiPSC lines were generated in the laboratory of Dr. S Duncan, MCW by transducing human foreskin fibroblasts with lentiviruses encoding OCT4 (octamer-binding transcription factor 4; also known as POU5F1 (POU class 5 homeobox 1)), SOX2 (sex determining region Y-box 2), NANOG (Nanog homeobox) and LIN28 (lin-28 homolog).(10) These cells are morphologically similar to ESCs and express pluripotency markers OCT4 and SSEA4 and exhibit alkaline phosphatase activity. They also form teratomas and display normal karotypes. Cells can be differentiated into derivatives of all three germ layers, including neuronal tissue, sebaceous tissue, columnar epithelium, gut-like epithelium, striated muscle, blood cells, adipose tissue, cartilage and immature bone. DNA fingerprinting showed that lines completely match human foreskin fibroblasts that were used for reprogramming.(10)

hiPSCs. Until recently, lines of pluripotent cells derived from human embryos, hESCs were the most widely investigated. However, the potential for therapeutic use of ESCs is hampered by ethical issues, immune rejection of allogeneic grafts, and oncogenic risk. The first two of these issues may have been recently resolved by the ability to generate iPSCs from human dermal fibroblasts, via transduction of pluripotency factors encoded in retroviral vectors.(8, 9) iPSCs provide the possibility of performing cell replacement therapy with patient-matched cells. In addition, disease-matched iPSC-lines will enable studies to determine the influence of genetic background on the differentiation of specific cell types, which has already been accomplished in the instance of a congenital heart defect that causes hypertrophic cardiomyopathy.(13)

hiPSCs are probably the fastest developing field in stem cell research and among the most promising areas of science, translational research and clinical medicine.(14, 15) Ever since the first description of the iPS cells in 2006, the field rapidly grew and it is on a verge to become one of the driving forces of translational research.(6) hiPSCs also have the immense potential in regenerative medicine, circumventing considerable problems that accompany the use of hESCs.(15) However, our knowledge about the applicability of hiPSCs for these purposes is limited and there is still uncertainty whether hiPSCs have comparable potential in cardiomyogenesis as hESCs. Takahashi and Yamanaka successfully derived iPSCs from adult mouse fibroblasts through ectopic coexpression of four genes OCT4, SOX2, KLF4 (Kruppel-like factor 4 (gut)) and c-MYC (c-avian myelocytomatosis viral oncogene homolog).(16) Very soon human fibroblasts were also converted into pluripotent stem cells followed by successful reprogramming of variety of other cells including adipose stem cells,

neural stem cells, stomach cells liver cells, melanocytes, keratinocytes and other cell types.(15, 17) Also, it was demonstrated that other combinations of stemness factors can be used for successful reprogramming, like OCT4, SOX2, LIN28, NANOG or OCT4, SOX2, KLF4 without c-MYC. At the beginning, reprogramming required transduction of cells using retroviral vectors which introduced potential side effects like the induction of inflammation or caused cell rejection after transplantation. Moreover, insertion of viral genome at unknown locations can generate oncogenic response.(15)

Recently, it was shown in several studies that iPSCs can be generated by using non-integrating viruses or without using viral vectors at all.(15) Variety of growth factors and chemical compounds have been found to improve the efficiency of iPSC induction, like 5-azacytidine and valproic acid. There is a series of molecular hallmarks which distinguish fully reprogrammed iPSCs from those only partially reprogrammed. They include OCT4, SOX2, NANOG, telomerase gene and SSEA1.(15) Genome-wide epigenetic reprogramming is essential for generating cells which are fully reprogrammed and thus comparable to ESCs. iPSCs which originate from different cell sources show similar gene expression patterns in the pluripotent state, but they also more readily differentiate toward the tissue of origin. Therefore, the memory could be epigenetic. The evaluation of methylation at the promoters of the pluripotency genes and of the genes responsible for driving differentiation is a way of measuring the success of reprogramming.(15)

Ethical issues and regulatory acts. Experiments carried out in this thesis are performed in accordance with European Science Foundation and International Society for Stem Cell Research issued rules and guidelines to ensure transparent, ethical and responsible research (please see link http://www.isscr.org/docs/default-source/hesc-guidelines/isscrhescguidelines2006.pdf). The major ethical concern of this thesis is the use of human embryonic stem cells (hESCs). However, this research is in agreement with the widely accepted guidelines of hESCs research, i.e. only commercially available hESCs were utilized. We did not generate new lines of hESCs or in any other way use human embryos or their parts. We did not use pre-implantation human embryos, do any transplantations into living human subjects or generate chimeric animals. Each research project using hESCs should take in consideration potential scientific questions, examine the current ethical concerns and estimate whether this research would advance towards clinical application.

European Science Foundation (ESF) published in 2002 a Science Policy Briefing (SPB) entitled *Human Stem Cell Research: Scientific Uncertainties and Ethical Dilemmas*.

Since then there have been many significant advances in this field of research, therefore the European Medical Research Councils (EMRC) at the ESF established a High-Level Expert Group to reflect the rapidly changing scope of the field. These members made specific recommendations ensuring that the research is conducted in accordance with accepted principles of research ethics. Furthermore, the International Society for Stem Cell Research (ISSCR) has formed the Task Force which has formulated the *Guidelines for the Conduct of Human Embryonic Stem Cell Research* that articulate ethical principles and rules of behavior for the performance of human stem cell research. ISSCR calls for due consideration and appropriate oversight of human stem cell research to ensure transparent, ethical and responsible performance of scientific experiments. To ensure that stem cell research is proceeding with due consideration ISSCR proposes specific categories of research, which are following:

1. Category 1: Experiments that are permissible after review under existing mandates and by existing local committees, and are determined to be exempt from full Stem Cell Research Oversight (SCRO) review. These will include experiments with pre-existing hESC lines that are confined to cell culture or involve routine and standard research practice, such as assays of teratoma formation in immune-deficient mice.
2. Category 2: Forms of research that is permissible only after additional and comprehensive review by a specialized mechanism or body established to address the issues pertinent to stem cell research.
3. Category 3: Research that should not be pursued at this time because of broad international consensus that such experiments lack a compelling scientific rationale or raise strong ethical concerns.

Experiments proposed in the present thesis include only the use of human embryonic stem cells that are confined to cell culture, can be commercially purchased and involve routine and standard procedures, such as *in vitro* differentiation into derivatives of embryonic layers, immunocytochemistry or PCR experiments. These experiments are determined by Category 1 from ISSCR's *Guidelines for the Conduct of Human Embryonic Stem Cell Research.* We are not performing any of the experiments described in categories 2 or 3 which include procedures like derivation of new human pluripotent cell lines, use of pre-implantation human embryos, transplantations into living human subjects or generation of chimeric animals.

Importance of Stem Cell Biology. Stem cell research has an important role in applied science, including: 1) disease modeling and studying pathogenesis of human diseases; 2)

embryonic development and tumor pathogenesis and 3) basic studies of regeneration of tissues and organs.

In his recent article, Dr. Francis S. Collins, the Director of National Institutes of Health pointed out that the current process of development of new drugs, which is based on translation findings from animal models to clinical practice, is costly, takes too much time and has a high rate of failure.(6) It takes 13 years to develop one drug with the cost of more than a billion dollars and the failure rate of 95%. This process is inefficient in large part since results obtained on animals do not reliably reflect the nature of human disease and drug safety in humans. He identified the use of cells derived from hESCs and hiPSCs as a promising strategy that may circumvent these problems and serve as a complementary experimental model. The use of human stem cell-based models is called "clinical trial in a dish". In this dissertation we tested cardiomyogenesis in hESCs and hiPSCs and validate it by testing their potential to yield cells with a phenotype of functional cardiac cell, i.e. cardiomyocytes that can rhythmically contract and express organized contractile machinery-sarcomeres. This characterization was crucial for our ability to understand the potential of hiPSCs to be used as pharmacological model of human cells.

An important aspect of stem cell research includes cancer stem cells.(18) In the past several years, impressive studies showed existence of cancer stem cells as a specific subpopulation of tumor cells, which play the key role in cancer development, metastasizing and resistance to chemo- and radiotherapy.(18) Furthermore, studies show that tumor cells exhibit characteristics that are genetically-driven, not just by random mutation, but with activation of complex programmed processes that in many aspects resemble those in normal stem cells, suggesting activation of stem cell genes in cancer stem cells.(19) Therefore, cancer stem cells open exciting new horizons in studying tumor development and designing effective targeted therapies.

Probably the most groundbreaking aspect of human stem cell research is their application for regenerative medicine. With the increase in the number of patients suffering from chronic diseases and increasing ageing of population, the requirement for transplantation of organs is in constant rise, while the number of suitable organ/tissue donors is insufficient. For many terminal organ insufficiencies (e.g. heart and liver failure) transplantation is the only therapy. Potential applications of stem-cell-based therapy that are vigorously investigated include cardiac muscle repair following myocardial infarction, treatment of neurodegenerative disorders like Parkinson's disease and the replacement of the non-functioning cells of the Langerhans islets in diabetes mellitus. Mouse liver can be regenerated

with hiPSCs.(10) Clinical trials have been initiated to examine the potential of using stem cell for regeneration of various organs.

The loss of terminally differentiated cardiomyocytes is replaced by proliferation of connective tissue that diminishes contractile function leading to heart failure. The only true and efficient therapy of infarcted myocardium would be replacing cardiomyocytes with new cardiomyocytes. Thus, considerable research efforts and funds are aimed at testing the potential of stem cells in regeneration of dysfunctional heart. However, at present, this approach is hampered by poor engraftment of transplanted cells.(20) This dissertation may advance the field of transplantation medicine since it may offer new possibility to increase the efficiency of stem cell engraftment by protecting them with anesthetic preconditioning (APC). Ethical issues and the immune rejection of allogenic transplant hamper the transplantation potential of hESCs. On the other hand, hiPSCs offer promising alternative since they originate from patient's own cells, like fibroblasts, and thereby are not burdened with ethical issues nor immune reaction.(8)

Shinya Yamanaka is a Nobel Prize laureate for 2012 for the discovery of iPSCs that was published in the *Cell* in 2006.(16) In 2013, nearly 20,000 manuscripts related to stem cells have been published in PubMed database. The ClinicalTrials.gov website has more than 4,500 listed clinical trials related to stem cells. The importance of stem cells research and organ recovery is recognized by world's leading biomedical institutions that have established centers or institutes for stem cells and regenerative medicine.

The Development of the Heart and Cardiomyocyte Differentiation

Among other possible implementations pluripotent stem cells have contributed to developmental biology research through generation of an in vitro model which enables studying normal and pathological development of an early mouse and human embryos.(21-23) Several studies have shown that mouse ESCs recapitulate important early cardiogenic events in vitro.(24) Upon the derivation of the human ESCs it has been shown that they can give rise to the functional cardiomyocytes.(11, 12)

Circulatory system, comprised of a heart, blood cells and blood vessels is the first functional unit in the developing embryo providing nourishment to the growing organism. In the amniote vertebrates, the cells which will later form the atrial and ventricular musculature, the Purkinje conducting fibres and the endothelial cells of the heart originate in the early primitive streak, migrate through it and form the cardiogenic mesoderm at each side of the Hensens node.(25, 26) Induction of the specification of the cardiac precursor cells comes

from the endoderm adjacent to the heart and includes BMP and FGF signaling pathways.(24, 25) Heart precursor cells are specified by the overlap of Wnt (wingless-type MMTV integration site family) antagonists and BMPs.(24) Upon and in parallel with specification heart precursor cells migrate towards the lateral walls of the anterior gut tube. The signals for this migration come from the foregut endoderm. Upon the formation of the foregut by inward folding of the splanchnic mesoderm the two cardiac tubes are brought together.(25, 27) While migrating, the cells of the cardiogenic mesoderm are instructed by the BMP signals to synthesize the NKX2.5 (NK2 homeobox 5) transcription factor which acts as a transcriptional activator of atrial natriuretic factor in cooperation with GATA4 (GATA binding protein 4) and probably other members of GATA and MEF2 (myocyte enhancer factor 2) families.(24, 27) These transcription factors activate the expression of cardiac specific genes which encodes proteins such as cardiac actin, titin, atrial natriuretic factor and myosin heavy chains.(24) The heart cells differentiate in the two separate heart primordia migrating toward each other. Their merging is forming an epithelium which will give rise to myocardium. The fusion of the two heart primordia occurs at about 3 weeks in human gestation, but the pulsations of the heart begin before the fusion is completed. The pulsations can occur upon the development of functional sodium-calcium exchange pump in the muscle cell membrane. In 5-week human embryo the heart is composed of one atrium and one ventricle. Looping of the heart is designated within the heart primordium while the NKX2.5 regulates the HAND1 and HAND2 transcription factors. HAND1 is restricted to the future left ventricule and HAND2 to the right as the looping progresses.(24, 26, 27)

It has been demonstrated that cardiac precursor cells are present in the pre-streak embryos, before the process of gastrulation in which the three germ layers are formed.(24-26) Upon the formation of primitive streak a group of epiblast cells undergoes epithelial-to-mesenchymal transition and formes the transient layer - the mesendoderm.(27) The segregation of endoderm and mesoderm from mesendoderm is a key event in defining the cardiac lineages. It has been shown that GATA factors 4, 5 and 6 play an immportant role in specification of both endoderm and mesoderm.(27) Furthermore, it has been shown both in embryos and hESCs that members of the TGFβ family (transforming growth factor beta) Nodal and activin are acting in accordance with Wnt/b-catenin pathway in defining the mesendoderm formation.(24) Another member of TGFβ family, BMP2 which is secreted by both visceral and pharyngeal endoderm is responsible for further specification of mesendoderm into cardiac specific direction.(27) Transcription factor OCT4 enables normal cardiogenesis through its target gene SOX17 required for specification of cardiac mesoderm.

Mesendodermal transient cell status depends on an equilibrium of Nodal/BMP and Wnt pathways and it is crucial in determination of cardiac cell fate. The anterior part of the primitive streak contains precursors of endocardium, myocardium and epicardium. These mesodermal cells express GATA 4,5 and 6, HAND1 and 2, WT1 (Wilms tumor 1) and NKX2.5.(24, 25, 27) The signals which induce the migration of cardiogenic mesodermal cells remain to be investigated but it is suggested that the transcription factor MESP1 has a role in this process. It has been shown that MESP1 is immportant for epithelial-to-mesenchymal transition and for delamination and migration of cardiac progenitors from the primitive streak.(27) Several cell lineages originate from the lateral mesoderm including haematopoietic and cardiac cell lineages. These two lineages are segregated early during the migration of epiblast through the primitive streak. A study done by the Duncan′s laboratory shows an essential role of GATA 4 and 6 in the cardiac transcriptional pathway.(28) A recent study also shows the immportant role of FGFs in favouring the cardiac mesoderm at the expense of hematopoietic one.(24) In cardiogenic mesoderm the transcription factor NKX2.5 repress the haemangiopoietic program in part by repressing GATA1 which further antagonises the cardiac and hematopoietic cell lineages. Next, a population of cardiac progenitors migrates into the heart field with subsequent differentiation into either endocardial or myocardial cells.(24, 26, 27) Several genetic lineage tracing studies suggest that endocardial and myocardial cells share a common FLK1+ progenitor.(29) In vitro studies utilizing ESCs have suggested the presence of two cardiac cell lineages, first (ISL1-,TBX5+) and second (ISL1+, TBX1+, RALDH2+, FOXH1+) which can be segregated from the MESP1+ ESC-derived mesodermal cells.(24)

Disease Modeling Based on Stem Cell Differentiation

iPSCs are generated by reprogramming fibroblasts or other mature, differentiated cells.(8) Reprogramming enables cells to return to the state of pluripotency and subsequently to be re-differentiated into cardiac, neuronal, hepatic or other lineages.(10, 30, 31) Fibroblasts can be conveniently isolated from small (several millimeter) skin samples from the patient. Reprogramming is based on various techniques of delivery of pluripotency factors or genes. Cells differentiated from reprogrammed fibroblasts maintain original genetic material (nuclear and mitochondrial DNA) of the patient and cellular characteristics (phenotype)-driven by the genotype. An increasing number of publications in high-impact journals show that iPSCs obtained from a skin sample of a patient with genetically-driven disease can be differentiated into cardiomyocytes, neurons or other cell types that exhibit

characteristics of that disease (Figure 1).(32, 33) Such proof-of-principle studies demonstrate that human iPSCs can be used for studying pathogenesis of human disease and development of new drugs and other therapeutic interventions using human experimental models instead of animal that are not completely compatible to human genetically-driven diseases. For example, human iPSC-cardiomyocytes were derived from patients with various arrhythmias with long QT syndromes or catecholaminergic polymorphic ventricular tachycardia, human iPSC-neurons were generated from patients with Parkinson's disease and Best disease.(33-36) iPSCs were also reprogrammed from mice with lysosomal storage diseases.(33, 37) All these models exhibit characteristic electrophysiological, metabolic and other characteristic phenotypes. Other diseases can also be modeled with iPSC approach, including neurons for Alzheimer's disease or hepatocytes for hereditary metabolic diseases of the liver.(31, 38) iPSCs are also used for in vitro studies of human embryonic development, which is a new powerful tool in efforts to understand developmental disorders. The latest and exciting breakthrough is a study that demonstrates *in vitro* genesis of "cerebral organoids", a 3D culture system that recapitulates cortical development with formation of discrete brain regions, including organized mature cortical neuron subtypes.(39) Authors also demonstrated successful modeling of microcephaly using the same model.

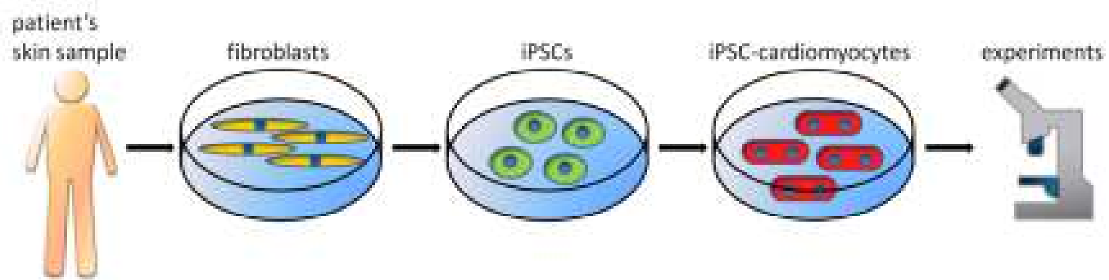


Figure 1. Disease modeling using patient-derived hiPSCs. Human iPSCs can be generated by introducing pluripotency factors into fibroblasts. Obtained iPSC colonies can be further differentiated into cardiomyocytes or other cell types and subsequently used for experiments. The patient's original genetic material is preserved from isolated fibroblasts, iPSCs down to iPSC-cardiomyocytes. Therefore, the phenotype of such iPSC-cardiomyocytes is in part driven by patient's genetic background. This allows examination of these genotype-driven characteristics in strictly controlled experimental environment. Moreover, interactions among environmental and genetic factors can be examined. Such approach is particularly useful for experiments with human cardiomyocytes since these cells are almost unavailable for experimental purposes.

Myocardial Infarction and Ischemia-Reperfusion Injury

Cessation of blood flow through coronary arteries (ischemia) inevitable causes cardiomyocyte death, i.e. myocardial infarction, unless the blood flow is reestablished. However, it has been well documented that reperfusion following relatively short ischemia induces more cell death than the ischemia itself.(40, 41) This seeming paradox has been described as reperfusion injury, which encompasses multiple noxious stimuli.(41-43) Myocardial infarction may lead to acute and chronic heart failure and death. Life threatening complications include various arrhythmias, like ventricular fibrillation, rupture of papillary muscle with mitral valve insufficiency, ventricular aneurism and wall rupture causing cardiac tamponade, and cardiogenic shock due to reduced contractility. Death of cardiomyocytes during ischemia-reperfusion is a consequence of complex intracellular processes that can be attenuated by various interventions, including preconditioning.(44, 45)

Reactive oxygen species. The burst of reactive oxygen species (ROS) production during the first couple of minutes of reperfusion is considered as the most important stressor of the reperfusion.(46, 47) Attenuation of this ROS burst by different strategies has led to improved cardiomyocyte survival in various *in vitro* and *in vivo* experimental models.(48, 49) For example, superoxide dismutase (SOD) overexpression reduces infarct size in transgenic mice.(50) Different preconditioning strategies attenuate excessive ROS production during reperfusion *in vivo* and during simulated reperfusion *in vitro* and thereby protect cardiomyocytes.(51, 52) However, clinical trials attempting to introduce antioxidants during coronary reperfusion in humans did not show any protective effects.(53) A plausible explanation for this disappointing result is that the burst of ROS production in reperfusion occurred before antioxidants were able to diffuse into cardiac mitochondria, which is the main site of ROS production in injured cardiomyocytes.(47)

ROS can damage numerous cellular molecules and structures, including lipid peroxidation, protein oxidation and carbonylation.(54, 55) Opening of the mitochondrial permeability transition pore (mPTP) by ROS is the critical event triggering cell death pathways, and protective effects of antioxidants are well correlated with attenuation of mPTP opening.(56, 57) An important source of excessive ROS production during reperfusion are inflammatory cells that infiltrate injured myocardium attracted by necrotic cells.(58) However, in early reperfusion of the heart, ROS are predominantly produced by postischemic cardiomyocytes, which is stimulated by sudden repletion of oxygen and increase in mitochondrial membrane potential.(47, 59) Alterations in the respiratory chain precipitated by ischemia in the form of decreased activity of the complex III, lower cytochrome c content

and decreased respiration through cytochrome oxidase also contribute to reperfusion ROR burst.(60-64) Other sources of ROS within cardiomyocytes include xanthine oxidase and NADPH oxidase.(65)

The extent of ROS generation during ischemia is controversial. Although some *in vitro* studies show increase in ROS production during ischemia, these results are questionable primarily due to the fact that during ischemia oxygen levels are far too low to provide substantial amount of substrate for superoxide generation.(42, 66) In these experiments cardiomyocytes were in contact with atmospheric air, which is not the case *in vivo*, and which could have served as the source of oxygen for ROS generation, probably causing observed artifact.(42)

Calcium. The burst of ROS production during reperfusion also stimulates accumulation of cytosolic calcium by stimulating its release via the ryanodine receptor on the sarcoplasmic reticulum.(67-69) However, intracellular calcium starts to rise also during ischemia, when ROS concentration is probably negligible (as discussed above).(70) A drop in intracellular ATP concentration during ischemia is responsible for calcium overload in ischemic cardiomyocytes by several different mechanisms. First, ATP-ases that actively pump calcium from the cytoplasm stop working due to low ATP.(42) This refers to plasma membrane calcium ATP-ase (PMCA) and sarcoplasmic/endoplasmic reticulum calcium ATP-ase (SERCA). Moreover, a decrease in intracellular pH due to anaerobic lactate production activates sodium/hydrogen exchanger that imports sodium into the cell.(71) This in combination with slowing down of sodium/potassium exchanger due to low ATP decreases the transmembrane sodium gradient that in turn reverses the action of sodium/calcium exchanger, leading to calcium import into the cell.(72) Last, depolarization of sarcolemma facilitates opening of voltage-sensitive L-type calcium channels whose opening leads to further calcium inflow.(42, 73) Calcium-induced calcium release, where calcium stimulates its own release from the sarcoplasmic reticulum by the ryanodine receptor exacerbates calcium accumulation.(74)

A rise in cytosolic calcium will facilitate mitochondrial calcium uptake, driven by the mitochondrial membrane potential ($\Delta\Psi_m$), via mitochondrial calcium uniporter.(75, 76) In mitochondria, calcium stimulates respiration and thereby can enhance ROS production during early reperfusion.(77) Moreover, calcium and ROS stimulate opening of the mPTP in mitochondria which can further stimulate ROS production and contribute to calcium overload by releasing intramitochondrial calcium.(78, 79) Hence, accumulation of calcium and excessive ROS production depend on multiple interconnected processes, which can serve as

target for cardioprotective strategies. APC may directly attenuate excessive ROS production by slightly depolarizing mitochondria, but it may also indirectly attenuate intramitochondrial and cytosolic calcium overload by reducing ROS-induced calcium release from the sarcoplasmic reticulum.(80) Decrease in intracellular pH during ischemia has protective effects, since protons compete with calcium for the binding site on the mPTP, thereby preventing calcium-induced mPTP opening.(81)

In addition to opening of mPTP, calcium overload will lead to activation of various enzymes including endonucleases and proteases, such as calpain that will cleave intracellular structural and other proteins contributing to necrotic cell death.(42, 82)

Anesthetic Preconditioning

In 1986 Murry and colleagues demonstrated that several brief periods of ischemia and reperfusion reduced infarct size when dogs were subjected to prolonged ischemia and reperfusion.(45) This has been named ischemic preconditioning. Later on various other agents were shown to induce preconditioning and protect the heart from ischemia and reperfusion injury. Volatile anesthetic, such as isoflurane, sevoflurane and desflurane are among those agents and this has been called anesthetic preconditioning (APC).(83) APC is a cardioprotective strategy that increases resistance to ischemia and reperfusion (I/R) by eliciting innate protective mechanisms.(83, 84) Preconditioning protects other organs, as well, like brain liver and kidney.(85-87) Volatile anesthetics may reduce reperfusion injury if applied at the onset of reperfusion, which is called anesthetic postconditioning, and it is clinically more applicable strategy(88). Postconditioning recruits RISK (Reperfusion Injury salvage Kinases) and SAFE (Survivor Activating Factor Enhancement) pathways.(89) RISK pathway is somewhat similar to preconditioning signaling cascade. Induction of ischemic preconditioning of a limb can also protect the heart and this is called remote preconditioning.(90) A randomized, single-blinded, placebo-controlled prospective study demonstrated that combination of isoflurane/sufentanil anesthesia and remote preconditioning by intermittent upper arm ischemia reduced serum troponin I in patients undergoing coronary artery bypass graft (CABG) surgery.(91) This study further corroborated clinical value of cardioprotection by volatile anesthetics and demonstrated importance of unraveling mechanism of cardioprotection and finding optimal protocols for clinical practice.

<u>General principles of APC.</u> It is now recognized that preconditioning activates endogenous cytoprotective mechanisms in cells rendering them more resistant to ischemia-reperfusion injury and oxidative stress, which is a hallmark of reperfusion injury.(80, 83) The

signaling phase of preconditioning, including APC, includes signal transduction cascade initiated by preconditioning stimulus (brief ischemic episode, application of volatile anesthetic or hydrogen peroxide) that results in activation of effectors of preconditioning.(92, 93) These effectors remain active even after removal of the anesthetic, i.e. during prolonged ischemia and reperfusion, which is called memory phase.(92) There are two windows of protection during memory phase, the first lasting for couple of hours and second starting 12h after the stimulus and ending 24-48h later.(94) The first window of protection is characterized by activation of effectors of protection by posttranslational modifications, such as phosphorylation or by translocation of crucial regulatory proteins, including translocation of protein kinase C to mitochondria.(93) Some of the identified effectors of protection are mitochondrial and sarcolemmal K_{ATP} channels.(95-97) The former depolarizes mitochondria and attenuates mitochondrial ROS production and calcium accumulation.(83) The later hyperpolarizes sarcolemma reducing deleterious calcium influx.(83)

Signal transduction cascade of APC involves several parallel and independent pathways.(92) The important components of this cascade are extracellular signal-regulated kinases (ERKs), phosphatidylinositol 3-kinase (PI3-kinase)/protein kinase B (Akt) pathway, protein kinase C. Activation of adenosine receptors (A1 and A3) by released adenosine also transmits preconditioning signals.(84, 93) It has been noticed in clinical work that myocardial infarction is smaller in patients who experienced attacks of angina pectoris 2h prior to coronary occlusion that in those who did not have prior chest pain.(98) This is considered as clinical correlation of ischemic preconditioning. Over the past years, the laboratory of Dr. Bosnjak has made a substantial progress in understanding fundamental processes responsible for the development of cardiac ischemia-reperfusion injury and the protection against it by APC. Mitochondria emerged as central organelles involved in pathological processes during ischemia-reperfusion, but also a key site of preconditioning signal and effectors.(80, 83)

Cardioprotection by volatile anesthetics is now accepted in clinical practice. Namely, the American Cardiology Society and the American Heart Association recommend the use of volatile anesthetics due to their cardioprotective properties in surgeries where heart damage can be expected.(99) However, ageing and diabetes mellitus adversely affect the ability of APC to exert cardioprotection.(100, 101) This urges for further research aimed at understanding molecular principles of APC and treatments required to restore APC in such patients.

Preconditioning cannot be elicited by intravenous anesthetics, such as barbiturate, which is than conveniently used for animal anesthesia in preconditioning studies.

Preconditioning protocols include administration of volatile anesthetic either to animals by inhalation or to isolated cells by superfusion with solution containing equipotent concentration of anesthetics. Most of the protocols involve application of anesthetics from 10 to 30 minutes followed by anesthetic washout prior to exposing cells to various forms of injury (ischemia-reperfusion, hypoxia-reoxygenation, metabolic inhibition-"reperfusion" or oxidative stress by hydrogen peroxide). The anesthetic washout period is important to distinguish preconditioning effects from possible direct protective effects of anesthetics (postconditioning). As liposolubile substances, volatile anesthetics need to be sonicated that facilitates their dissolution. A 0.5 mM isoflurane corresponds to 1 minimal alveolar concentration and this concentration is usually used in APC studies.

APC in stem cell regenerative medicine. Stem cells and their derivatives have potential to be utilized for regeneration of organs, including heart, in addition to their use for disease modeling. Dr. Murry's group showed that the regeneration of myocardium damaged by acute ischemia-reperfusion by ESCs can be increased by two strategies: first, by using differentiated cardiomyocytes instead of undifferentiated stem cells, and second, by protecting engrafted cells from the noxious environment in the host tissue using a cocktail of pro-survival factors.(5) This was the first study that demonstrated the critical importance of actively protecting engrafted cells from the conditions in the infarcted myocardium. The ability of implanted hESC-derived cardiomyocytes to repair I/R-injured myocardium critically depends on their ability to survive the stressful environment within the host tissue, which can be improved by enhancing their resistance to activation of cell death pathways using a pro-survival cocktail.(5) Interestingly, some components of the pro-survival cocktail have comparable effects to APC: inhibition of mPTP opening, (102) anti-apoptotic pathway activation (103) and opening of ATP-sensitive potassium channels.(83) Unfortunately, it can be anticipated that the use of cocktail of agents in the clinical practice could be troublesome since each component needs to be approved by regulatory agencies and multiple drug treatments are likely to cause adverse drug interactions. However, since majority of these pro-survival agents act similarly to a single treatment with APC, (80, 83), and since volatile anesthetics are already in the clinical use, APC could serve for protection of stem cell-derived cardiomyocytes in clinical practice.

Reactive Oxygen Species in Anesthetic-induced Preconditioning

Although excessive production of ROS causes damage to cardiomyocytes during reperfusion, a moderate increase in ROS production prior to prolonged ischemia-reperfusion

triggers endogenous cytoprotective mechanisms that protect the cell from the subsequent burst of ROS production.(52, 104) ROS are crucial mediators of preconditioning signal, since antioxidants abrogate both ischemic- and anesthetic preconditioning.(52, 105, 106) Moreover, ROS can directly elicit preconditioning.(107) Volatile anesthetics induce production of ROS by altering electron flux along the respiratory chain and thereby slightly increasing electron leak and incomplete reduction of oxygen molecules.(104) Generated superoxide radical is precursor of other ROS.

Volatile anesthetic isoflurane partially inhibits complex I of the respiratory chain in isolated cardiac mitochondria and isolated cardiomyocytes.(104, 108, 109) This action increases ROS production, which is corroborated by observations that complex I inhibitor rotenone also elevates ROS production.(110) In addition, isoflurane directly induces mitochondrial depolarization by opening mitochondrial K_{ATP} channels, which tends to accelerate the rate of electron flux along the respiratory chain.(111, 112) Accelerated electron flux along the respiratory chain in combination with complex I inhibition may further enhance ROS production to reach levels sufficient to trigger preconditioning signals.(95, 104) A preconditioning potency of two volatile anesthetics, desflurane and sevoflurane is in direct correlation with the amount of ROS generated during triggering phase of preconditioning with each anesthetic.(52) This finding is another piece of evidence that ROS species are crucial mediators of preconditioning signaling cascade.

Protein kinase C (PKC) is well characterized downstream target of ROS in preconditioning signaling cascade.(113) It is a redox sensitive kinase that becomes activated after oxidation or calcium binding to its regulatory subunit.(113) Inhibition of PKC by non-selective inhibitor chelerythrine or by selective inhibitor of epsilon isoenzyme (myristoylated PKC-epsilon V1-2 peptide) abolishes APC-induced cytoprotection.(102) This indicates that protein kinase C, as a downstream target of ROS signaling, is another critical component of signal transduction cascade. A study has demonstrated that upon activation protein kinase C translocates to mitochondria where it activates mitochondrial K_{ATP} (mitoK_{ATP}) channels.(114) ROS signaling and redox activation of protein kinase C is not only important for preconditioning, but it also underlies increased resistance of cancer cells.(113) Therefore, this cytoprotective signaling seems to be part of general mechanism allowing cells to survive harsh conditions associated with hypoperfusion, hypoxia and oxidative stress.

Mitochondrial ATP-sensitive Potassium Channels in Anesthetic-induced Preconditioning

Inhibition of mitoK_{ATP} channels by 5-hydroxydecanoate (5-HD) either during signaling or memory phase of preconditioning abrogates APC.(97, 112) This demonstrates that opening of these channels is a part of preconditioning signal transduction cascade, and also an important effector of cytoprotection. During the signaling phase of APC opening of mitoK_{ATP} channels can slightly increase ROS production.(95, 115) However, in stressed cells that are preconditioned (memory phase of APC) mitoK_{ATP} channel opening may prevent excessive generation of ROS due to partial depolarization of mitochondria.(80) Namely, it has been demonstrated that small changes in mitochondrial membrane potential have substantial effect on ROS production at near-normal mitochondrial potentials.(80, 116, 117) Therefore, during reperfusion mitochondrial depolarization by mitoK_{ATP} channel opening may reduce the burst of ROS and therefore contribute to increased cell survival elicited by APC.

Opening of mitoK_{ATP} increases potassium flux into mitochondria lowering mitochondrial membrane potential (normal is estimated between 160 and 200 mV).(118-120) High mitochondrial membrane potential impedes electron flux along the respiratory chain thereby increasing electron leak and ROS formation. Restoration of mitochondrial membrane potential during early reperfusion facilitates ROS burst in combination with rapid electron flux stimulated by oxygen repletion.(121) Therefore, opening of mitoK_{ATP} channels can attenuate this ROS burst in early reperfusion by slightly depolarizing mitochondria.

This partial mitochondrial depolarization can also attenuate mitochondrial calcium overload during ischemia-reperfusion, since calcium is driven into mitochondria by mitochondrial membrane potential.(76, 111, 122, 123) Mitochondrial calcium overload together with ROS burst facilitate opening of mPTP that further exacerbates cell injury.(57, 64, 102) Attenuation of ROS production by slight mitochondrial depolarization can also attenuate calcium release from the sarcoplasmic reticulum by ryanodine receptor and also improve cellular calcium expulsion by sarcoplasmic reticulum calcium ATP-ase.(67, 124) Both of them are redox sensitive.(125) This decreases cytosolic and mitochondrial calcium overload. Beneficial effects of partial mitochondrial depolarization for cell survival during simulated reperfusion injury have been demonstrated with the use of 2,4-dintrophenol.(80) Namely, when 2,4-dintrophenol was used in s dose that mimics mitochondrial depolarization elicited by APC, it produced substantial cytoprotective effects that included attenuation of excessive ROS production, attenuation of cytosolic and mitochondrial calcium accumulation, delay in mPTP opening and improved cell survival. These cytoprotective effects were

quantitatively similar to those elicited by APC. This was direct evidence that partial decrease of mitochondrial membrane potential, likely by mitoK_{ATP} channel opening, is a key effector of cytoprotection by APC.

Mitochondrial Permeability Transition Pore Opening in Anesthetic-induced Preconditioning

Opening of the mPTP is the crucial step during ischemia reperfusion that exacerbates cell injury and initiates cell death pathways, necrosis and apoptosis.(56, 57) Excessive production of ROS and intramitochondrial calcium accumulation are two main triggers of mPTP opening, which results in rapid and complete collapse of mitochondrial membrane potential.(126) The affected mitochondrion stops producing ATP thereby accelerating progression towards necrotic cell death.(126, 127) Pore opening also allows mitochondrial influx of various solutes, leading to mitochondria swelling and rupture of outer mitochondrial membrane that has a smaller surface area than the inner mitochondrial membrane. The rupture of the outer mitochondrial membrane allows the release of cytochrome c from the intermembrane space, which will initiate apoptotic pathways by initiating cascade caspase activation.(126, 127) Moreover, second mitochondria-derived activator of caspases (SMAC/DIABLO) and apoptosis-inducing factor (AIF) are also released from mitochondria further stimulating caspase-dependent and caspase-independent apoptotic pathways, respectively.(126, 128) Although mPTP opening initiates apoptotic cell death, the number of unaffected mitochondria by the mPTP opening, i.e. the amount of ATP in the cell, will determine whether the cell will undergo energy-dependent apoptosis or energy independent necrosis.(126)

The structure of the mPTP has been debated over the long time and its constituents are still unclear. Initially, it was thought that the pore was formed jointly by the adenine nucleotide translocator (ANT) on the inner mitochondrial membrane and the voltage dependent anion channel (VDAC) on the outer membrane.(129) However, knockout studies showed that the pore can be formed in the absence of either component although a greater amount of stress was required to induce pore opening.(130-132) The important regulator of the mPTP is cyclophilin D, whose inhibitor cyclosporine A is the most widely accepted experimental pore inhibitor.(129) Although the pore structure is still under investigation, it is known that the pore allows free passage of solutes up to 1.5 kDa in size.(126) Direct inhibition of the pore by cyclosporine A attenuates infarct size in vivo demonstrating its

critical role in ischemia-reperfusion injury.(133, 134) Several studies demonstrated that APC induces cardiac cytoprotection by delaying opening of the mPTP.(80, 102)

2. HYPOTHESIS

The general hypothesis of the doctoral dissertation is: human pluripotent stem cell-derived cardiomyocytes exhibit competent responses to preconditioning with volatile anesthetic isoflurane and therefore can be used as a valid model to study APC *in vitro*.

3. AIMS

The overall objective of this doctoral dissertation is to investigate cardiomyogenesis in human pluripotent stem cells lines and to investigate competence of these cardiomyocytes as an experimental model for studying APC.

<u>Specific Aim 1:</u> To optimize methodology for differentiation of cardiomyocytes from human pluripotent stem cells.

<u>Specific Aim 2:</u> To investigate feasibility of using human cardiomyocytes differentiated from pluripotent stem cells for studying mechanisms of APC *in vitro*.

<u>Specific Aim 3:</u> To investigate the process of cardiomyogenesis and efficiency of cardiomyocyte differentiation in hiPSC lines.

The doctoral dissertation focused on the differentiation of cardiomyocytes from two types of human pluripotent stem cells, hESCs and hiPSCs. Approach called “directed differentiation” was utilized in this dissertation to generate hESC-derived cardiomyocytes and hiPSC-derived cardiomyocytes with high efficiency. It relies on the induction of cardiomyogenesis by application of endoderm-secreted growth factors that have been shown to favour cardiac differentiation and thereby can improve efficiency of cardiomyogenesis. Commercially available lines of hESC were utilized, and experiments with these cells were conducted at the Medical College of Wisconsin. hiPSC lines were obtained from the laboratory of Dr. S. A. Duncan, MCW. The efficiency of APC in human pluripotent stem cell-derived cardiomyocytes was studied by testing characteristic mediators of APC previously identified in human adult cardiomyocytes.

4. MATERIALS AND METHODS

Human embryonic stem cell and human induced pluripotent stem cell culture

Human ESC lines H1 and H9 (135) were purchased from the National Stem Cell Bank (NSCB; WiCell, Madison WI). hiPSC lines C2a and C6a were induced from human foreskin fibroblasts (ATC #CRL2097) as previously described (10) by transduction with lentivirus-encoded Oct4, Sox2, Nanog and Lin28. Pluripotent cells were maintained under hypoxic conditions (4% O_2, 5% CO_2) on mouse embryonic fibroblasts (MEFs) inactivated with mitomycin C (#M0503; Sigma-Aldrich, St Louis, MO), in medium consisting of DMEM/F12 (#11330-032, Invitrogen, Carlsbad, CA) supplemented with 20% knock-out serum replacement (#10828-028; Invitrogen), 1% non-essential amino acids, 1% penicillin-streptomycin (#15140-148, Invitrogen), L-glutamine (#25030-081, Invitrogen), 100 µM ß-mercaptoethanol (#M-6250, Sigma-Aldrich) and 4 ng/mL human recombinant basic fibroblast growth factor (#PHG0026, AA 10-155, Invitrogen). Colonies of ESCs and iPSCs were passaged every five days by manual microdissection with a 27-gauge needle to create ~500 μm^2 clumps, followed by a 1 → 4 split.

Cardiac differentiation of hESCs and hiPSCs

To differentiate pluripotent cells into cardiomyocytes, we used "directed differentiation" previously described. A schematic representation of differentiation protocol is depicted in Figure 2. Briefly, colonies were mechanically dissected into small clumps that were plated onto wells of a 6-well dish (without MEFs) that had been pre-coated with Reduced Growth Factor Matrigel (354230; BD-Biosciences, San Jose, CA) at a final concentration of 0.05 mg/ml in DMEM/F12; counting of cells dissociated from clumps estimated that each well contained 250,000 cells. For the next seven days pluripotency was maintained under hypoxic conditions using human ESC medium that had been conditioned by MEFs during the previous 24-hours period (i.e. MEF-conditioned medium). To induce differentiation, medium was exchanged for RPMI Medium 1640 (#22400, Invitrogen) fortified with B-27 Serum-Free Supplement (#17504-044, Invitrogen, which contains a proprietary amount of insulin), supplemented with activin-A (50 ng/ml; #338-AC, R&D Systems, Minneapolis, MN) and BMP4 (10 ng/ml; 314-BP-010 R&D Systems) and the cultures were placed in normoxic conditions; this time point is referred to as "day 0". After five days' induction with activin-A and BMP4, these growth factors were removed and

differentiation was continued in RPMI/B27/insulin alone. Cultures were monitored daily for the onset of spontaneous contractions.

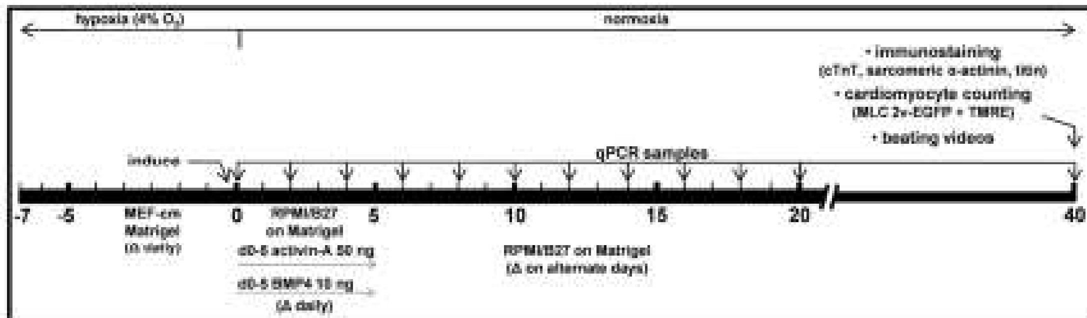


Figure 2. Experimental scheme for inducing the cardiomyogenic lineage in clumps of pluripotent hESCs and hiPSCs. hESCs, human embryonic stem cells; hiPSCs, human induced pluripotent stem cells; MEF, mouse embryonic fibroblasts; BMP4, bone morphogenetic protein 4; cTnT, cardiac troponin T 1. Published in (136).

Microdissection and single cell dissociation

After 90 days from treatment with growth factors, the beating cell clusters were mechanically dissociated from the remaining cell aggregates under a dissecting microscope (SMZ1000, Nikon, Japan) and treated with 0.05 % trypsin-EDTA (Invitrogen) for 4 min to dissociate individual cells, which were plated onto Matrigel-coated coverslips.

Immunostaining

Cardiomyocytes derived from ESCs and iPSCs were characterized by immunostaining 40 days after induction of differentiation. Cells were fixed in 1% paraformaldehyde for 1 hr and permeabilized with 0.5% Triton X-100 for 30 min. Cultures were rinsed 3x with PBS, blocked with 3% bovine serum albumin/PBS, followed by 3x rinses with PBS. Primary antibodies prepared in 1% BSA/PBS were applied overnight at 4˚C. The following primary antibodies were used: anti-α-actinin (1:100; A37732, Sigma) and anti-troponin T (1:100; AMPA6687, Thermo Scientific, Rockford, IL) and anti-titin (1:200; 9D10, Developmental Studies Hybridoma Bank, Iowa City, IA). Primary antibodies were rinsed 3x with PBS, followed by application of appropriate secondary antibody labeled with Alexa Fluor 488 (1:1000; A21202 Invitrogen). Secondary antibodies diluted in 1% BSA/PBS were applied for 2 hr on a rotator. Cells were rinsed 3x with PBS and nuclei were stained with TOPRO-3 (1:1,000; T3605, Invitrogen) for 10 min. Images were acquired using a laser-scanning confocal microscope (Eclipse TE2000-U; Nikon, Tokyo, Japan) and analyzed using MetaMorph 6.2 software (Universal Imaging, West Chester, PA).

Identification of Cardiomyocytes in Beating vs. Non-beating Areas

Percentages of cardiomyocytes in beating and non-beating areas of the culture dishes were determined as follows. Cells in each area were isolated using a 27-gauge needle under the dissecting microscope (SMZ 1000; Nikon, Tokyo, Japan) and enzymatically dispersed in 0.05 % trypsin-EDTA (Invitrogen) for 4 min, after which trypsin was inactivated by adding DMEM (SLM-021-B; Millipore) containing 20% FBS (16000-044; Invitrogen). Dissociated cells were pelleted (3 min @2,800 g), resuspended in RPMI/B27, counted and plated onto Matrigel-coated glass coverslips at low density (~1,000 cells/cm^2). Two days later, the cells were transduced with lentiviral particles encoding human MLC 2v-driven EGFP. Four days later the transfected cells were counter-stained with TMRE (T669, Invitrogen), a mitochondrial marker used to identify all cells in the population, followed by enumeration of EGFP-positive cells as a percentage of TMRE-positive cells as previously described;(137) a

minimum of 500 randomly selected TMRE-positive cells were enumerated on each coverslip. The MLC 2v-EGFP lentiviral construct was a generous gift from the laboratory of Dr. Lior Gepstein (Technion-Israel Institute of Technology, Haifa, Israel.(138) Prior to use, the MLC 2v-EGFP cassette was subcloned into transfer plasmid pHR(+)c.Ub.MCSoligo.R(-)W(+) (which does not contain a CMV promoter), followed by production and titering of lentiviral vector as described previously.(139, 140)

Laser-scanning confocal microscopy

Four days after lentiviral vector transduction, imaging was performed using a confocal microscope (Eclipse TE2000-U, Nikon) and data were analyzed with MetaMorph 6.1 software (Universal Imaging, West Chester, PA). Living hESC- and hiPSC-derived cardiomyocytes were identified by detecting fluorescence of MLC 2v-driven EGFP-positive cells and experiments were conducted in Tyrode's solution (in mM: 132 NaCl, 10 HEPES, 10 glucose, 5 KCl, 1 $CaCl_2$, 1.2 $MgCl_2$, pH 7.4) at room temperature. The percentage of hESC-derived cardiomyocyte death was determined after exposing cells to oxidative stress induced by 10 mM H_2O_2 (Calbiochem) applied for 50 min, followed by perfusion with Tyrode's solution for 10 min. The cells stained with red-fluorescent propidium iodide (2 μM, Sigma-Aldrich) were considered dead.(47) hESCs- and hiPSC-derived cardiomyocytes were preconditioned with 0.5 mM isoflurane (APC) applied for 15 min, followed by 5 min isoflurane washout. After each experiment, a gas chromatography was used to test isoflurane concentrations, which varied ±10% of reported values. The tetramethylrhodamine ethyl ester (TMRE, 30 nM, Invitrogen) was used to detect $\Delta\Psi_m$ in hESC-derived cardiomyocytes. ROS production was monitored in cells loaded with dihydroethidium (10 μM, Invitrogen). Opening of the mPTP was assessed as previously described in our laboratory,(102) a method based on mPTP induction by photoexcitation-generated oxidative stress.(79, 141-143) The mPTP opening was detected by rapid dissipation of $\Delta\Psi_m$, observed as loss of TMRE fluorescence, which is sensitive to mPTP opening inhibition.(102) The mPTP opening was assessed in hESC-derived cardiomyocytes and hiPSC-derived cardiomyocytes.

Electrophysiology

Membrane potential (E_m) was measured in microdisscted beating clustures using 3M KCl-filled borosilicate glass (FHC) microelectrodes (impedance 40-60 MΩ) in RPMI/B27 medium. Data were processed using a Grass RPS7C polygraph (Astro-Med/Grass Inc) and Superscope II digital data acquisition system (GW Instruments)

Oxygen consumption

Spontaneously and rhythmically contracting cell clusters of hESC-derived cardiomyocytes were separated by microdissection and respiration of cell clusters that predominantely contain cardiomyocytes was measured using an oxygen electrode (Hansatech Instruments, Norfolk, UK) at 37ºC. Isoflurane was delivered at incremental steps and the rate of oxygen consumption after each isoflurane addition was normalized to baseline values.

Real-Time PCR

Expression of genes expressed during cardiomyogenic differentiation was assessed by real-time quantitative PCR (qPCR). Beginning at the time of induction (day 0), cells were harvested on alternate days during the first 20 days, followed by harvest of a terminal time point on day 40. RNA purification and qPCR were performed as previously described.(137) Briefly, cells were lysed, vigorously pipetted and stored at -80˚C until purification. Immediately after thawing, total RNA was purified using the RNeasy Mini Kit (#74104, Qiagen, Valencia, CA), with Qiashredder (#79654, Qiagen), per the manufacturer's instructions. Following quantification at A_{260}, 1.0 µg total RNA from each sample was reverse-transcribed (#C-03 RT^2 First Strand Kit, SABiosciences, Frederick, MD). qPCR was performed by adding 10 µl of each RT product to 90 µl master mix (#PA-011 RT^2 SYBR Green/Fluorescein qPCR Master Mix, SABiosciences) in wells of 96-well plates containing custom pre-arrayed primer pairs (Custom RT^2 Profiler PCR Array, SABiosciences) to amplify the mRNAs described in Figure 14. PCR reactions were performed using an iCycler (BioRad, Hercules, CA). Each time point in Figure 14 is the average of duplicate dishes.

Statistical analyses

Data are presented as mean ± SD. Each experimental group comprises hESC-derived cardiomyocytes from at least 3 different differentiations, where n indicates the number of independent experiments. Statistical comparisons were performed using one-way analysis of variance with Bonferroni's post hoc test. Differences at $P<0.05$ were considered significant. The *t* test was used to analyze differences in experiments with only two groups.

5. RESULTS

In the first part of the dissertation we investigated whether cardiomyocytes derived from hESCs can be used as a complimentary experimental model of human cardiomyocytes to study APC. We also analyzed whether hiPSC-derived cardiomyocytes exhibit similar responses to APC as hESC-derived cardiomyocytes in terms of the opening of the mPTP.

To identify the possibility that hESC-derived cardiomyocytes have the competent endogenous protective mechanisms against oxidative stress to be used as an experimental model for APC, we investigated whether preconditioning with the anesthetic isoflurane elicits distinct mediators of protection in these cells: reactive oxygen species (ROS) and opening of mitochondrial ATP-sensitive potassium (mitoK_{ATP}) channels as signal mediators, and a delay in mPTP opening as an endpoint of protection. The model was validated by comparing obtained results to our previous work using adult human and adult animal cardiomyocytes.

Differentiation and characterization of hESC-derived cardiomyocytes

The presence of cardiomyocytes after cardiac differentiation of hESCs was observed as occurrence of spontaneously and rhythmically beating areas of contiguous cells in culture dishes, beginning approximately 10 days after treatment with activin-A and BMP4 and lasting up to a one year (please see link: http://links.lww.com/ALN/A624). As shown in Figure 3, immunostaining revealed an abundance of cells positive for cardiac specific sarcomeric proteins, organized in characteristic striated pattern (Figure 3A, B, C, D). Following the dissociation from culture dishes, cardiomyocytes retained their striated appearance (Figure 3F), with some continuing to exhibit spontaneous rhythmic contractions (please see link: http://links.lww.com/ALN/A625). Short (<130 ms) atrial-like action potentials, and long (>250 ms) ventricular-like action potentials, were recorded in dissociated, contracting cell clusters, indicating electrical activity and existence of functional sarcolemmal ion channels in these cardiomyocytes.

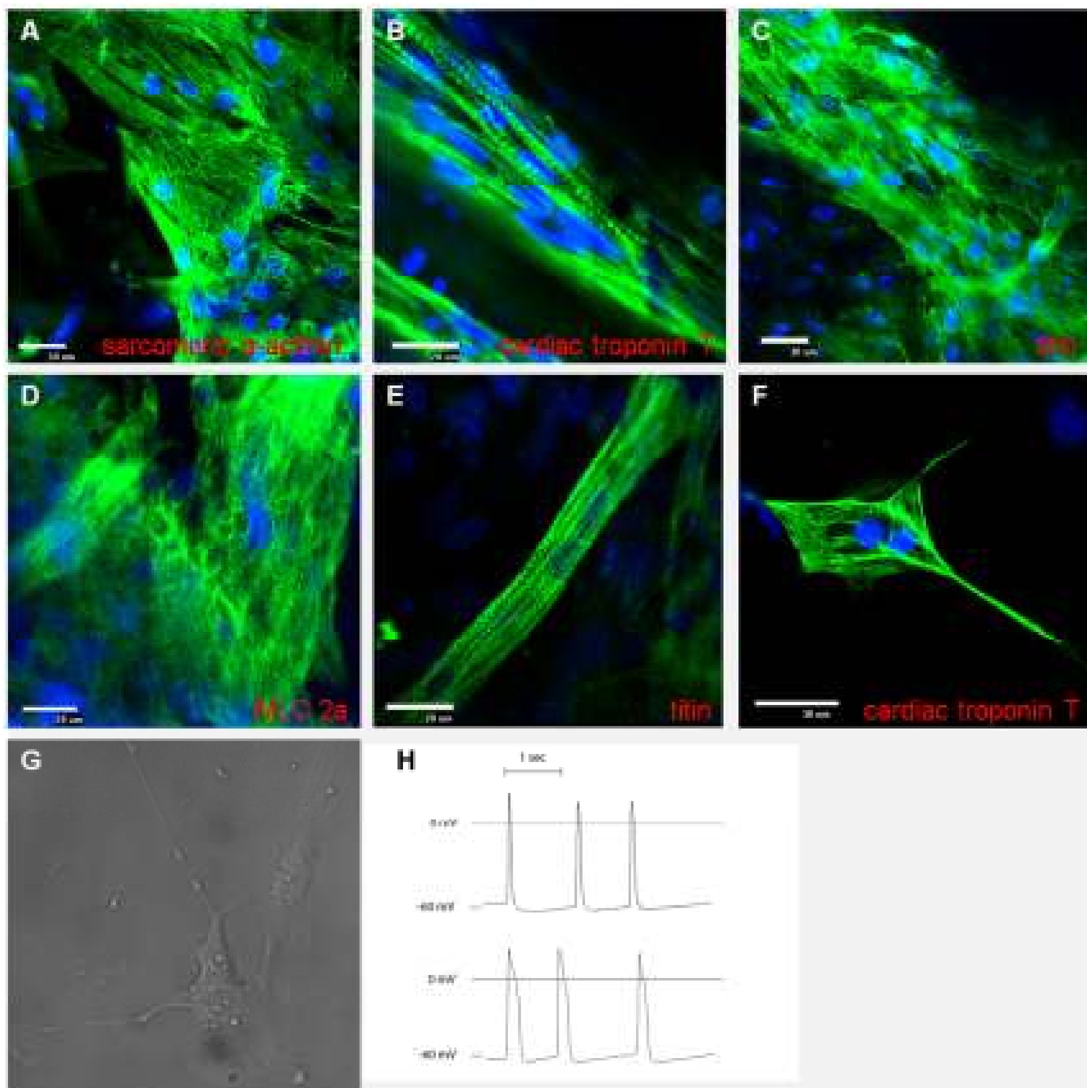


Figure 3. Immunolabeling and electrophysiological characterization of hESC-derived cardiomyocytes. Confocal images of non-dissociated cells after 90 days from treatment with growth factors, and stained for cardiac sarcomeric proteins: (**A**) sarcomeric α-actinin, (**B**) cardiac-specific cTnT, (**C**) titin, and (**D**) cardiac-specific MLC 2a. A large percentage of positive cells are observed, including striated patterns indicating highly organized sarcomeres. (**E**) Occasional occurrence of cells with rod-shaped morphology that resemble adult cardiomyocytes. (**F**) Dissociated cell stained for cTnT showing that cells maintain sarcomeric organization after dissociation. (**H**) A dissociated cell that is spontaneously and rhythmically contracting. (**G**) Representative recordings of action potentials: shorter, atrial-like (*upper trace*) and longer, ventricular-like (*lower trace)*. Published in (137).

Genetic marking and labeling of live hESC-derived cardiomyocytes using a lentiviral vector

To identify living hESC-derived cardiomyocytes and determine differentiation efficiency, differentiated cells were genetically marked using a self-inactivating lentiviral vector containing a cardiac-specific promoter MLC 2v driving the expression of EGFP (Figure 4A). Lentiviral transduction efficiency, determined with ubiquitin-driven EGFP, was 98±1% (Figure 4B). The 85±3% of cells were MLC 2v-EGFP-positive, i.e. ventricular myocytes, indicating high efficiency of our differentiation protocol (Figure 4B,C).

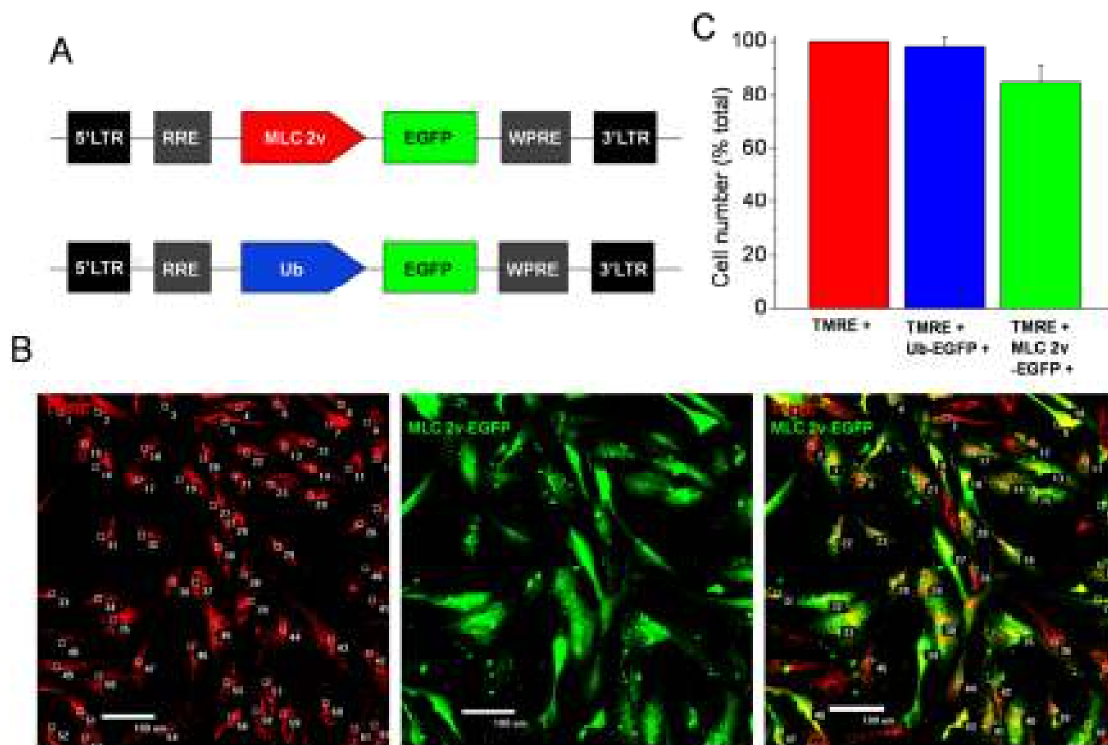


Figure 4. Labeling and counting of hESC-derived cardiomyocytes using lentiviral vector. (**A**) Schematic representation of lentiviral vectors, pHR(+)c.MLC 2v.EGFP.R(-)W(+) and pHR(+)c.Ub.EGFP.R(-)W(+) used for identifying cardiomyocytes and determining transduction efficiency, respectively. (**B**) To determine the total cell number in beating clusters, after dissociation cells were loaded with TMRE (red) to visualize cell bodies. MLC 2v-EGFP-positive cells were counted by detecting green fluorescence, giving the number of ventricular myocytes. (**C**) Summarized data from five separate differentiation experiments show high percentage of hESC-derived cardiomyocytes. Published in (137).

APC protects hESC-derived cardiomyocytes from oxidative stress

To test whether APC protects the hESC-derived cardiomyocytes from oxidative stress-induced cell death, the preconditioned and control cells, were exposed to H_2O_2. The cardiomyocytes were identified as MLC 2v-EGFP-positive cells (green-fluorescent cells in Figure 5A). APC attenuated the hESC-derived cardiomyocyte death compared to control, 32±7%, vs. 58±7%, n=5, respectively (Figure 5B,C). This correlates with our previous study which showed that APC protects adult human atrial cells from oxidative stress.(100)

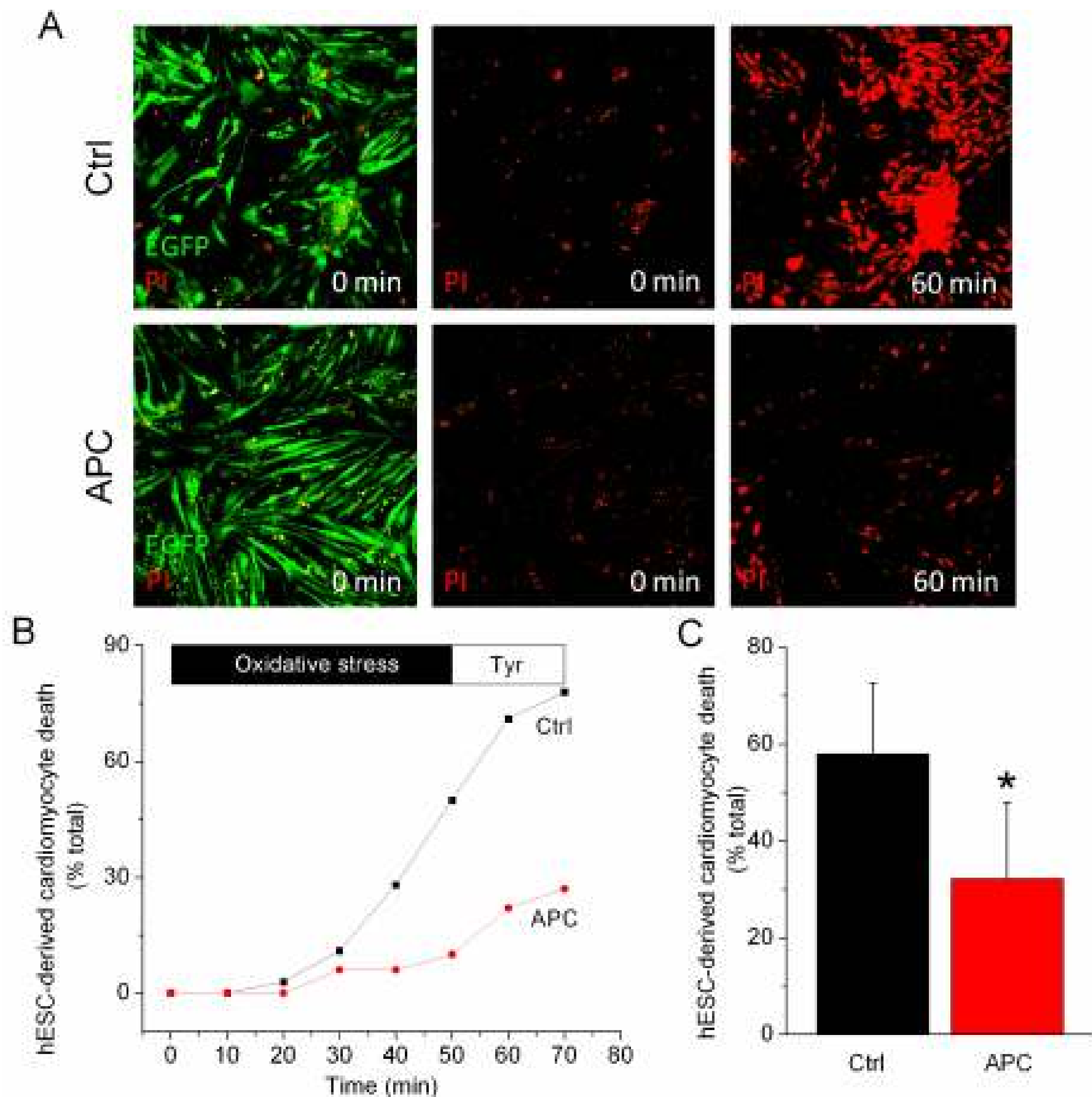


Figure 5. hESC-derived cardiomyocytes are protected from oxidative stress by APC. (**A**) MLC 2v-EGFP-positive cells, i.e. hESC-derived cardiomyocytes, were identified by green fluorescence using confocal microscopy. Following exposure to oxidative stress and 3compared to control (Ctrl), APC decreased the number of hESC-derived cardiomyocytes that stained positive for red-fluorescent propidium iodide (PI), an indication of cell death. (**B**) The rate of increase in number of PI-positive cells, expressed as percent of total number of hESC-derived cardiomyocytes, is attenuated in APC group compared to Ctrl. (**C**) Summarized values after the application of H_2O_2, following 10 min of perfusion with Tyrode solution. *$P<0.05$ vs. Ctrl. Published in (137).

Isoflurane depolarizes mitochondria in hESC-derived cardiomyocytes by opening mitoK_{ATP} channels

Using adult rat cardiomyocytes, we have previously shown that isoflurane induces opening of mitoK_{ATP} channels causing decrease in $\Delta\Psi_m$ and thereby eliciting cardioprotection.(111) In hESC-derived cardiomyocytes, diazoxide, an opener of mitoK_{ATP} channels,(144) decreased TMRE fluorescence intensity to 87.9±1.8% of baseline (n=11), indicating opening of mitoK_{ATP} channels (Figure 6) Application of 0.5 mM isoflurane decreased TMRE fluorescence intensity to 80.8±3.5% of baseline (n=11). This was attenuated in the presence of 5-hydroxydecanoate (5-HD), an inhibitor of mitoK_{ATP} channel opening (144) (91.5±1.0% of baseline, n=11), indicating that mitochondrial depolarization by isoflurane is, in part, mediated by opening of mitoK_{ATP} channels.

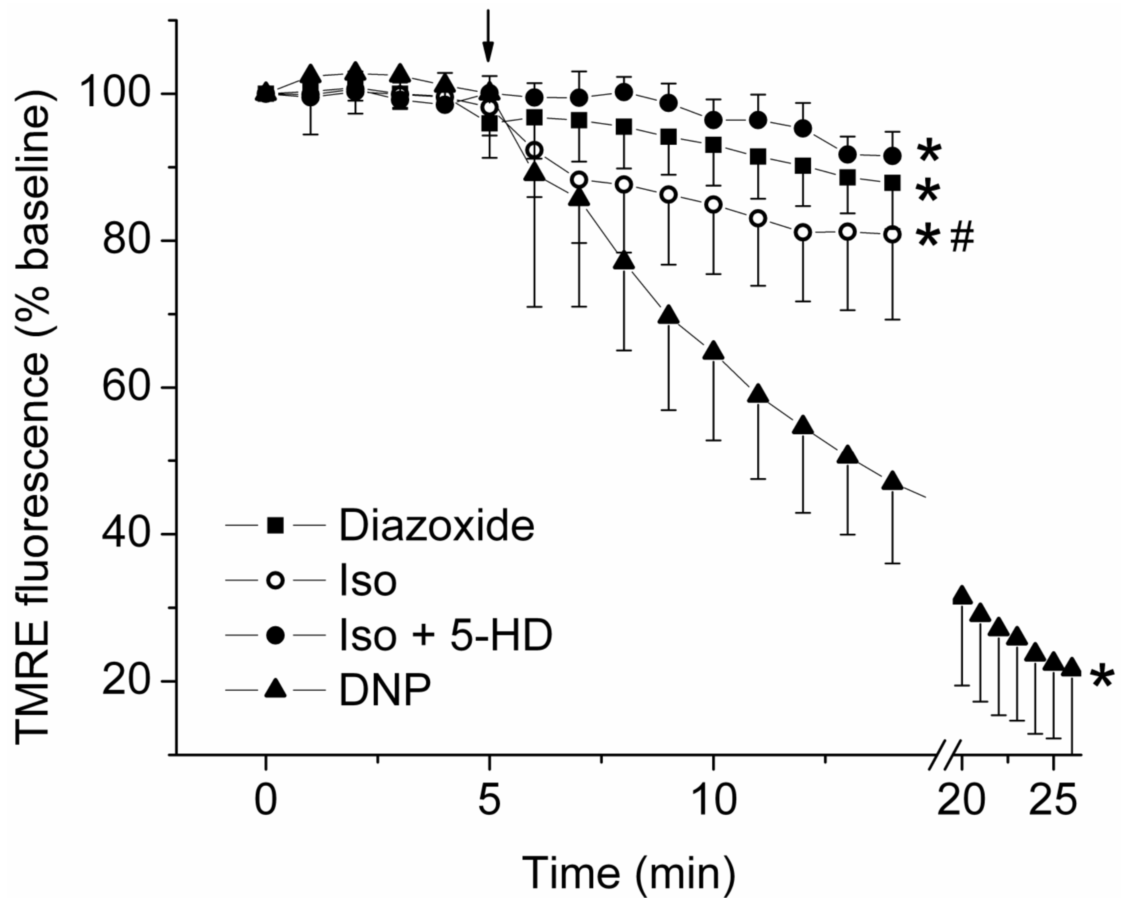


Figure 6. Isoflurane opens mitoK_{ATP} channels and depolarizes mitochondria in hESC-derived cardiomyocytes. $\Delta\Psi_m$ was monitored in dissociated hESC-derived cardiomyocytes using TMRE fluorescence. The time point when drugs were added is indicated by the arrow. Diazoxide (50 µM) decreased TMRE fluorescence intensity, indicating mitochondrial depolarization. Application of isoflurane (Iso) also decreased TMRE fluorescence intensity, an effect that was partly blocked with 200 µM 5-HD. 2,4-dinitrophenol (DNP; 100 µM) completely depolarized mitochondria. $^{*}P<0.05$ vs. baseline; $^{\#}P<0.05$ vs. Iso + 5-HD. Published in (137).

Oxygen consumption by cell clusters containing hESC-derived cardiomyocytes is inhibited by isoflurane

The rate of oxygen consumption of microdissected beating cell clusters that predominantly contained hESC-derived cardiomyocytes was monitored in the presence of incremental isoflurane concentration (Figure 7). At a concentration of 0.12 mM, isoflurane slightly, but not significantly increased oxygen consumption. However, by increasing isoflurane concentration to 0.25, 0.5 and 1.0 mM, the oxygen consumption progressively decreased to 98.1±6.8%, 88.7±6.6%, and 83.6±4.8% of baseline, respectively, (n=7). This correlates with our data from adult rat cardiomyocytes, indicating partial obstruction of the electron transport chain (ETC) by isoflurane.(104)

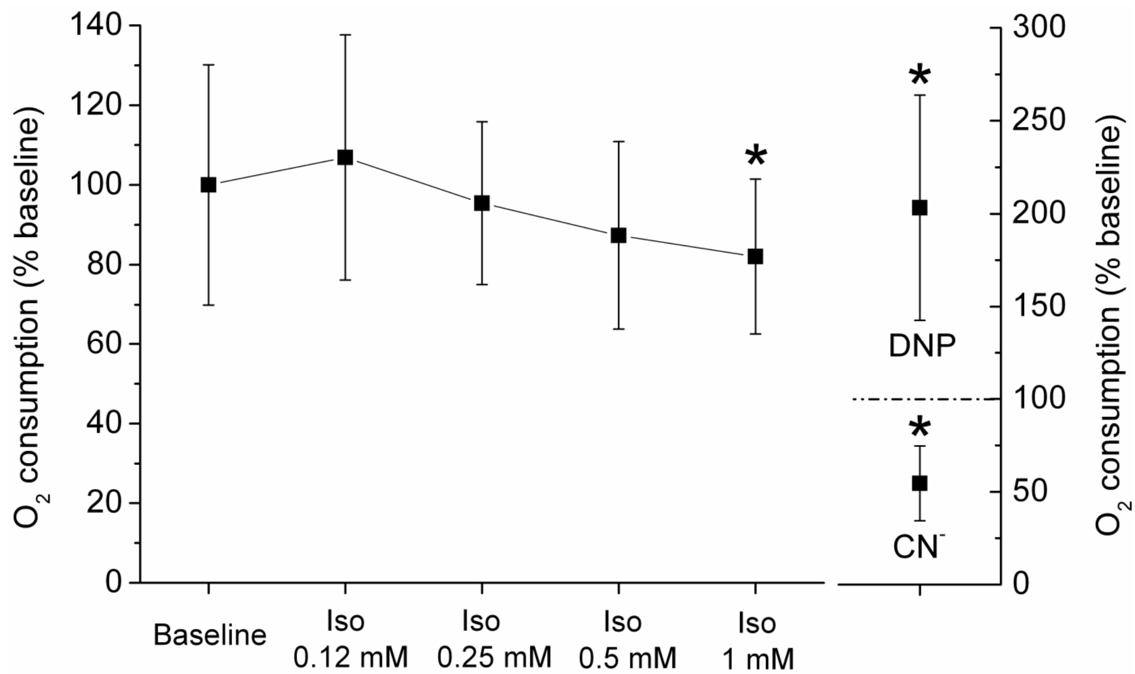


Figure 7. Isoflurane attenuates hESC-derived beating cell cluster oxygen consumption. The rates of oxygen consumption in hESC-derived contracting cell clusters after the addition of incremental isoflurane (Iso) concentration isoflurane were normalized to baseline values. At the higher concentrations, isoflurane attenuated oxygen consumption. DNP (100 μM) and cyanide (CN^-, 2 mM) were added to establish maximal and minimal rates of oxygen consumption. $^*P$<0.05 vs. baseline. Published in (137).

Isoflurane enhances production of ROS by hESC-derived cardiomyocytes

Production of ROS, important signaling molecules in preconditioning of adult cardiomyocytes,(52, 145) was monitored in hESCs-derived cardiomyocytes (Figure 8). Ten minutes after the addition of isoflurane (0.5 mM), Eth fluorescence increased to 120.0±3.3% of baseline (n=12) which is a significantly greater than in un-treated cells (101.5±0.7% of baseline, n=5). This indicated that isoflurane increases ROS production in hESC-derived cardiomyocytes.

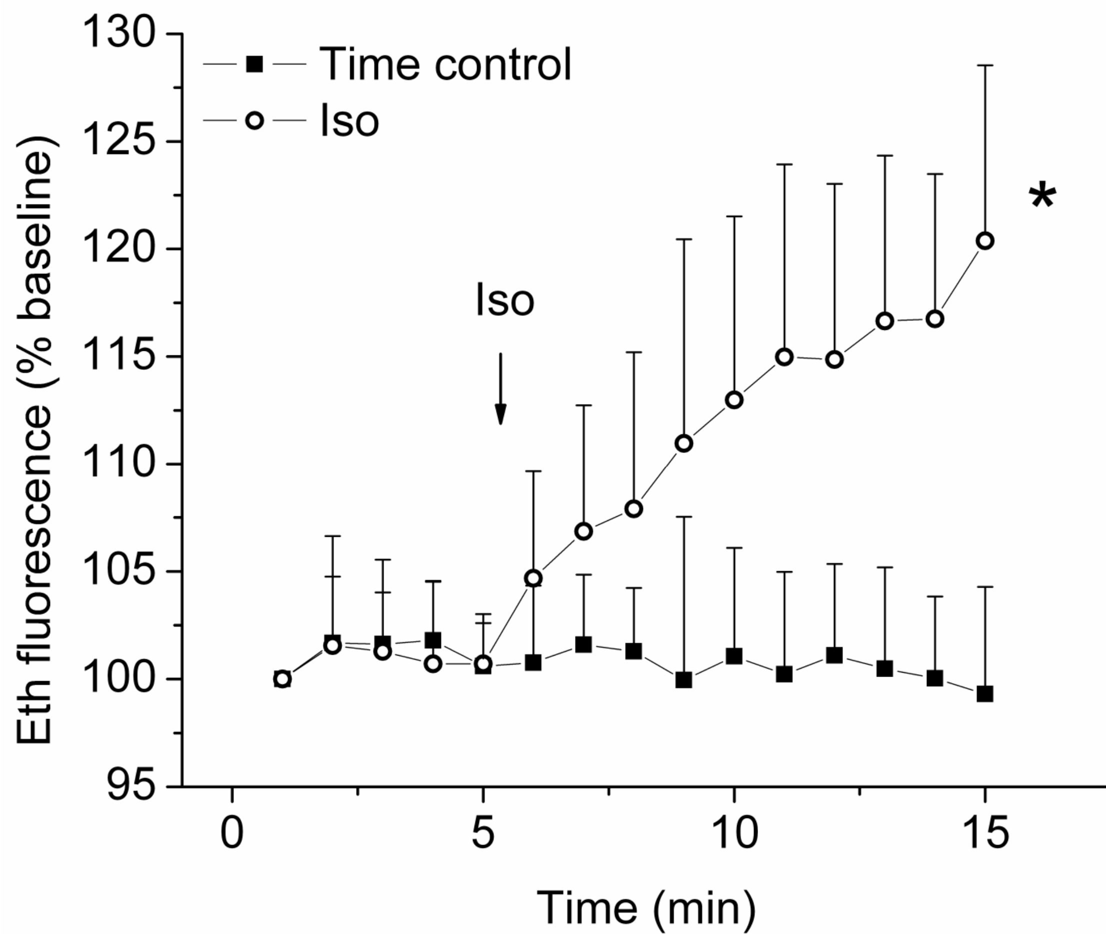


Figure 8. Isoflurane enhances ROS production in hESC-derived cardiomyocytes. ROS production in hESC-derived cardiomyocytes was measured by detecting fluorescence intensity of ethidium (Eth). Compared to the Time control, isoflurane (Iso) significantly increased Eth fluorescence intensity. $^{*}P<0.05$ vs. Time control. Published in (137).

Preconditioning with isoflurane or hydrogen peroxide delays opening of mitochondrial permeability transition pore in hESC-derived cardiomyocytes

Cardioprotective strategies, including APC delay opening of mPTP, a critical event in the transition towards cell death. mPTP opening-induced dissipation of $\Delta\Psi_m$ was monitored in hESC-derived cardiomyocytes exposed to oxidative stress (Figure 9). Similarly to mPTP inhibitor cyclosporine A (CsA, 1 μM), APC delayed the mPTP opening occurred (Figure 9B). APC and preconditioning with H_2O_2 increased arbitrary mPTP opening time to 139.4±10.1 (n=16) and 130.5±9.0% of control (n=12), respectively, indicating that each treatment elicited preconditioning and delayed mPTP opening (Figure 9C). Application of 5-HD together with isoflurane abrogated APC-induced increase in arbitrary mPTP opening time (95.3±2.9% of control, n=16) confirming the role of mitoK_{ATP} channel opening in signal mediation of APC.

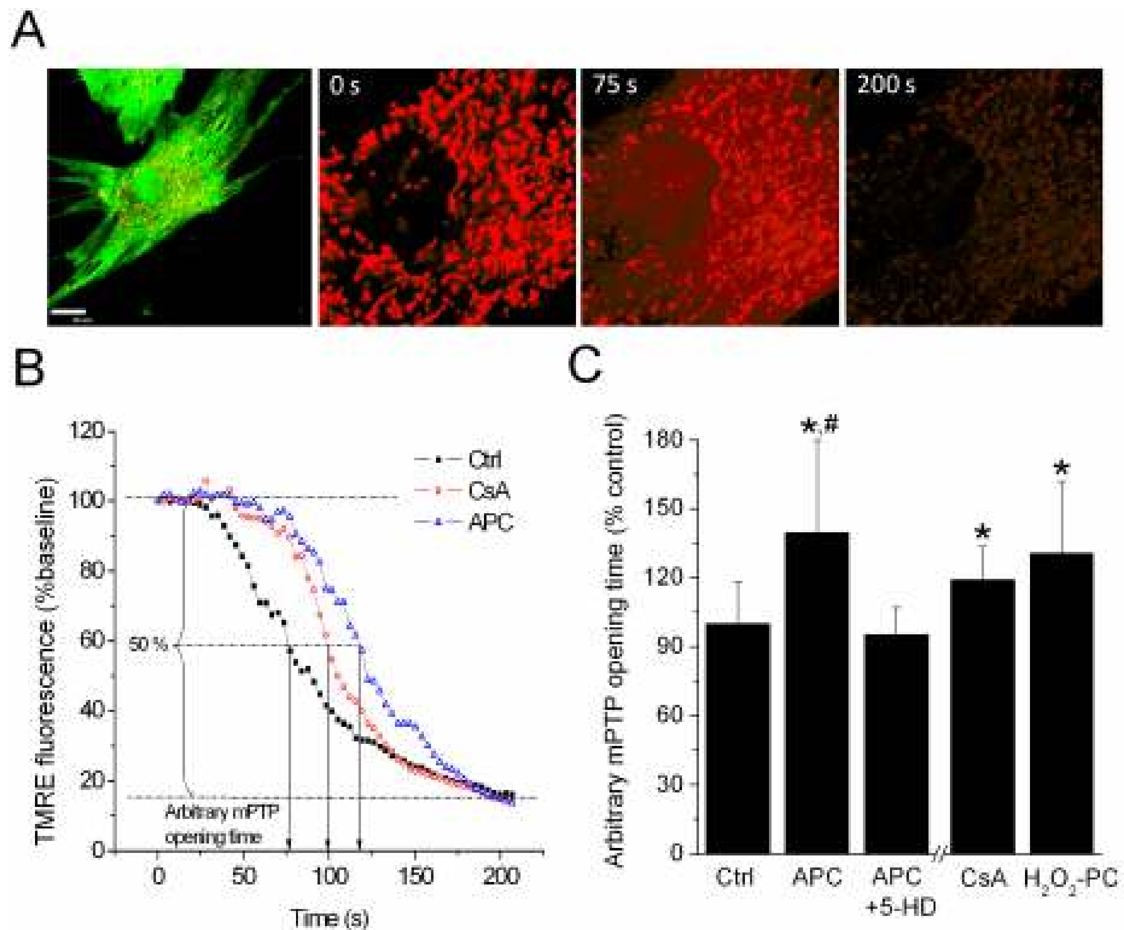


Figure 9. Preconditioning delays mPTP opening in hESC-derived cardiomyocytes. (**A**) mPTP opening was induced by photoexcitation-generated oxidative stress and detected by rapid dissipation of TMRE fluorescence. (**B**) Representative signal traces from control (Ctrl), CsA-treated cells, and APC. Arbitrary mPTP opening time was determined as the time when TMRE fluorescence intensity decreased by half between initial and residual fluorescence intensity. (**C**) mPTP blocker CsA and APC increased arbitrary mPTP opening time, which was blocked in presence of 5-HD (APC + 5-HD). Preconditioning with 40 μM H_2O_2 (H_2O_2-PC) also delayed mPTP opening. $^{*}P<0.05$ vs. Ctrl; $^{\#}P<0.05$ vs. APC + 5-HD. Published in (137).

Preconditioning with isoflurane delays opening of mitochondrial permeability transition pore in hiPSC-derived cardiomyocytes

mPTP opening time was determined in hiPSC-derived cardiomyocytes using a similar experimental protocol as for hESC-derived cardiomyocytes. The arbitrary mPTP opening time induced by oxidative stress was greater in hiPSC-derived cardiomyocytes exposed to APC (136.6±8.3% of control, n=5) than in control cells (102.4±23.8% of control, n=5), as shown in Figure 10. This indicated that preconditioning with isoflurane elicited effective endogenous cytoprotective mechanisms that induced the delay in mPTP opening in hiPSC-derived cardiomyocytes.

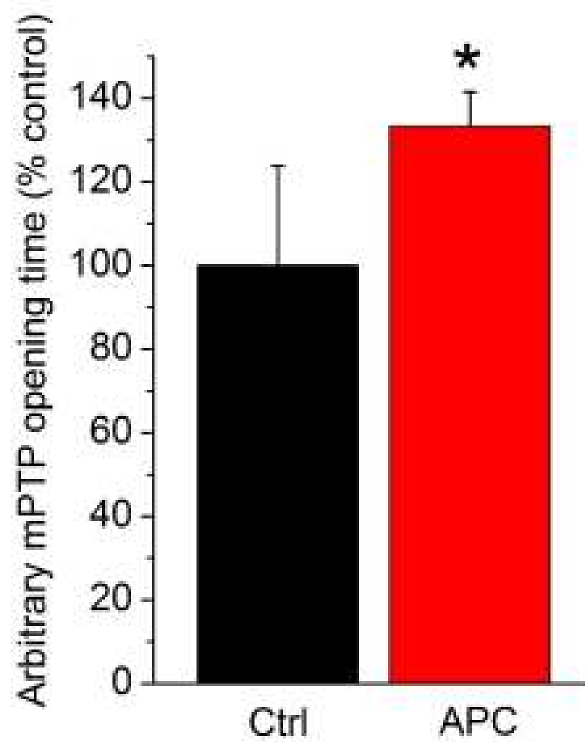


Figure 10. APC delays mPTP opening in hiPSC-derived cardiomyocytes. mPTP opening was induced by photoexcitation-generated oxidative stress. A drop in the initial TMRE fluorescence intensity by 50% was compared between experimental groups (as in Figure 9). Preconditioning with isoflurane increased arbitrary mPTP opening time compared to control. $^{*}P<0.05$ vs. Ctrl.

With the results presented so far we have demonstrated an efficient method to differentiate cardiomyocytes from hESCs, indicated by genetic labeling using lentiviral vectors, showing that ~85% of cells in beating clusters expressed cardiac-specific promoter MLC 2v. Efficient differentiation and a phenotype of functional cardiomyocytes was also indicated by observing globally contracting cell clusters that widely expressed highly organized, cardiac-specific sarcomeric proteins and generated action potentials resembling those of mature heart cells. Moreover, we showed that preconditioning with isoflurane attenuates cell death and elicits competent mechanisms of protection in ventricular hESC-derived cardiomyocytes against oxidative stress. These included characteristic and important mediators of cardioprotection: opening of mitoK_{ATP} channels, partial inhibition of the ETC, ROS as signaling molecules, and a delay of oxidative stress-induced mPTP opening, an end-point of protection. APC also induced the delay in mPTP opening in hiPSC-derived cardiomyocytes. Similar responses to APC between adult cardiomyocytes documented in our previous studies and hESC-derived cardiomyocytes demonstrated in this study indicate the feasibility of using hESC-derived cardiomyocytes as a model of human ventricular cardiac cells to study APC and potentially other treatments/diseases.

In the second set of experiments we tested and compared cardiomyogenic potential among hESC and hiPSC lines. This was achieved using the same directed differentiation approach.

Differentiation of Beating Cardiomyocytes is more Extensive in ESCs than in iPSCs

We simultaneously compared the ability of two widely used ESC lines (H1 and H9) and two iPSC (C2a and C6a) lines to differentiate into beating cardiomyocytes, using identical conditions of induction and cell culture. As described in Supplemental Figure 4 in the reference (10), pluripotent C2a and C6a iPSCs exhibit morphological, gene expression, and karyotype characteristics that are indistinguishable from pluripotent H9 ESCs. Moreover, C2a and C6a iPSCs form teratomas containing cells from all three germ layers; these iPSCs can reproducibly and efficiently differentiate into hepatocytes in stepwise, directed fashion by sequential treatment with growth factors that induce liver development in the embryo.(10)

In this experiment, all four lines were induced to differentiate in defined medium (RPMI/B27) supplemented with cardiomyogenic growth factors BMP4 and activin-A at time zero (day 0) for five days, after which the cells were treated with RPMI/B27 only, up to day 40, by which time cells in all cultures had become confluent and multilayered (Figure 11). Although rhythmic beating was observed by day 10 in both ESC lines, contractions were not

observed in the iPSC lines until the fourth week. In all lines, contractions were spontaneous and rhythmic, always occurring in cell clusters rather than in individual cells, which is characteristic of cardiomyocytes derived from pluripotent cells.(20, 146) Figure 11 shows characteristic morphologies of cultures derived from each cell-line at day 40. Corresponding videos showing the extent of rhythmic contractions throughout the culture dish on day 40 can be viewed by accessing the following link: http://www.mcw.edu/cellbiology/johnloughphd.htm. In some instances (H1 ESCs), contractions were sustained for a year or longer (not shown). Contractility was most extensive in H1 ESC cultures, wherein multilayered networks of contracting cells formed that were interconnected, nearly spanning the entire dish (Video Clip 1); in accord with our previous experience using H1 cells, this indicated that the contractile tissue was comprised of contiguous myocytes that were electrophysiologically synchronized.(137) H9 ESCs also exhibited contractile three-dimensional areas (Video Clip 2); however, these were neither as networked nor as extensive as in cultured H1 ESCs. By contrast, cultures containing iPSC lines C2a and C6a exhibited beating in only a few clumps of cells that were not interconnected (Video Clips 3 & 4); with time, contractile areas in iPSC cultures did not expand or become multilayered, in contrast to H1 and H9 ESCs.

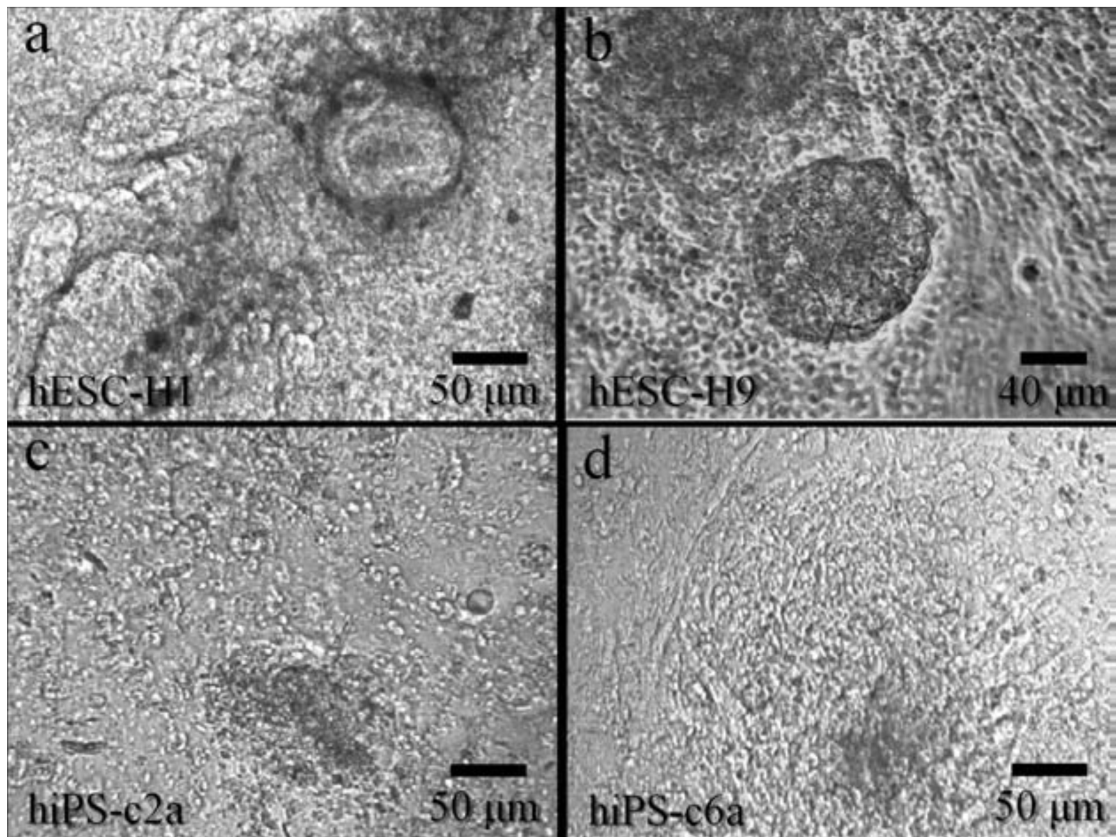


Figure 11. Phase-contrast images of differentiated hESCs and hiPSCs after 40 days in culture. Representative light microscope images of hESC- and hiPSC-derived cells at high magnification (400x) on day 40 of differentiation. Note that H1 and H9 hESCs formed multilayered confluent cluster of cells that exhibited spontaneous rhythmic contractions (viewed in Video Clips 1 and 2). hiPSC lines (C2a and C6a) also form multilayered and interconnected cell clusters that also spontaneously and rhythmically contracted (Video Clips 3 and 4); however, the hiPSC cells formed less confluent clusters than hESCs. Published in (136).

Sarcomere Differentiation is more Extensive in ESCs than in iPSCs

Immunostaining was performed 40 days after inducing differentiation to monitor presence of cardiac-specific proteins and their assembly into sarcomeres. All panels of Figure 12 show immunostaining patterns in rhythmically beating areas of the dish. This revealed that cTnT and sarcomeric α-actinin were organized into clearly striated patterns in H1 and H9 ESCs (Figure 12a-f). However, striations in both iPSC lines (Figure 12h,k) were less conspicuous, and definitely not as widespread as in the ESC cultures (not shown), consistent with the abundance of beating clusters shown in the Video Clips. It is also noteworthy that the titin immunostaining pattern was remarkably extensive only for H1 ESCs (Figure 12m,n), which displayed three-dimensional organization of sarcomeric structures. These characteristics were less evident in H9 ESCs, and were almost completely absent in contractile areas of C2a and C6a iPSCs (not shown).

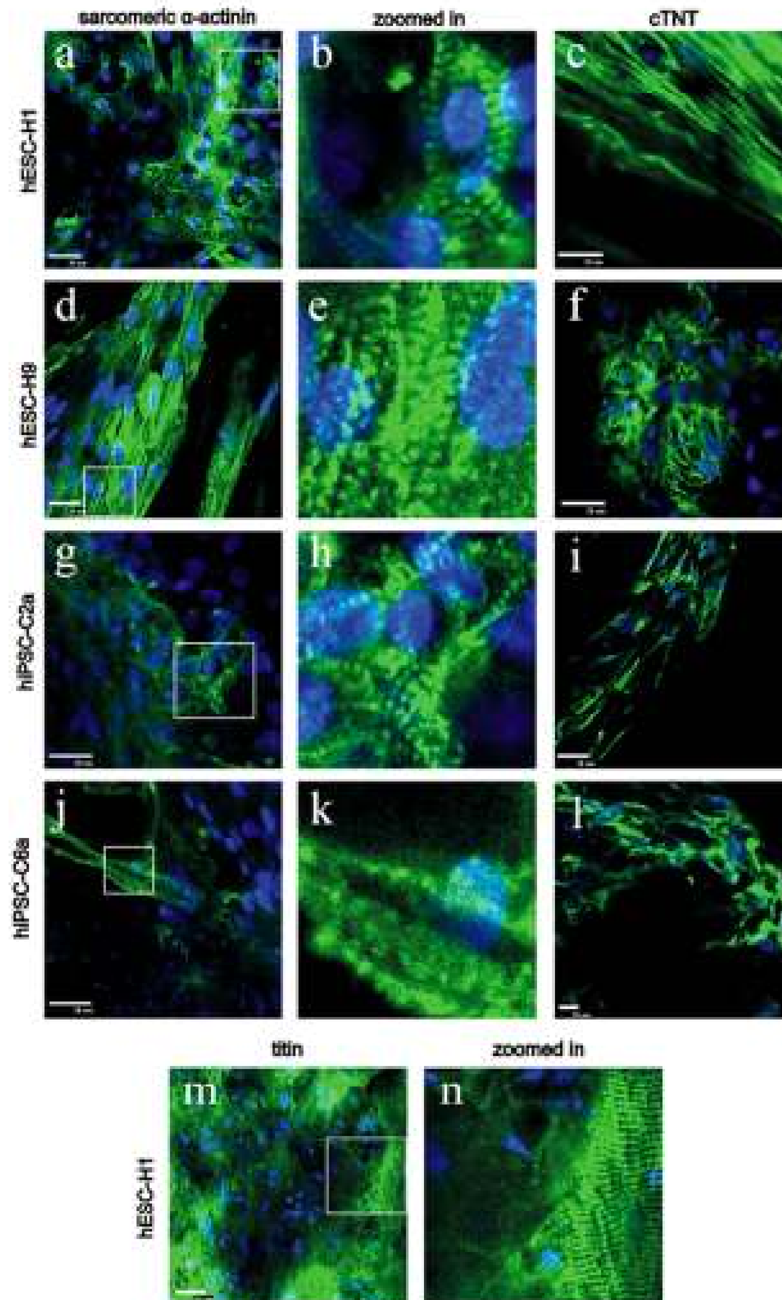
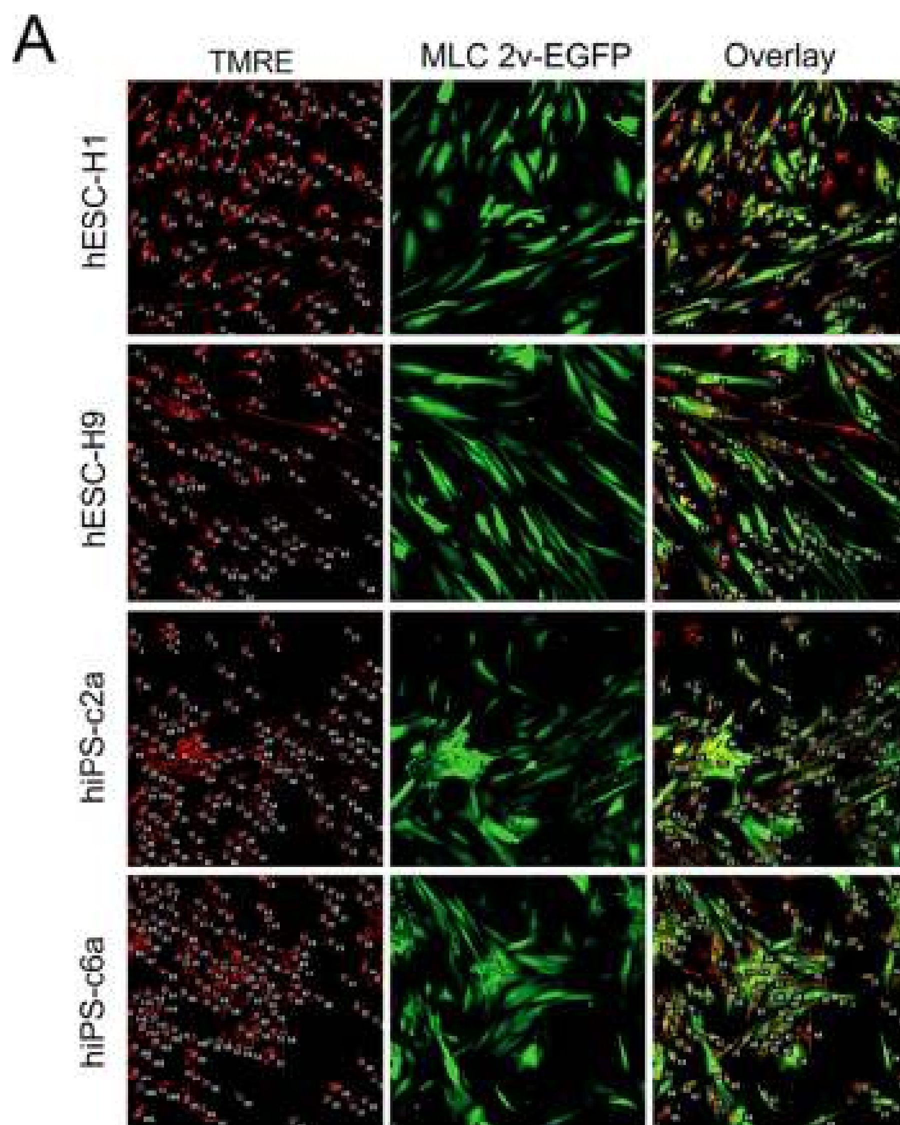


Figure 12. Immunostained cardiomyocytes derived from hESCs and hiPSCs. (a–l) Representative images of immunostained cardiac troponin T (cTnT) and sarcomeric a-actinin in all four cell lines at day 40. Striated staining patterns indicative of sarcomeres are seen in all hESC and hiPSC lines; however, striations are most extensive in H1 cells, wherein titin immunostaining (m, n) reveals a level or sarcomeric networking that was not observed in the other cell lines. Published in (136).

H1 ESCs Exhibit the most Extensive Cardiomyocyte Enrichment

By day 40, accurate counting of immunostained cells could not be performed due to extensive multilayering. As alternative, percentages of cells in ESC and iPSC cultures that were in the cardiomyogenic pathway were estimated from transduced MLC 2v-EGF. For these determinations, areas containing beating and non-beating cells were microdissected from each culture at day 40 and sub-cultured onto coverslips at low cell density, followed by transduction of a lentivirus-encoded MLC 2v-EGFP construct, which is activated at an early stage of ventricular myocyte differentiation. Four days later, cells containing MLC 2v-activated EGFP were imaged (Figure 13A) and enumerated (Figure 13B). Total cells were counted after counter-staining with TMRE, a supravital mitochondrial dye (see Figure 13A) we previously employed (137) to avoid having to fix cells on coverslips, which inexplicably caused extensive cellular losses. The filled bars in Figure 13B depict percentages of EGFP-positive cells from beating areas, revealing that the greatest percentage (86%) was from the H1 ESC cultures; by contrast, cells sub-cultured from beating clusters of the H9, C2a and C6a lines displayed only 65-67 percent EGFP-positive cells. The empty bars in Figure 13B depict percentages of EGFP-positive cells from the non-beating areas, which surprisingly was nearly as extensive as the percentages of EGFP-positive cells from the beating areas.


A
TMRE
MLC 2v-EGFP
Overlay
hESC-H1
hESC-H9
hiPS-c2a
hiPS-c6a

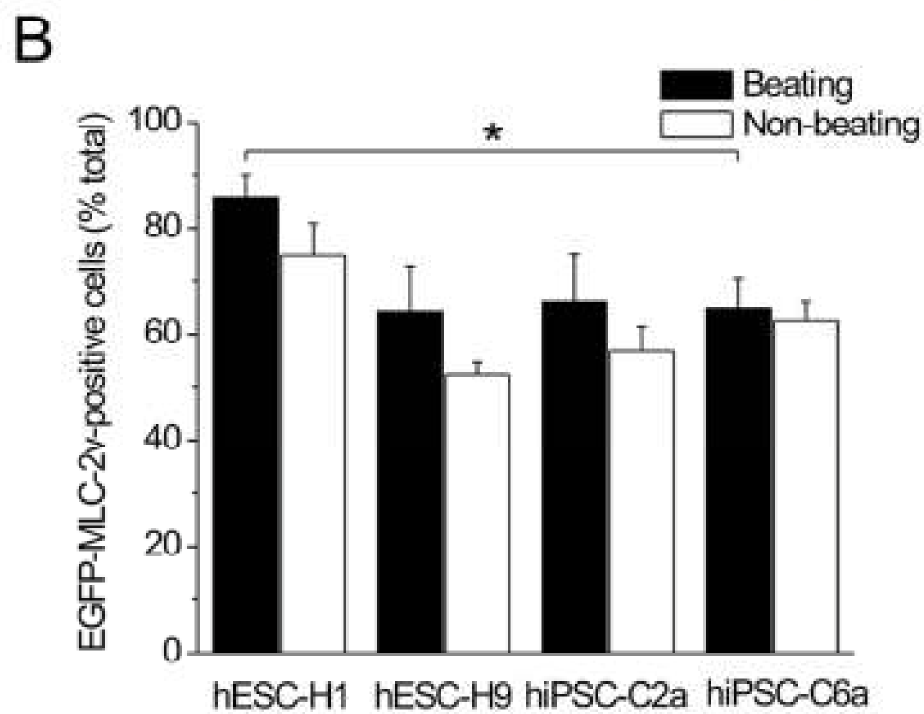

B
Beating
Non-beating
*
EGFP-MLC-2v-positive cells (% total)
100
80
60
40
20
0
hESC-H1
hESC-H9
hiPSC-C2a
hiPSC-C6a

Figure 13. MLC 2v activation in ESCs and iPSCs. Cells differentiated from all ESC and iPSC lines were sub-cultured and transduced with a lentivirus-encoced MLC 2v-eGFP construct at day 40. Four days later cells were imaged to detect EGFP and counter-stained TMRE. (A) fluorescent images of TMRE-positive (red fluorescence, left column) denoting total cells, and EGFP-positive (green fluorescence middle column) cells denoting cells in the cardiomyogenic lineage. (B) percentages of MLC 2v-EGFP-positive cells determined relative to numbers of total cells identified by TMRE mitochondrial staining. Each bar indicate the mean ± SEM of three determinations; $^{*}P<0.05$.

qPCR Monitoring of Marker Gene Expression

Expression of selected mRNAs was monitored by qPCR at multiple points during the course of differentiation (Figure 14). In all four lines, induction of differentiation at day 0 was followed by diminishing levels of the pluripotency marker OCT4, concomitant with an immediate peak in expression of the mesendoderm marker brachury (T), followed by decreases; this pattern was as predicted for these markers. The earliest phases of cardiomyogenic induction are characterized by expression of MESP1 (mesoderm posterior basic helix-loop-helix transcription factor 1), NKX2.5 and ISL1 (ISL LIM homeobox 1); expression of these markers was similar in all lines, except that expression of ISL1 was low in both iPSC lines but remarkably high in H9 ESCs. As cardiomyocytes begin to terminally differentiate, they express mRNAs including GATA4, MEF2C, TNNT2, TBX20 (T-box 20), and MYL7. Although these markers increased as expected during the second week of the 40 day culture period, expression of TNNT2, TBX20 and MYL7 was remarkably low in both iPSC lines. Expression of mRNAs encoding various ion channel genes (HCN4, SCN5A, CACNA1C, KCNH2, KCNJ2, KCND3) was also monitored, revealing that with the exception of HCN4 which peaked in all lines at day 6, all began to undergo steady, subtle increases late in the second week; however, no cell line-specific differences were noted. Finally, expression of mRNAs indicative of differentiation into ectodermal (paired box 6; PAX6) and endodermal (sex determining region Y-box 17; SOX17) lineages revealed that whereas all four lines exhibited strong SOX17 expression (300x-800x) during the first week, induction of ectoderm was minimal, with the exception of increased PAX6 levels in C2a iPSCs on days 4-12, and especially on day 40.

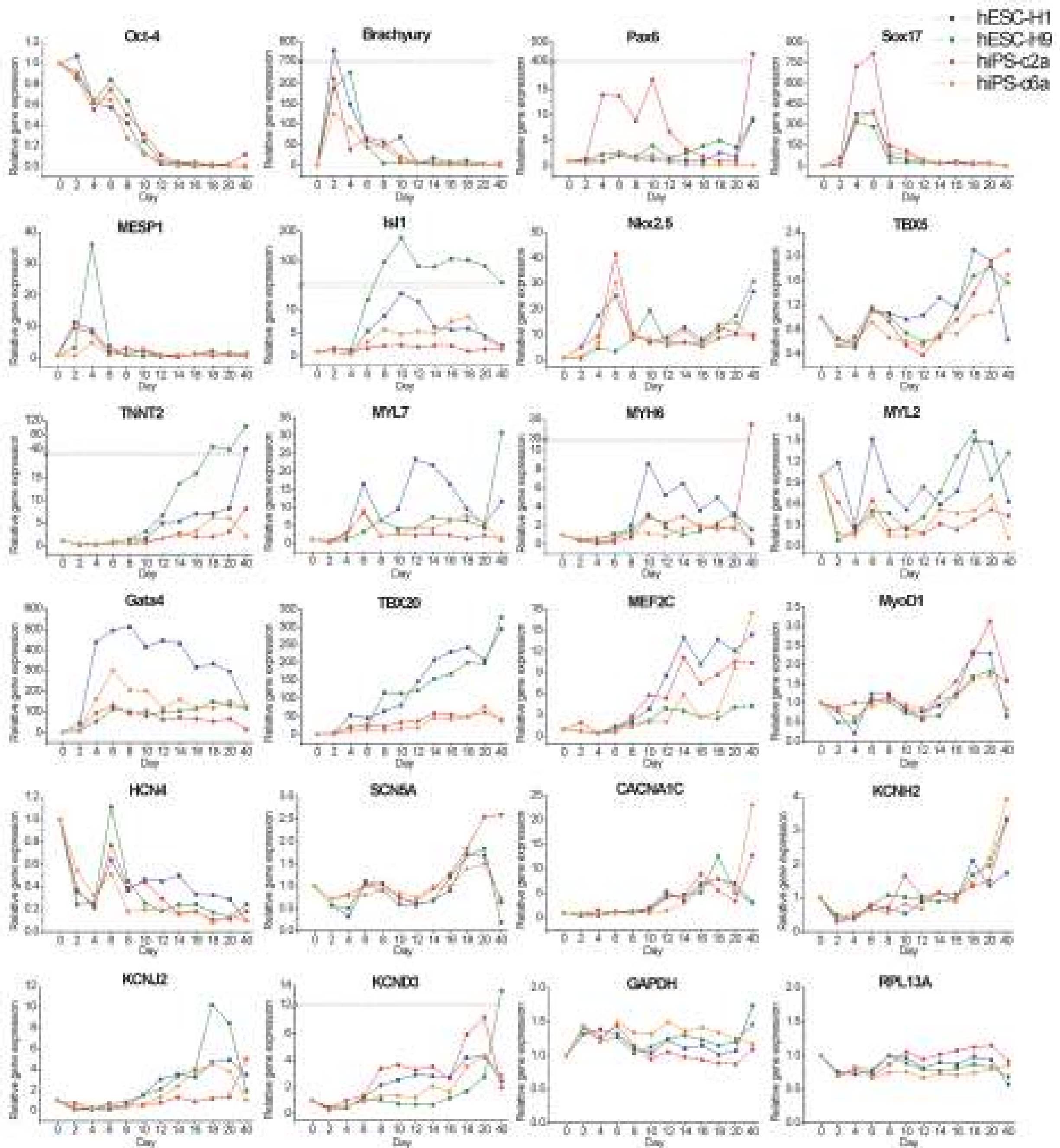


Figure 14. Gene expression during cardiomyogenesis in hESC and hiPSC lines. Expression of the indicated genes was monitored during the 40-day differentiation period by qPCR. Each data point is normalized to values at day 0 (induction of differentiation). Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and ribosomal protein L13a (RPL13A) served as loading controls. Each point represents the mean of duplicate samples; vertical lines indicate the range. Published in (136).

6. DISCUSSION

Summary of the obtained results

The results presented in this dissertation demonstrated that the undertaken directed differentiation of all tested human pluripotent stem cell lines results in high enrichment of cardiomyocytes especially in the beating areas. Differentiated hESC-derived cardiomyocytes exhibit many characteristics of mature heart cells, including spontaneous beating, expression of contractile myofibrils and generation of mature action potentials. These cells exhibit similar responses to APC as mature animal cardiomyocytes. Namely, volatile anesthetic isoflurane moderately increased ROS production by partly inhibiting respiratory chain and induced opening of the mitoK_{ATP} channels in these cells. Activation of these crucial signals of APC is qualitatively and quantitatively similar to previously published results using adult cardiomyocytes. Moreover, APC delayed opening of the mPTP and reduced cell death after exposure to oxidative stress, which is comparable to previously published results using adult human and animal myocardium. The results demonstrate the existence of competent endogenous mechanisms against oxidative stress, i.e. ischemia-reperfusion in hESC-derived cardiomyocytes and their responsiveness to APC, which indicates feasibility of using these cells as an *in vitro* model for testing APC. Thus, it can be concluded that hESC-derived cardiomyocytes can be used as a valid experimental model of human cardiomyocytes for studying APC. Our findings are in agreement with several other laboratories showing that stem cell derivatives are phenotypically comparable to mature cells and therefore could be used as a valid model for pharmacological testing.

The experiments in chapter 5 showed that cardiac differentiation of hiPSC lines recapitulates cardiomyogenesis in hESCs, which are considered gold standard for pluripotent stem cells. Similar to hESC, hiPSC lines exhibited high differentiation efficiency and the ability to generate contracting cell clusters. Cardiac sarcomeric proteins expressed striated pattern and the hiPSC-derived cardiomyocytes were positive for genetic labeling with cardiac-specific marker MLC 2v-EGFP. Temporal expression profile of pluripotency, early and late cardiac differentiation genes in hESC and hiPSC lines was in general comparable to previously published results, in spite of sporadic alterations in the expression profile for some individual genes and lines. Altogether, this indicated an appropriate cardiomyogenesis in both hESC and hiPSC lines. Cardiac differentiation efficiency was similar in hESC and

hiPSC lines, with the exception of H1 line that showed approximately 20% better results, which correlated with the greatest size of beating areas in this line. Similar to hESC-derived cardiomyocytes, hiPSC-derived cardiomyocytes also respond to APC by eliciting endogenous protective mechanisms against the oxidative stress that resulted in a delay in mPTP opening. All these results support the hypothesis that hiPSC-derived cardiomyocytes can be used for *in vitro* testing of APC, just like hESC-derived cardiomyocytes.

Cardiac differentiation of pluripotent stem cells

In this dissertation we achieved highly efficient differentiation of human cardiomyocytes from H1 hESCs, obtaining unprecedented levels of ~85% of cells positive for the cardiac-specific marker MLC 2v in beating cell clusters. An abundance of cardiomyocytes was corroborated by cardiac-specific immunostaining. Moreover, areas with spontaneously and rhythmically beating cells, a characteristic of cardiomyocytes, spanned the entire surface of the culture dish (please see link: http://links.lww.com/ALN/A624). hESC and hiPSC lines used here were previously tested for their pluripotency.(10-12) Cardiac differentiation of these cells was obtained by mimicking natural induction of cardiomyogenesis by endodermal signaling using growth factors activin-A and BMP4 in a RPMI/B27 medium. This approach is called directed differentiation.(147) Unlike commonly used EB approach that relies on spontaneous differentiation of different cell types, whereby cardiomyocyte enrichment is relatively poor, the directed differentiation yields a great number of cardiomyocytes.(11, 148)

We have applied BMP, FGF and activin-A, all endoderm-secreted growth factors, to direct differentiation into cardiac lineage, based on findings that endoderm-induced cardiomyogenic signaling regulates heart development in the embryo.(26) Previously published protocols utilizing same growth factors were modified in this study.(20, 29) Namely, pluripotent cells were exposed to activin-A at a relatively high level (50 ng/ml) for an extended period of time (five days). We speculate that this optimized the efficient production of mes-endoderm (149) leading to progressive differentiation into the cardiomyogenic lineage. Our results compare highly favorably with other studies and approaches to differentiate cardiomyocytes, especially with a widely used method involving spontaneous differentiation termed embryoid bodies that yields low percentage of cardiomyocytes (≤10 %).(150)

More than 55% and 65% of cardiomyocytes was obtained in non-beating and beating areas, respectively, for hiPSC and hESCs. The greatest cardiac differentiation was achieved in H1 hESC line where 75% and 85% of cardiomyocytes was detected in non-beating and beating areas, respectively. These results indicate that directed differentiation is superior methodology for obtaining enriched human cardiomyocytes than the EB approach. Other approaches targeting specific signaling pathways have also produced promising results and high cardiac enrichment. Wnt/β-catenine signaling pathway is important for cardiac differentiation. Its inhibition or stimulation at the specific time points of cardiomyogenesis can increases cardiomyocyte yield.(151) These seemingly contradicting data are further indicating complexity of the cardiac differentiation process whereby positive regulation by certain signaling pathways at the early stage of differentiation, such as Wnt/β-catenine pathway can become negative regulator at the later stages of differentiation. Therefore, it was very important in our protocol to restrict the treatment of human pluripotent stem cell lines with cardiomyogenic factors only to early differentiation stages and not throughout the differentiation process.

Tissues that build the heart are composed of three main mesoderm-derived cell lineages, the cardiomyocyte, the vascular smooth muscle and the endothelial cell lineages. There are four main steps which are necessary for derivation of cardiomyocytes from pluripotent stem cells: 1) formation of mesoderm; 2) commitment of mesoderm toward anterior mesoderm/cardiogenic mesoderm; 3) formation of cardiac mesoderm and 4) generation of early cardiomyocytes.(24, 27) The differentiation of pluripotent cells in vitro following these steps can be determined by characterization of the expression pattern of transcription factors characteristic for each step of the process. Pluripotent stem cells are characterized by the expression of OCT4 and NANOG which are diminishing upon the induction of the differentiation process.(22) The qPCR experiments presented here show that the expression of pluripotency factor OCT4 immediately decreases in all hESC and hiPSC lines, indicating the loss of pluripotency. Downregulation of OCT4 is accompanied with upregulation of SOX17, an endodermal marker, which is in line with the induction of endodermal signaling with activin-A and BMP4. The results also show early and transient increase in T/Brachyury expression in all tested lines. It is expressed during the specification of certain cells from primitive streak toward the mesoderm.

The expression of MESP-1 and ISL-1 is associated with specification of cardiac mesoderm and the expression of NKX2.5, TBX5, TBX20, GATA4, MEF2c and HAND1/2

with generation of cardiac progenitors.(24, 27) Here it was shown that early stages of cardiomyogenesis are characterized by transient increase in the expression of MESP1, NKX2.5 and ISL1, while GATA4 and TBX20 exhibited steady and progressively increasing pattern of expression, respectively. PAX6, an ectodermal marker, did not show any significant expression during the differentiation of cardiac myocytes except for the hiPSC-C2a cells. This could indicate less efficient cardiac mesodermal and later cardiac differentiation in this specific cell line. Cardiac development is a complex and dynamic process accomplished through the sequential expression of signal transduction proteins and transcription factors. The major signaling pathways involved in the development of heart are Wnts/Nodal, BMPs and FGFs.(24, 26, 27)

Electrical activity of cardiomyocytes. The most prominent evidence of cardiac differentiation of hESC and hiPSC lines was observation of spontaneously and rhythmically beating areas. This indicates the presence of electromechanically competent cardiomyocytes that form the functional syncytium, which allows them to respond to electrical stimuli and to contract synchronously. The spreading of action potentials among cardiomyocytes in culture dishes is enabled by the gap junctions, which represent channels connecting two cells. Two types of action potentials were detected in hESC-derived cardiomyocytes, shorter, atrial cardiomyocyte-like, and longer, ventricular cardiomyocyte-like.(152) These data are in correlation with another study where we demonstrated the presence of calcium transients in hiPSC-derived cardiomyocytes that by their duration corresponded to shorter and longer action potentials.(30) Therefore, these results suggest the presence of functional ion channels and transporters in both hESC- and hiPSC-derived cardiomyocytes that are capable of maintaining ion gradients across the sarcolemma which are prerequisite to action potentials. Moreover, these channels can respond to depolarization stimuli and follow programmed patterns of closing and opening in order to generate the action potential, i.e. timely fluxes of ions across the sarcolemma. In functional cardiomyocytes membrane depolarization caused by actins potentials is necessary to trigger calcium influx from T-tubules and sarcoplasmic reticulum.(153) Opening of sarcolemmal L-type calcium channels initiates influx of calcium that further induces calcium release from the sarcoplasmic reticulum via ryanodine receptor, which is called calcium-induced calcium release.(153) A rise in sarcoplasmic calcium allows the interaction among contractile filaments following the binding of calcium to troponin complex, which triggers the contraction of the cardiomyocyte. qPCR and immunostaining experiments confirmed the presence of cardiac troponin T in hESC and hiPSC lines. The

presence of calcium transients consisting of calcium influx (corresponding to heart systole) and calcium efflux (corresponding to heart diastole) in hiPSC-derived cardiomyocytes was shown in our previous study. This experiment provided the final proof of a proper excitation-contraction coupling and electromechanical competence of human pluripotent stem cell-derived cardiomyocytes.

qPCR experiments verified the presence of several ion channels that are found in cardiomyocytes and which underlie the observed excitability and electromechanical competence of stem cell-derived cardiomyocytes discussed in the paragraph above. This includes HCN4 (hyperpolarization-activated cyclic nucleotide-gated channel 4), which is a sodium and potassium channel found in adult sino-atrial node cells and also in the first heart field as a marker of cardiomyogenic progenitors.(154, 155) Unlike other ion channel genes analyzed here that showed greater expression in later phase of cardiac differentiation, HCN4A was found to have peak expression in early differentiation (day 6). A recent study demonstrated that maturation of hESC-derived cardiomyocytes is accompanied with increased association of HCN4 and caveolin-3, which is thought to be an important step in cardiac maturation.(156) Mutations in this gene have been linked to the sick sinus syndrome, which is manifested by arrhythmia, either as bradycardia, or tachycardia or combination of both in alternating pattern.(157, 158)

SCN5A (sodium channel, voltage-gated, type V, alpha subunit) is another sodium channel found to be increasingly expressed with cardiac differentiation of hESC and hiPSC lines here. The gene SCN5A encodes $Na_v1.5$, a sodium ion channel subunit., which is abundantly expressed in the human atrial and ventricular myocardium.(159) Opening of the channel is responsible for the initial upstroke of the action potential.(159) Mutations of this gene are found in several diseases, including Brugada syndrome, Romano-Ward syndrome, sick sinus syndrome and others.(158, 160) Brugada syndrome is caused by dysfunction of several different ion channels, where SCN5A mutation impairs normal actions potentials, especially in epicardial areas, leading to lethal ventricular fibrillations.(158) Romano-Ward syndrome is a form of long QT syndromes that predisposes heart to arrhythmia.(160)

CACNA1C (calcium channel, voltage-dependent, L type, alpha 1C subunit) encodes $Ca_V1.2$., a subunit of L-type calcium channel found in different cell types, especially in cardiac and brain cells.(161) As described in the chapter 1, opening of this channel leads to calcium influx into the cardiomyocyte causing a plateau in the action potential and also

initiation interactions among contractile filaments. The experiments presented here showed a progressive increase in the expression of this channel in all four lines, indicating its important role in the mature heart cells. The mutation of this gene causes Timothy syndrome that affects many parts of the body and causes long QT syndrome.(162, 163) Cardiac dysfunction in Timothy syndrome is characterized by prolonged calcium influx with arrhythmia and cardiac malformations.(162)

All three analyzed potassium channels KCNH2 (potassium voltage-gated channel, subfamily H (eag-related), member 2), KCNJ2 (potassium inwardly-rectifying channel, subfamily J, member 2) and KCND3 (potassium voltage-gated channel, Shal-related subfamily, member 3) exhibited a tendency for a stabile increase in the expression with cardiomyocyte maturation. KCNH2 is found in cardiomyocytes, neurons and microglia.(164) In excitable cells, channel made from alpha KCNH2 subunits is responsible for repolarization after initiation of the action potential.(160, 164) Mutations of this gene have been described in Romano-Ward syndrome, short QT syndrome and other diseases.(165) KCNJ2 is expressed in cardiac and skeletal muscle and is also responsible for cell repolarization.(166) Mutations are associated with Andersen-Tawil syndrome, short QT syndrome and familial atrial fibrillation.(166, 167) Andersen-Tawil syndrome is one of the subtypes of long QT syndromes. KCND3 is expressed in variety of cells, including cardiomyocytes. Its dysfunction is associated with lethal ventricular arrhythmias.(168) qPCR experiments demonstrated the presence of sodium, potassium and calcium channels that play the critical role in generation of action potentials and calcium influx and are principal structures that mediate observed action potentials and contractions in our human pluripotent stem cell-derived cardiomyocytes. Altogether, cell beating, action potentials and expression of cardiac ions channels indicates electromechanical competence of human pluripotent stem cell-derived cardiomyocytes tested in this dissertation.

<u>*Contractile machinery in cardiomyocytes.*</u> Regular beating pattern of cell clusters in all four lines, hESC and hiPSC, suggested the presence of the functional contractile machinery in these cells, which was verified with immunostaining experiments. Laser-scanning confocal microscopy allowed examination of sarcomeres with a high spatial resolution. Regular striated pattern was observed for two tested sarcomeric proteins, sarcomeric α-actinin and cTnT in all hESC and hiPSC lines. cTnT is found solely in cardiomyocytes, while sarcomeric α-actinin is less specific and is expressed in the Z lines of skeletal and cardiac muscle(150).(169) Additional testing was performed in H1 line for

myofilament proteins, titin and MLC 2a (myosin light chain 2a) which corroborated these results, i.e. they were also expressed in the striated pattern. Our results are in agreement with other studies showing enhanced expression of MLC 2v and MLC 2a and other sarcomeric proteins in a striated pattern in cardiomyocytes differentiated from human pluripotent stem cells.(170)

Extensive analysis of myofilament gene expression at various stages of cardiomyogenesis supported immunostaining experiments. A progressive increase in the expression with cardiac maturation was found for most of the tested genes. TNNT2 (troponin T type 2, cardiac) is normally found solely in the heart where it regulates the intensity of interaction of actin and myosin filaments and thereby heart contractility. The results presented here show continuous increase in the expression of this gene as it was expected due to cardiomyocyte maturation.(171) Both hESC lines exhibited greater expression of TNNT2 than the hiPSC lines in later stages of cardiomyogenesis, which is in correlation with the observation that differentiation of hESC lines yielded greater number of contracting areas. Mutations in this gene are found in familial hypertrophic cardiomyopathy.(172) MYL7 (myosin light chain 7), another contractile protein, was predominately expressed in a later phase of differentiation only in hESC lines, while in hiPSC lines its expression was increased, but steady throughout cardiomyogenesis. As for TNT2, MYL7 was also more expressed in hESC lines than hiPSC lines in later stages of cardiomyogenesis.

The obtained results are in agreement with a previously published whole-genome microarray analysis conducted in a hESC subjected to cardiomyogenic directed differentiation over a period of 12 days showing an increase in the expression of TNNT2, MYL7 and other myofilament genes.(171) The same study presented similar results when gene expression analysis was performed in human fetal heart obtained from voluntary abortion. This was an important finding since it demonstrated that human stem cell-derived cardiomyocytes exhibit similar gene expression pattern as the human fetal heart.

Genetic labeling of living cardiomyocytes. Although directed differentiation yields high percentage of cardiomyocytes, other cell types are also present in these culture dishes. In this dissertation, cell were genetically labeled with the reporter construct MLC 2v-EGFP that is expressed only in cardiac cells in order to conduct experiments only on cardiomyocytes. Following the directed differentiation and prior to experiments, cells were enzymatically dissociated and transduced with the lentiviral vector carrying MLC 2v-EGFP construct. This

construct allowed the expression of EGFP (that is detected by green fluorescence) only in cells that have cardiac specific promoter MLC 2v. Such approach has been previously used in Dr. Gepstein's laboratory to identify differentiated cardiomyocytes and the same group provided the MLC 2v-EGFP construct for our experiments.(138) The identification of cardiomyocytes with this approach is particularly useful as it allows identification of living cells that can be subsequently used for experimentation.

Cardiomyogenesis in hESC vs. hiPSC lines

We simultaneously compared the cardiomyogenic potential of two human iPSC lines with two well-characterized human ESC lines, under controlled conditions using a directed differentiation protocol. We show that cells from all four pluripotent stem cell lines can differentiate toward a cardiomyocyte fate. However, H1 ESCs exhibited the most overt cardiomyogenic competence, forming extensive areas of rhythmically contractile cells (Supplemental Video Clips 1-4 in reference (136)) that contained organized sarcomeres (Figure 3). Accordingly, in terms of biochemical differentiation, H1 cells from both beating and non-beating areas of the culture dish expressed the highest percentage of EGFP-positive cells (Figure 13), and, qPCR revealed that H1 cells were the most robust in terms of cardiomyogenic gene expression (Figure 14).

Several groups have reported that human and mouse iPSCs possess cardiomyogenic competence.(173-178) Among these findings, those of Zhang et al. (178) are particularly noteworthy because these investigators compared differentiation of H1 and H9 ESCs with two lines of lentivirus-induced human iPSCs, observing that sarcomere organization and expression of cardiomyogenic genes were indistinguishable in iPSCs and ESCs although the ability to contract was highest in H9 ESCs. Despite similarities in approach it is difficult to compare these findings with those described here since Zhang et al. (178) used an EB rather than a directed differentiation format, and because their gene expression analysis was based on a single endpoint determination. Regarding competence of H1 and H9 ESCs, differences in cardiomyogenic potency have previously been noted, attributed to epigenetic marks;(179, 180) reports now indicate that the most cardiomyogenic ESC line is either H1 (this paper and (137)), or H7 (20), or H9 (178). These differences are possibly related to differences in spontaneous differentiation that were noted during pluripotent expansion and passaging of the cell-lines used in this study. Specifically, spontaneous differentiation in H1 ESCs was minimal during more than 50 passages, in contrast with H9 ESC colonies which exhibited differentiation after only seven passages. Both iPSC lines displayed even greater propensity

to spontaneously differentiate, requiring the establishment of fresh colonies after every fourth passage. Although epigenetic factors may have influenced these results, differences in cell culture format and induction conditions, for example directed differentiation vs. the EB format, certainly effected differentiated outcomes. Hence, while the results described here indicate that both of the ESC and iPSC lines can enter the cardiomyogenic pathway but that only the ESC lines exhibited substantial terminal differentiation, such a conclusion must remain tentative until universal conditions are established to efficiently, reproducibly and rapidly induce pluripotent cells to defined terminally differentiated cardiomyogenic endpoints.

Regarding the relative inability of C2a and C6a iPSCs to develop into functional cardiomyocytes, the possibility that differentiation is suppressed due to the integration of pluripotency genes in lentivirus-induced iPSCs was previously noted.(178) However, the decline of OCT4 expression in C2a and C6a cells was essentially equivalent to that observed in H1 and H9 ESCs (Figure 14). Moreover, evidence that C2a iPSCs are differentiation-competent was recently demonstrated by the equivalent ability of C2a iPSCs and H9 ESCs to undergo growth factor-directed differentiation into mature hepatocytes, with efficiency exceeding 80%.(10) Although the relative inability of iPSCs to undergo terminal myocyte differentiation is unexplained, it is noted that the highly efficient differentiation of hepatocytes was performed in monolayer culture format wherein all cells are exposed to equivalent growth factor levels, in contradistinction to the induction of pluripotent cells organized in three-dimensional clumps as employed here. It is therefore speculated that the relative inability of iPSCs to terminally differentiate may indicate that while initial cardiomyogenic signals in clumped cells were sufficient to overcome the presence of integrated pluripotency genes, signals that regulate terminal differentiation were somehow compromised. It is nonetheless encouraging that most C2a and C6a cells, whether derived from beating or non-beating areas, exhibited activation of transduced MLC 2v (Figure 13), suggesting that these cells occupy a position in the cardiomyogenic pathway from which improved signaling may support terminal differentiation. In this regard we are currently inducing pluripotent cells maintained in monolayer culture, with the goal of establishing conditions that mimic the high levels of efficiency that are reproducibly achieved during hepatocyte differentiation.(10)

APC in human pluripotent stem cell-derived cardiomyocytes.

The results discussed so far demonstrate that hESC- and hiPSC-derived cardiomyocytes reconstitute crucial steps in cardiomyogenesis and that they exhibit key functions characteristic for adult cardiomyocytes, i.e. they form syncytium and synchronously beat in the culture dish. In the subsequent series of experiments it was tested whether human pluripotent stem cell-derived cardiomyocytes have developed competent endogenous mechanisms that can be elicited by the APC and which afford protection against the oxidative stress, a hallmark of reperfusion injury.

We showed here that isoflurane partly depolarized mitochondria in a 5-HD-sensitive manner, suggesting opening of mitoK_{ATP} channels, crucial mediators of cardioprotection.(97, 111) This effect was almost identical to isoflurane-induced opening of mitoK_{ATP} channels demonstrated with the similar approach in our previous study using adult rat cardiomyocytes,(111) indicating a comparable response between adult and hESC-derived cardiomyocytes. We also showed here that isoflurane moderately enhanced production of ROS, an important component of preconditioning signaling cascade.(52, 112) Our previous study demonstrated that desflurane and sevoflurane induce similar extent of ROS production in adult rat cardiomyocytes.(52) The importance of ROS was confirmed here by showing that a low dose of H_2O_2 induces preconditioning and delays mPTP opening, which is in agreement with a study by Hanouz et al. that indicated critical importance of ROS signaling in APC using adult human atrial trabeculae.(145) Anesthetic-induced increase in ROS production has been attributed to the opening of mitoK_{ATP} channels,(112) but it can be also induced by partial obstruction of the ETC,(181) another effect of volatile anesthetics.(109) Indeed, in this study we showed that isoflurane induces partial obstruction of the ETC, indicated by attenuation of oxygen consumption in beating cell clusters by isoflurane. This effect is similar to our results obtained in adult rat cardiomyocytes using identical approach, which we correlated to the ETC inhibition.(104) The importance of mitoK_{ATP} channel opening for inducing cardioprotection by APC was verified here by showing that inhibition of channel opening by 5-HD abrogates the APC-induced delay in mPTP opening.

In chapter 5 it was shown that volatile anesthetic isoflurane inhibits oxygen consumption of isolated beating cell clusters that have high hESC-derived cardiomyocyte enrichment. This result is in a good correlation with our previous study demonstrating similar effect in adult rat cardiomyocytes.(104) In that study, an extensive investigation including non-permeabilized cells and isolated mitochondria demonstrated that observed inhibition of

respiration originated from the partial inhibition of the complex I of the respiratory chain by isoflurane. This finding is also verified by other studies.(109) It has been shown that the partial inhibition of complex I, for example with rotenone, increases generation of ROS.(110) A partial inhibition of the complex I of the respiratory chain disrupts the normal electron flow within the complex increasing electron leak at the complex I. Resulting incomplete reduction of oxygen molecule by a single electron at the complex I yields a superoxide anion ($O_2^{\bullet}$).(44, 182) Normally, during oxidative phosphorylation, two electrons reduce a single oxygen molecule yielding water at the complex IV. These experiments suggested that partial complex I inhibition by isoflurane may result in a moderate increase in ROS production that in turn triggers preconditioning signaling cascade. Indeed, results shown in Figure 8 verified that clinically relevant concentration of isoflurane (0.5 mM, corresponding to 1 minimal alveolar concentration) moderately increased production of ROS in hESC-derived cardiomyocytes. The importance of this moderate increase in ROS production is best demonstrated in a study showing that elimination of this signaling ROS by ROS scavenger Trolox completely blocked preconditioning by two volatile anesthetics sevoflurane and desflurane.(52) Several other studies also showed that ROS scavenging during triggering phase of either ischemic preconditioning or isoflurane-induced APC blocks cytoprotection.(183-185) Although it may seem paradoxical that ROS exerts beneficial effect for protecting cells from oxidative stress and ischemia-reperfusion injury, it is now known that moderate amount of stress in the form of ROS serve as the crucial signal that initiates preconditioning and prepares the cell for upcoming greater amounts of oxidative stress occurring in the reperfusion. This was verified by the experiment in this dissertation showing that small amounts of exogenous H_2O_2 triggered preconditioning and protected cells from the excessive amounts of oxidative stress causing cell death. Downstream targets of this signaling ROS that have been identified so far include PKC and the hypoxia-induced factor-1alpha (HIF-1α) in cancer cells.(113, 186)

In the chapter 5 it was also shown that isoflurane depolarizes mitochondria, which was attenuated by inhibition of mitoK_{ATP} channels with 5-HD, and which was mimicked by mitoK_{ATP} channel opening with diazoxide. This finding indicated that isoflurane induced opening of mitoK_{ATP} channels, which was previously shown in many studies to be important event for preconditioning.(95, 97, 111, 112) Opening of the mitoK_{ATP} channels plays a significant role both in the triggering phase of preconditioning, and as an effector of cytoprotection.(97) In the triggering phase, opening of the mitoK_{ATP} channels may contribute

to enhanced ROS production, which augments preconditioning stimulus by stimulating the flux of electrons along the respiratory chain and thereby electron leak. Studies showed that mitoK_{ATP} channels remain open during the memory phase of preconditioning and attenuate ischemia-reperfusion injury by decreasing mitochondrial calcium overload, which is one of potent inducers of mPTP opening.(111, 126) Partial inhibition of the respiratory chain with a moderate increase in ROS production and opening of mitoK_{ATP} channels are fundamental events required for eliciting cytoprotective pathways. The obtained results are well matched with previous results and indicate that APC elicits adequate signals in hESC-derived cardiomyocytes.

The final proof of effectiveness of APC in human pluripotent stem cell-derived cardiomyocytes were experiments with the mPTP opening and the cell survival. Our results indicated that preconditioning with isoflurane induced a delay in opening of the mPTP both in hESC- and hiPSC-derived cardiomyocytes. Preconditioning with 0.5 mM isoflurane increased the arbitrary mPTP opening time compared to control in hESC-derived cardiomyocytes exposed to photoexcitation-generated oxidative stress. This indicated that APC elicited innate mechanisms that increased the threshold for mPTP opening. It has been shown before that cardioprotective treatments increase the amount of stress required for induction of mPTP opening.(80, 102) This has a significant functional importance since the opening of mPTP has been recognized as the crucial event in the transition towards cell death during I/R injury.(42). Moreover, mPTP inhibition reduces infarct size *in vivo*.(133, 134) Cardioprotective strategies, including APC were found to induce delay in mPTP opening and mPTP blockers may decrease infarct size by 30-50%.(187) This is in correlation with the observed delay in mPTP opening by mPTP inhibitor cyclosporine A, as shown in Figure 9 here.

Inhibition of mitoK_{ATP} channel opening abrogated APC, while preconditioning with the exogenous H_2O_2 delayed mPTP opening. These experiments verified an important role of mitoK_{ATP} channels and small amounts of ROS for induction of cytoprotection by APC in hESC-derived cardiomyocytes. Animal *in vivo* experiments show that inhibition of mitoK_{ATP} channels by 5-HD abrogates APC,(112) while preconditioning with diazoxide, a mitoK_{ATP} channel opener reduces infarct size.(188)

During I/R, opening of mPTP dissipates $\Delta\Psi_m$, preventing oxidative phosphorylation and initiates death pathways in the cell.(42) Using an identical approach as in this dissertation, we previously demonstrated that APC induces a similar delay in mPTP opening

in adult rat cardiomyocytes,(102) which further correlates with another study from our laboratory demonstrating that APC elicits cellular and mitochondrial protective mechanisms against oxidative stress in human adult cardiomyocytes.(100) The inhibition or the delay in mPTP opening is associated with improved cell survival since mPTP opening is a critical step in the initiation of apoptotic and necrotic death pathways.(56, 57, 64, 126, 189) Inhibition of mPTP opening can postpone cell death pathways by preventing the release of intramitochondrial proteins, such as apoptosis-inducing factor (AIF), or by preventing the rupture of outer mitochondrial membrane and the release of proapoptotic cytochrome c, or by delaying cessation of ATP production that is accelerated with the collapse of $\Delta\Psi_m$ due to mPTP opening.(126) It is also shown here that APC induced the delay in mPTP opening in hiPSC-derived cardiomyocytes, suggesting existence of equivalent cytoprotective mechanisms in hESC-derived cardiomyocytes and hiPSC-derived cardiomyocytes. A strong correlation between mPTP opening and cell death was confirmed with cell survival experiments in Figure 5 showing that APC-induced delay in mPTP opening is paralleled with a decrease in the number of dead hESC-derived cardiomyocytes exposed to oxidative stress. This was another proof that hESC-derived cardiomyocytes have developed competent cytoprotective machinery that protects cells against oxidative stress in a similar fashion as in adult cardiomyocytes freshly isolated from the rat heart.

Taken altogether, all tested parameters indicate similar responses of adult cardiomyocytes and hESC-derived cardiomyocytes to APC.

Studies using hESC-derived cardiomyocytes to regenerate dysfunctional myocardium after I/R injury have had limited success due to factors including inefficient differentiation, poor engraftment and survival within injured myocardium.(190) However, Laflamme et al. demonstrated that the use of a pro-survival cocktail during implantation of hESC-derived cardiomyocytes improved cell engraftment and functional recovery of the heart.(5) This cocktail protected graft cells from the stressful environment of (post)ischemic myocardium by blocking cellular death pathways. Interestingly, APC involves inhibition of the same cellular death pathways as pro-survival cocktail.(102, 103, 112) This suggests that APC could improve cardiomyocyte engraftment, with the added advantage that, unlike the components of the cocktail, volatile anesthetics are approved for the clinical application. In fact, the recent Guidelines by the American College of Cardiology and the American Heart Association recommended the use of volatile anesthetics based on the findings of 15 randomized trials in patients undergoing coronary bypass surgery showing that volatile anesthetics decrease

cardiac troponin release and improve ventricular function in comparison with intravenous anesthetics.(99)

Relevance of the doctoral dissertation and prospects

The key scope of this dissertation proposal is to contribute to the development and the use of human pluripotent stem cell-derived cardiomyocytes, especially hiPSC-derived cardiomyocytes for investigation of APC. Preconditioning by volatile anesthetics is a clinically applicable therapy that may ameliorating cardiac ischemia-reperfusion injury.(99) Human cell models based on the differentiation from stem cells have been recognized as an attractive approach for studying many other disease models based on different cell types.(6, 31, 33, 38, 39) Therefore, this dissertation, by showing feasibility of utilizing human stem cell models for testing APC will also aid and encourage the use of this approach for studying other diseases. The current process of new drug discovery is relatively expensive and lengthy in part due to inefficient translation of findings from animals to human subjects.(6) Human stem cell-based models hold a great promise in upgrading translational research with a "clinical trial in a dish" approach.(6) Along these lines, Dr. Collins stated: "The use of animal models for therapeutic development and target validation is time consuming, costly, and may not accurately predict efficacy in humans." He sees the solution in: "more reliable efficacy models that are based on access to biobanks of human tissues, use of human embryonic stem cell and induced pluripotent stem cell models". Thus, by contributing to new trends for accelerating and advancing translational research, this dissertation may have an important impact on people's health and pharmaceutical industry. Other investigators have demonstrated that hESC- and hiPSC-based models reliably recapitulate certain normal and disease phenotypes, including "cerebral organoids" as a 3D model of early brain development or long QT syndrome cardiac model exhibiting electrophysiological properties characteristic for cardiomyocytes of patients with the disease.(33, 39)

hiPSC models are of particular value for disease modeling since these cells carry identical genotype as the person who donated skin fibroblasts or other cells for reprogramming. This can be exploited in a sense that by selecting patients with specific disease with genetic component, derived hiPSC lines and differentiated cells exhibit phenotypic characteristics driven by the disease genotype (Figure 1). This provides a unique opportunity to dissect genetic and environmental factors of that disease using a cell model in a highly controlled experimental environment. Based on the ground work laid by this

dissertation, our group has already utilized diabetic hiPSC-derived cardiomyocytes to study underlying mechanisms of inefficient APC in a diabetic heart.(191) This pioneering manuscript has demonstrated for the first time that both genetic and environmental factors (high glucose) interfere with APC in diabetic cardiomyocytes. Although in humans we can correlate genes with diseases like type 2 diabetes mellitus, our understanding of phenotypic significance, mechanistic aspects and quantitative nature of pathological processes related to identified genes requires experimental perturbations that are almost exclusively performed in animal models. Since animal models cannot consistently emulate human (patho)physiology, hiPSC-derived cardiomyocytes would allow phenotypic characterizations circumventing ever-present issue of species differences. hiPSC-derived cardiomyocyte would allow not only to distinguish contribution of genetic and environmental factors to inefficient preconditioning in diabetics, but they allow us to "rescue" preconditioning by overexpressing or silencing genes of interest. hiPSC-derived cardiomyocytes can widen our perspectives in studying absence of cardioprotection by APC in diabetics. This aspect of future use of the hiPSC-derived cardiomyocyte model is epidemiologically and clinically very important. On one hand, the incidence of diabetes mellitus is increasing. On the other hand, clinical reports show that cardiovascular incidents like myocardial infarction are particularly severe in diabetics,(192) while APC is ineffective in animal models of ischemia-reperfusion injury.(193) Due to limited availability of human subjects and materials for research purposes and the use of less reliable animal models, mechanisms by which diabetes and other factors modify preconditioning are not completely understood. Extensive studies are required to identify defective pathways of APC in human diabetic myocardium in order to design targeted therapy. hiPSC-derived cardiomyocytes seem as a new promising tool in this quest, and in other clinically relevant problems, such as ageing, where APC is ineffective or requires improvements.

Cardioprotection by APC has been first described in 1988 at the Department of Anesthesiology, MCW, two years after initial discovery of ischemic preconditioning by Murry and colleagues.(194) These cardioprotective properties of volatile anesthetics have been recognized and promoted in the Guidelines of the American College of Cardiology and the American Heart Association, which is the best indicator of relevance of APC for clinical practice.(99) Modulation of myocardial infarct size by pharmacological agents either as preconditioning or postconditioning strategies are potentially very valuable for clinical practice.

7. CONCLUSION

Several lines of evidence were presented in this dissertation to support the hypothesis that human pluripotent stem cell-derived cardiomyocytes exhibit similar responses to APC as adult animal cardiomyocytes and human myocardium and represent a feasible model for studying APC in human cell model *in vitro*: 1) directed differentiation yields high percentage of cardiomyocytes both in hESC and hiPSC lines, 2) cardimyogenesis in hESC and hiPSC lines recapitulates temporal gene expression profile seen in normal cardiac differentiation, 3) differentiated cardiomyocytes are excitable cells that exhibit functional contractile machinery and are capable of performing regular contractions, 4) volatile anesthetic isoflurane elicits crucial mediators of preconditioning signal transduction pathways and effectors of cytoprotection in human pluripotent stem cell-derived cardiomyocytes resulting in improved cell resistance to oxidative stress. Thereby, it can be concluded that human pluripotent stem cell-derived cardiomyocytes can be used for studying mechanisms of APC *in vitro*. This can be used as a new complementary experimental model that may accelerate translation of results obtained in basic research to clinical practice.

We showed that hESC-derived cardiomyocytes phenotypically resemble functional human cardiomyocytes by showing that these cells spontaneously and rhythmically contract, generate action potentials that, by shape and morphology resemble functional human cardiomyocytes. Moreover, these cardiomyocytes exhibit highly organized sarcomeric structures, indicated by immunostaining for cardiac-specific cTnT and MLC 2a and non-specific sarcomeric α-actinin and titin. This is in accord with other laboratories that have described structural properties of hESC-derived cardiomyocytes.(150, 195, 196) The presence of spontaneously and rhythmically beating cell clusters that extend throughout the culture dishes (please see link: http://links.lww.com/ALN/A624), indicate presence of cardiomyocytes that form a functional syncytium which exhibits synchronized action potential propagation.(150, 197) Other laboratories demonstrated electrophysiological and functional competence of hESC-derived cardiomyocytes by electrophysiological recordings, measurements of Ca^{2+} transients, as well as appropriate chronotropic responses to β- and muscarinic receptor stimulation.(150, 195, 197-201) However, the extent of these cells' maturity requires further investigation.(190) At minimum, studies describe hESC-derived

cardiomyocytes as cells having characteristics of embryonic cardiomyocytes that may, with extended time in culture, differentiate into a mature cardiomyocyte phenotype.(202)

We have demonstrated molecular mechanisms by which preconditioning with isoflurane elicits competent defense against oxidative stress in hESC-derived cardiomyocytes. These protective mechanisms have been previously characterized in our and other laboratories using animal (52, 102, 103, 111) and, to lesser extent, human myocardium.(100, 145) However, it was unclear whether hESC-derived cardiomyocytes have adequate phenotype to resist oxidative stress, a hallmark of I/R injury.(41) In this study, we showed that APC successfully protects hESC-derived cardiomyocytes from oxidative stress, which is in accordance with the results of our previous study using adult human atrial cardiomyocytes.(100) To investigate the phenotypic similarity of the cardioprotective mechanisms by APC between adult cardiomyocytes and hESC-derived cardiomyocytes, we tested some of the most relevant mediators of protection.

In conclusion, our study shows for the first time that preconditioning with anesthetic isoflurane elicits competent defensive mechanisms against oxidative stress in ventricular hESC-derived cardiomyocytes. A similar APC-induced delay in mPTP opening in hESC- and hiPSC-derived cardiomyocytes suggested existence of similar cytoprotective mechanisms in both hESC-derived cardiomyocytes and hiPSC-derived cardiomyocytes. The similarity in responses to APC between adult cardiomyocytes documented in our previous work and hESC-derived cardiomyocytes demonstrated here implies that these cardiomyocytes can be used as a valuable experimental human model to study APC, and possibly other human diseases/treatments. As a complimentary model of human cardiomyocytes, hESC-derived cardiomyocytes offer new experimental advantages, overcoming previous limitations when using human myocardium from patient surgeries. Moreover, APC may be a promising tool for protecting hESC-derived cardiomyocytes during engraftment, thereby increasing regeneration of injured myocardium. Importantly, our study also demonstrates a simple and efficient modification of previously published protocols to differentiate human cardiomyocytes from hESCs.

Although biochemical evidence of cardiomyogenesis was similar in all four hESC and hiPSC lines, terminal differentiation into cardiomyocytes exhibiting well-defined sarcomeres and rhythmic contractions was most pronounced in H9, and especially in H1 ESCs. However, a considerable cardiomyocyte enrichment obtained in hiPSC lines suggests feasibility of using these cells for *in vitro* testing of APC.

8. ABSTRACT OF DISSERTATION

The lack of human myocardium for research purposes represents a great problem in studying pathogenesis of human cardiac disease. The doctoral dissertation is designed to test whether cardiomyocytes differentiated from human pluripotent stem cells can be used for studying anesthetic preconditioning (APC), a cardioprotective strategy. Human embryonic stem cell (hESC) lines, H1 and H9, and human induced pluripotent stem cell (hiPSC) lines, C2a and C6a were differentiated into cardiomyocytes by directed differentiation. All experiments with hESCs were conducted at the Medical College of Wisconsin using commercially available lines and their use was approved by the local Ethical Committee. hiPSC were obtained from the laboratory of Dr. S.A Duncan, MCW. Cardiac differentiation of hESC and hiPSC lines was accompanied with the occurrence of spontaneous contractions in cell clusters. Cardiac enrichment was greater than 65% in beating clusters of all cell lines. Living differentiated cardiomyocytes were labelled with a genetic construct, MLC 2v-EGFP, delivered by a lentiviral vector. Immunostaining for sarcomeric proteins, sarcomeric-α actinin and cardiac troponin T revealed that sarcomeres in hESC- and hiPSC-derived cardiomyocytes exhibited a proper striated pattern. The time course of expression of genes involved at various stages of cardiomyogenesis was analyzed by qPCR in all hESC and hiPSC lines. It revealed that the loss of expression of pluripotency and later mesodermal gene was accompanied by the increase in expression of genes of early phase of cardiac differentiation. Genes characteristic for terminal cardiac differentiation were expressed during the second week of cardiomyogenesis. APC of hESC- and hiPSC-derived cardiomyocytes induced a delay in opening of the mitochondrial permeability transition pore (mPTP) in stressed cells, which is a critical mediator of protection by APC. Increase in the survival of preconditioned cardiomyocytes correlated with the delay in mPTP opening in hESC-derived cardiomyocytes. Isoflurane activated characteristic mediators of preconditioning in hESC-derived cardiomyocytes. It induced opening of mitoK_{ATP} channels and moderately increased production of reactive oxygen species by inhibiting respiratory chain.

In conclusion, functional human pluripotent stem cell-derived cardiomyocytes can be efficiently differentiated *in vitro*. These cells exhibit competent responses to APC and therefore could be used as a reliable experimental models for studying APC.

Keywords: human pluripotent stem cells, differentiation, cardiomyocytes, anesthetic preconditioning, reactive oxygen species, mitochondrial permeability transition pore.

9. SADRŽAJ NA HRVATSKOM JEZIKU

NASLOV DISERTACIJE:

Kardiomiociti diferencirani iz ljudskih pluripotentnih stanica kao eksperimentalni model za prekondicioniranje potaknuto anesteticima

AUTOR: Ana Šepac

Zagreb, 2015

SADRŽAJ DISERTACIJE

Uvod: Postoje brojne prepreke u translacijskim istraživanjima i njihovoj sigurnoj i efektivnoj kliničkoj primjeni, kao što je upotreba animalnih modela koji su skupi, zahtijevaju velik utrošak vremena i ne mogu predvidjeti točan odgovor u ljudi. Ipak, istraživanja koja testiraju učinke lijekova i patogenezu brojnih patoloških stanja i bolesti, uključujući i bolesti srca, se većinom provode na životinjskim modelima i zahtijevaju ocjenu valjanosti na ljudskim modelima. Priznate alternative su ljudski modeli dobiveni iz banaka ljudskog tkiva, ljudske embrionalne matične stanice (hESC) i ljudske inducirane pluripotentne matične stanice (hiPSC). Anesteticima-potaknuto prekondicioniranje (APC) je strategija koja potiče endogene zaštitne mehanizme u stanicama srca koji mogu povećati preživljenje ovih stanica tijekom ishemijsko-reperfuzijske ozljede. Doktorska disertacija je osmišljena kako bi se testiralo mogu li se ljudske pluripotentne matične stanice diferencirati u kardiomiocite koji bi se koristili kao eksperimentalni model za istraživanje APC-a.

Materijali i metode: Predložena disertacija se bazira na istraživanjima provedenim na dvije vrste ljudskih pluripotentnih matičnih stanica, i to na hESC linijama i hiPSC linijama. Prethodno je pokazano da se ove pluripotentne matične stanice mogu diferencirati u derivate svih triju embrionalnih zametnih listića, uključujući i kardiomiocite. U istraživanju se koristilo komercijalno dostupne hESC linije, H1 i H9, a eksperimenti su se izveli na Medical College of Wisconsin-u uz odobrenje lokalnog etičkog povjerenstva. hiPSC linije su dobivene

iz laboratorija dr. S. Duncana. Ove stanice su generirane iz fibroblasta kože prepucija nakon transdukcije s čimbenicima OCT4, NANOG, SOX2 i LIN28. Pokazano je da ekspresija tih proteina uzrokuje dediferencijaciju već diferenciranih fibroblasta u pluripotentno stanje. Laboratorij dr. Duncana je potvrdio sve karakteristike pluriopotentnosti ovih stanica i pokazao da se mogu efikasno diferencirati u hepatocite. Kardiomiociti su diferencirani iz pluripotentnih stanica metodom usmjerene diferencijacije. Da bi se eksperimenti provodili isključivo na kardiomiocitima stanice su se obilježile zelenim flourescentnim proteinom (EGFP) pod transkripcijskom kontrolom gena za miozinski laki lanac 2v (MLC 2v) koji je specifičan za srčane stanice. Ovakvo obilježavanje se provelo nakon usmjerene diferencijacije tako da su stanice bile transducirane lentiviralnim vektorom koji sadrži MLC2v-EGFP konstrukt. Ovaj konstrukt omogućuje da EGFP (koji se detektira zelenom fluorescencijom) bude izražen samo u stanicama koje istovremeno imaju i MLC-2v transkripcijski čimbenik, odnosno u kardiomiocitima. Lentiviralni vektor je dobiven iz laboratorija dr. F. Parka, Medical College of Wisconsin. Svi eksperimenti u kojima se koristio ovaj virus su provedeni na Medical College of Wisconsin-u. Kardijalna diferencijacija se testirala imunocitokemijskom detekcijom sarkomerskog α-aktinina, kardijalnog troponina T i titina. U disertaciji se testiralo mogu li diferencirani kardiomiociti odgovarati na APC na isti način kao i zreli kardiomiociti, a dobiveni podatci su se usporedili s rezultatima prethodnih studija. Ispitalo se nekoliko dobro okarakteriziranih i značajnih komponenti APC-a. Testiralo se može li APC povećati preživljenje kardiomiocita diferenciranih iz hESC nakon izlaganja oksidativnom stresu koji je induciran uz pomoć vodikovog peroksida. Zatim se testiralo ima li inhalacijski anestetik izofluran, koji potiče APC, utjecaja na proizvodnju slobodnih kisikovih radikala, što je izmjereno u hESC-diferenciranim kardiomiocitima uz pomoć fluorescentnog indikatora dihidroetidina i konfokalnog mikroskopa. Važni izvršitelji kardioprotekcije APC-om su također testirani. Ovo uključuju otvaranje ATP-ovisnih kalijevih kanala u mitohondrijima (mitoK_{ATP}) i odgodu u otvaranju mitohondrijske permeabilizacijsko-tranzicijske pore (mPTP). Otvaranje mPTP-a je inducirano oksidativnim stresom a detektirano je mjerenjem brze i potpune depolarizacije mitohondrija konfokalnim mikroskopom uz pomoć fluorescentnog indikatora TMRE. Kvantitativni PCR se koristio da određivanje izražaja gena koji su značajni u različitim stadijima kardijalne diferencijacije. Potrošnja kisika u izoliranim nakupinama diferenciranih kardiomiocita je mjerena uz pomoć Clarkove elektrode, što se koristilo za ispitivanje protoka elektrona kroz respiratorni lanac i neizravno za procjenu moguće opstrukcije respiratornog lanca od strane izoflurana.

Rezultati: Usmjerena diferencijacija rezultira je visokim udjelom kardiomiocitima u dijelovima koji spontano i ritmično kucaju, pri čemu je najviše kardiomiocita detektirano u H1 hESC liniji (85%). U ostalim linijama taj broj se kretao oko 65%. Kardiomiociti su izražavali sarkomerične proteine, kardijalni troponin T, sarkomerični α-aktinin i titin. U hESC-kardiomiocitima su detektirani akcijski potencijali koji morfološki odgovaraju zrelim ventrikularnim i atrijskim srčanim stanicama. APC u hESC-kardiomiocitima potiče protektivne mehanizme što se očitovalo poboljšanim preživljenjem nakon izlaganja oksidativnom stresu te odgođenim otvaranjem mPTP-a. mPTP je ključni događaj koji inicira različite puteve koji dovode do smrti stanice Pokazano je da izofluran djelomično inhibira respiratorni lanac u hESC-kardiomiocitima što dovodi do umjerenog porasta u proizvodnji kisikovih radikala koji igraju ključnu ulogu u unutarstaničnoj signalizaciji tijekom prekondicioniranja. Izofluran je doveo i do otvaranja mitoK_{ATP} kanala, koji je također važan medijator prekondicioniranja. APC je odgodilo otvaranje mPTP-a i u hiPSC-kardiomiocitima. Kvantitativni PCR je pokazao da usmjerena diferencijacija dovodi do sličnog obrasca izražaja različitih gena tijekom diferencijacije hESC i hiPSC linija. Pokazano je da tijekom diferencijacije prvo dolazi do smanjenog izražaja gena pluripotentnosti (OCT4), nakon čega slijedi kratkotrajni porast izražaja mezodermalnog markera (brachyury). Rana faza kardijalne diferencijacije je obilježena izražajem nekoliko gena, MESP1, NKX2.5 i ISL1. Terminalna faza kardijalne diferencijacija je obilježena izražajem GATA4, MEF2C, TNNT2, TBX20 i MYL7. Ionski kanali SCN5A, CACNA1C, KCNH2, KCNJ2 i KCND3 su uglavnom bili izraženi tijekom kasne faze diferencijacije.

Zaključci: Ova disertacija je ponudila nekoliko dokaza koji podupiru hipotezu da se kardiomiociti diferencirani iz ljudskih pluripotentnih matičnih stanica mogu koristiti za proučavanje APC-a *in vitro*. Ovo uključuje: 1) učinkovitu diferencijaciju velikog broja kardiomiocita, 2) kardiomiogenezu koja rekapitulira vremenski obrazac izražaja ključnih gena važnih za normalnu srčanu diferencijaciju, 3) diferencijaciju kardiomiocita koji su ekscitabilne stanice, koji imaju funkcionalne sarkomere, te mogu izvoditi kontrakcije i 4) kompetentne odgovore na APC uz aktivaciju karakterističnih medijatora prekondicioniranja.

Ključne riječi: ljudske pluripotentne matične stanice, diferencijacija, kardiomiociti, anesteticima-potaknuto prekondicioniranje, slobodni radikali kisika, mitohondrijska permeabilizacijsko-tranzicijska pora.

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11. BIOGRAPHY

Dr. Ana Šepac was born in Zagreb in 1981. After obtaining an MD degree in 2005 at the University of Zagreb School of Medicine, she completed a clinical internship. In 2007 she joined Dr. Bosnjak's laboratory at the Department of Anesthesiology, Medical College of Wisconsin, USA, as a postdoctoral fellow. During that time she helped in establishing the first stem cell laboratory in the Department. In 2010 she returned to Croatia and in 2011 she joined the Department of Pathology, University of Zagreb School of Medicine as a junior researcher. In 2014 Dr. Šepac has started a pathology residency. In 2014 she received an award "For Women in Science" given by the L'Oreal, UNESCO and the Ministry of Culture of the Republic of Croatia for the best female senior graduate students in Croatia. At the moment Dr. Šepac has seven manuscripts listed at the PubMed database and several more in the preparation. She has participated as a postdoctoral fellow in two NIH grants.

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