Aus dem Zentrum Physiologie und Pathophysiologie der Universität zu Köln

Institut für Neurophysiologie

Geschäftsführender Direktor: Universitätsprofessor Dr. med. J. Hescheler

Xeno free Culture Conditions for Human Pluripotent Stem Cells (ES and iPS cells) for Investigations of Cardiomyogenic Effects of Small Molecules

Inaugural-Dissertation
zur
Erlangung der Würde eines
doctor rerum medicinalium
der Hohen Medizinischen Fakultät der Universität zu Köln

vorgelegt von

IGNATIUS GUNASEELI JESUDOSS

aus

Paramakudi, TN, Indien

Promoviert am: 01.Juni 2011

Dekan:

Universitätsprofessor Dr.med.Dr.h.c.Th.Krieg

1.Berichterstatter:

Professor Dr. rer. nat. A. Sachinidis

2.Berichterstatter:

Universitätsprofessor Dr. med. W. Krone

Erklärung

Ich versichere, dass ich die von mir vorgelegte Dissertation selbständig angefertigt, die benutzten Quellen und Hilfsmittel vollständig angegeben und die Stellen der Arbeit- einschließlich Tabellen und Abbildungen-, die anderen Werke im Wortlaut oder dem Sinn nach entnommen sind, in jedem Einzelfall als Entlehnung kenntlich gemacht habe; dass diese Dissertation noch keiner anderen Fakultät oder Universität zur Prüfung vorgelegen hat; dass sie- abgesehen von unten angegebenen beantragten Teilpublikationen- noch nicht veröffentlicht ist, sowie, dass ich eine Veröffentlichung vor Abschluss des Promotionsverfahrens nicht vornehmen werde. Die Bestimmungen dieser Promotionsordnung sind mir bekannt. Die von mir vorgelegte Dissertation ist von Prof. Dr. Agapios Sachinidis , Prof.Dr. Hescheler betreut worden.

01.04.2010

Köln den Ignatius Gunaseeli Jesudoss

Acknoweldgement

I am deeply indebted to my supervisor and guide **Prof. Agapios Sachinidis** for his excellent guidance, invaluable suggestions, stimulating discussions, critical review, timely help and constant support without which I could not have found a shape to my project as it stands now.

I express my gratefulness to Prof.Jürgen Hescheler for deciding me to be a part of Crystal project (sanctioned by the EU consortium) and eventually the inestimable benefit that I have gained from that.

I value highly 'the being-with' of Dr. Kurt Pfannkuche throughout with his technical and moral support.

Dr. Johannes Winkler, Dr. Shuhua Chen and Dr. Dimitry Spitkovsky were so kind enough to extend their scientific hold up as colleagues.

Mrs.Rita Altenburg was so helpful with her friendly advice and technical support. I am also thankful to Mrs. Cornelia Böttinger for providing CF1 feeder cells and END-2 cell line in time along with her personal concern.I cannot but show my appreciation to Daniel Derichsweiler for his care and timely technical help at critical situation, Sven Baumgartner for his Electro physiological study and Mr. Alex Müller, Ms. Angela Buckermann from the Vegetative Physiology team for their assistance in using the machineries.

I was able to work pleasantly and concentrate on my project since my lab mates Ms.Rabea Niemann,Ms.Sania,Mr.PhillipTreskes,Mr.SureshKumarP.S,Mr.GabrielPeinkofer,Mr.Johnanto ny, Ms.Mirjam,Mr.Moritz Haustein, Dr.Markus Khalil,Mr.Tobias Hannes,Ms.Anna-Lena Weiß, Ms. Evmorphia Daglidu,Dr. Marilenna Lupu,Dr. Osama ,Dr. Wahl and Mr. Martin Hubach, in creating a very friendly and pleasant atmosphere.

I acknowledge sincerely and express my gratitude to Mrs. Ilona Borsos, Mrs.Susan Rohani, Mrs.Annette köster, Manoj kupta, Matthias matzkies and Shiva Potta for their direct and indirect assistance and guidance throughout my course in various forms. I take this opportunity to remember Vilas Wagh, Kesavan Meganathan, for their solidarity as colleagues.

I would like to thank Mrs.Suzanne Wood, Mrs,Andrea Karadag and Mrs. Elke Lieske in a special way for their kind and well-timed administrative support. It is my pleasure to thank Mr.Frank Strassen and Mr. Michael Döweling for their excellent computer support and Mr.Metzner and his team for technical assistance.

Last but not least, I express my special thanks to my **Parents** for allowing me to stay in Germany to do my doctoral study and their unconditional love and support. Moreover, I thank my **Brother** for all supports that he has rendered towards me with his constant encouragement and love which have brought me to this level in my scientific career.

I thank the Almighty God for His providential care.

J. Ignatius Gunaseeli

Table of Contents

Abbreviations used

hESC	human embryonic stem cells
hiPSC	human induced pluripotent stem cells
SSEA-3	Stage-specific embryonic antigen-3
ROCKi	inhibitor of Rho kinase
IL6	Interleukin 6
IGF1	Insulin-like Growth Factor
EBs	Embryoid bodies.
μl	micro litre
ng	nanogram
TGF	Transforming growth factor
α-MHC	α-Myosin Heavy Chain (*Myh6*)
nm	Nanometer
μm	micrometer
bFGF	Basic Fibroblast gowth factor
PCR	Polymerase Chain Reaction
RT-PCR	Reverse Transcriptase- Polymerase chain reaction
cDNA	Complimentary deoxy ribonucleic acid
DNA	Deoxy ribonucleic acid
RNA	Ribonucleic acid
qPCR	Quantitative PCR
END-2	mouse visceral endoderm-like cell-line
CF1 Strain	Mouse Embryonic Fibroblasts or MEF
FBS	Fetal bovine serum
FCS	Fetal calf serum

1.Introduction

Pluripotent human embryonic stem cells (human embryonic stem cells (hESCs) and human induced pluripotent stem cells (iPS cells)) have the capacity to differentiate into all of the somatic cell types and therefore hold great promise for regenerative medicine. One key issue that needs to be addressed in guiding hESC/iPSC technology from “bench” toward “bedside” is developing defined cell culture systems for culture of hESCs, and differentiation of such cells into therapeutically relevant cells using clinically compliant systems.

1.1 Human embryonic stem cells:

1.1.1 Derivation of hES cells:

During human development, the fusion of sperm and egg gametes during human fertilization establishes a diploid zygote and this occurs in the oviduct, near the ovary. After fertilization, the zygote makes its way to the uterus, a journey that takes five to seven days in humans. As it travels, the zygote divides. The first cleavage produces two identical cells and then divides again to produce four cells. If these cells separate, genetically identical embryos result, the basis of identical twinning.

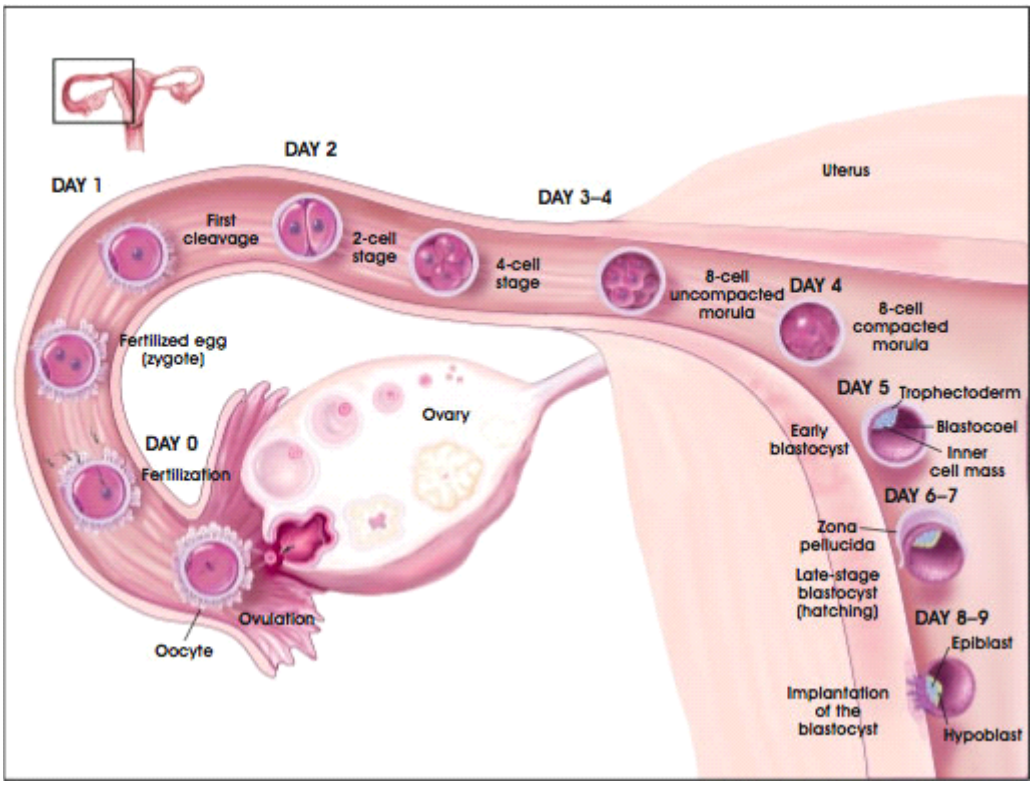


Figure 1. Development of the Preimplantation Blastocyst in Humans. [Courtesy: © 2001 Terese Winslow].

Usually, however, the cells remain together, dividing asynchronously to produce 8 cells, 16 cells, and so on. *A*t about the eight-cell stage, the embryo compacts, meaning that the formerly "loose" ball of cells comes together in a tight array that is interconnected by gap

junctions. By the 16-cell stage, the compacted embryo is termed a morula. By embryonic days 5 to 6, the embryo develops a cavity called the blastocoel. It fills with a watery fluid secreted by trophectodermal cells and transported in from the exterior. As a result of cavitation and the physical separation and differentiation of the trophectoderm from the inner cell mass, the morula becomes a blastocyst. Its chief structural features are the outer sphere of flattened trophectoderm cells (which become the trophoblast), the small, round cells of the inner cell mass, and the fluid-filled blastocoel. Between 5 to 7 days postfertilization in humans, the blastocyst reaches the uterus. It has not yet implanted into the uterine wall and is therefore still a pre-implantation embryo. When it arrives in the uterus, the blastocyst "hatches" out of the zona pellucida, the structure that originally surrounded the oocyte and that also prevented the implantation of the blastocyst into the wall of the oviduct. It is at this stage of embryogenesis—near the end of the first week of development in humans—that embryonic stem (ES) cells can be derived from the inner cell mass of the blastocyst.

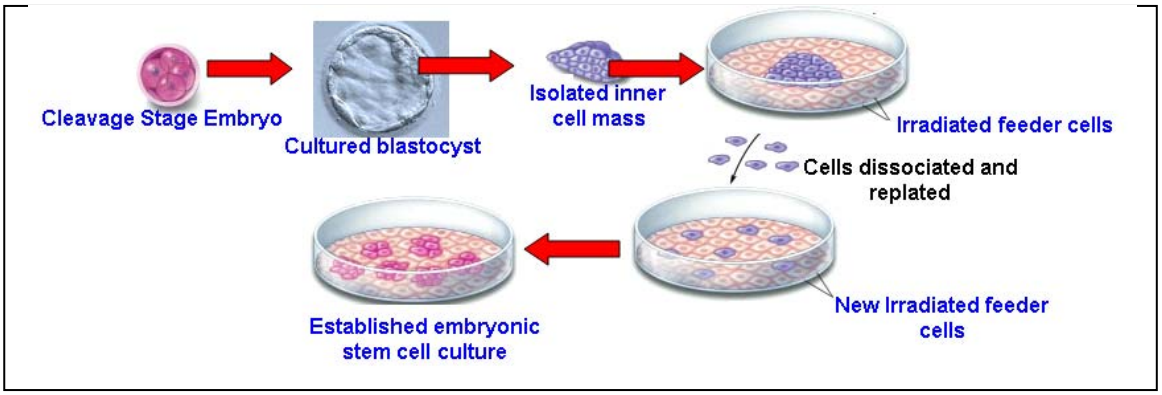


Figure 2. Derivation of human embryonic stem cells

To generate human ES cell cultures, cells from the inner cell mass of a human blastocyst were cultured in a multi-step process. The pluripotent cells of the inner cell mass were separated from the surrounding trophectoderm by immunosurgery, the antibody-mediated dissolution of the trophectoderm. The inner cell masses were plated in culture dishes containing growth medium supplemented with fetal bovine serum on feeder layers of mouse embryonic fibroblasts that had been gamma-irradiated to prevent their replication. After 9 to 15 days, when inner cell masses had divided and formed clumps of cells, cells from the periphery of the clumps were chemically or mechanically dissociated and replated in the same culture conditions. Colonies of apparently homogeneous cells were selectively removed, mechanically dissociated, and replated. These were expanded and passaged, thus creating a human ES cell line.

Human ES cells are derived from embryos generated through *in vitro* fertilization procedures and donated for research. An embryo at this stage of development *in vivo* would not yet be physically connected to the uterine wall; it would still be a preimplantation embryo.

ES cells, per se, may be an *in vitro* phenomenon. Some scientists argue that the apparent immortality of ES cells occurs only in a laboratory culture dish [13]. ES cells that are grown in the laboratory most closely resemble cells of the epiblast [5], but ES cells are not identical to epiblast cells[35]. The term epiblast refers to all the pluripotent cell populations that follow the formation of the primitive endoderm and precede the formation of the gastrula. Like the epiblast cells of the embryo, ES cells in culture have the potential to give rise to all the cell types of the body. However, unlike the epiblast cells of the embryo, ES cells *in vitro* cannot give rise to a complete organism. They do not have the three-dimensional environment that is essential for embryonic development *in vivo*, and they lack the trophectoderm and other tissues that support fetal development *in vivo*.

1.2 human induced pluripotent stem cells

These cells are the second type of pluripotent stem cells which do not require the use of an embryo. The landmark discovery that lineage-restricted somatic cells can be reprogrammed directly to a state of pluripotency has opened a new frontier in the field of regenerative medicine and drug discovery. Induced pluripotent stem (iPS) cells, as they were termed by Shinya Yamanaka, have now been derived from mouse and human somatic cells through the ectopic forced expression of OCT4 and SOX2 with either the combinations of KLF4 and MYC or NANOG and LIN28 [38,43] . iPS cells resemble pluripotent embryonic stem (ES) cells in morphology, proliferation, surface antigens, gene expression, epigenetic status of pluripotent cell-specific genes, telomerase activity and their potential to differentiate into a spectrum of adult somatic cell types. The revolutionary facets of iPS involve their ability to bypass the limitations of immune rejection in existing stem cell therapy approaches unlike the ES cells. The iPS cell discovery is less than 3 years old, yet iPS cell hold great promise for both basic research and therapeutic applications.

1.2.1 iPS cell derivation

iPS cells have now been derived from mouse and human somatic cells through the ectopic forced expression of OCT4 and SOX2 with either the combinations of KLF4 and MYC or NANOG and LIN28 [3,38,43] (Figure 3). Current reprogramming strategies involve retroviral, lentiviral, adenoviral and plasmid transfection to deliver reprogramming factor transgenes [4,24,37,38]. In humans, iPS cells are commonly generated from dermal fibroblasts and recently from human keratinocytes isolated from plucked hair and also from mobilized human $CD34^+$ peripheral blood cells [1,21]. However, it remains unclear whether hair cells will be a faithful source for reprogramming since the growth and quality of the hair follicles are dependent on the age, genotype, and the medical conditions of the human donors. Blood cells represent a source of cells that obviate the need for skin biopsies, and require minimal maintenance in culture prior to reprogramming.

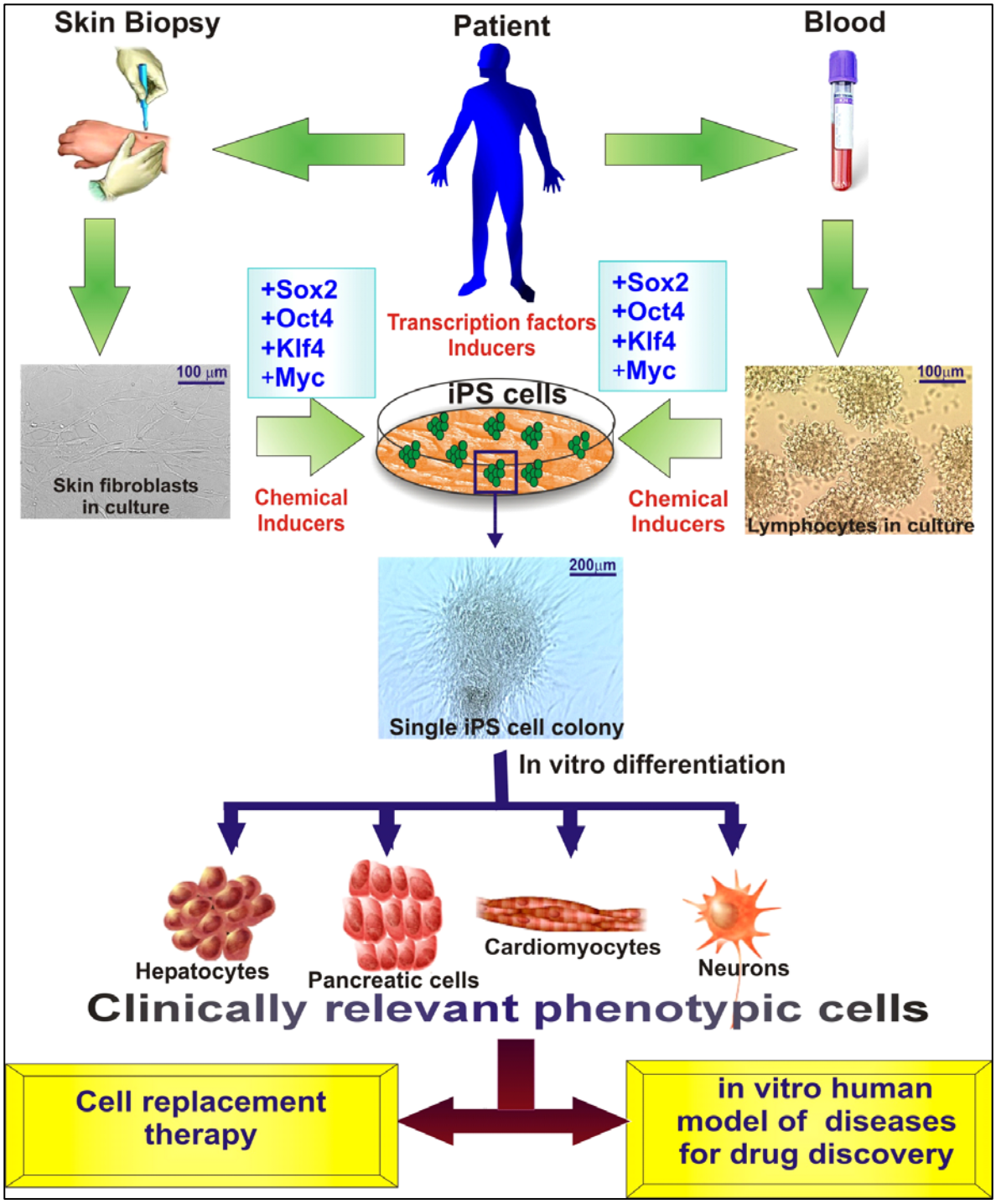


Figure 3. Scheme of derivation of iPS cells and possible therapeutically applications

1. 3 Therapeutic Clinical Application of Human Pluripotent Stem Cells

Both human ES cells and iPS cells have already been differentiated into various functional clinically relevant cell phenotypes such as neurons, cardiomyocytes and hematopoeitic cells [10,14,22]. Various types of somatic cells derived from pluripotent stem cells could be used in regenerative medicine to repair tissues damaged through disease or injury [12] (Figure 4).

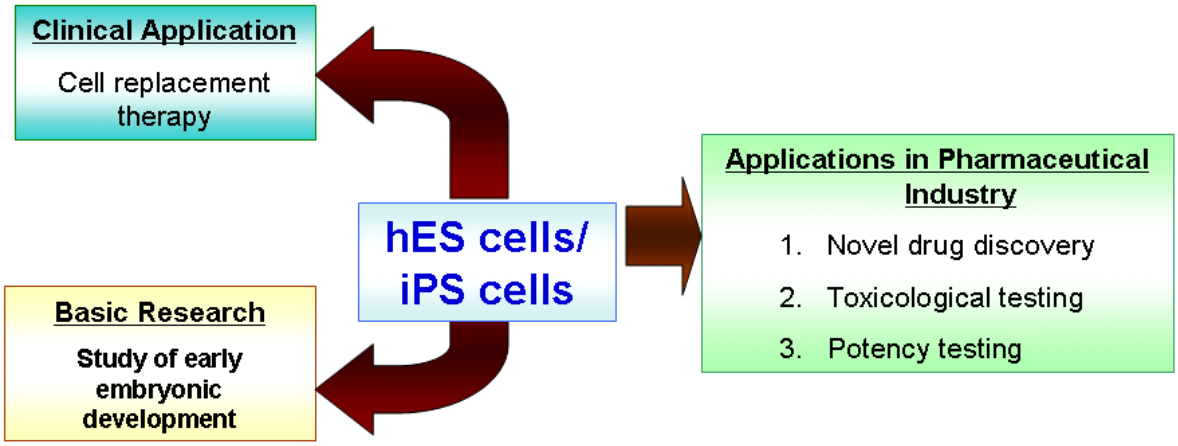


Figure 4. Clinical applicability of human pluripotent stem cells

1.3.1 Cell Replacement therapy

The pluripotency and indefinite proliferative capacity of pluripotent stem cells make them a promising candidate for the cell replacement therapy where the damaged cells are replaced by the functional cells derived from stem cells *in vitro*. Emerging results with human ES cells for the cell replacement therapy are encouraging, but this approach is still in its infancy and is under extensive investigation. The daily upcoming experimental observations are reinforcing the solid hope that ESC will be the potential source for use in cell replacement therapy. Although this demands serious considerations ethically and on practical applicability, the newly upcoming discoveries show that the adult human fibroblasts can be reprogrammed to embryonic stem cell like cells (called induced pluripotent stem cells) which can be used for cell replacement therapies. Remarkably, this obviates the need for the embryo destruction and overcomes related immunological problems which are the long time hurdles for the ES cell based cell replacement therapy.

Monogenic and polygenic Diseases

Creation of iPS cell lines from patients with single-gene disorders allows experiments on disease phenotypes *in vitro*, and an opportunity to repair gene defects *ex vivo*. The resulting cells, by virtue of their immortal growth in culture, can be extensively characterized to ensure that gene repair is precise and specific, thereby reducing the safety concerns of random, viral-mediated gene therapy. Repair of gene defects in pluripotent cells provides a common platform for combined gene repair and cell replacement therapy [11,12] for a variety of genetic disorders, as long as the pluripotent cells can be differentiated into relevant somatic stem cell or tissue populations. The generation of iPS cells from patients with a variety of genetic diseases with either Mendelian or complex inheritance has been described. These diseases include adenosine deaminase deficiency-related severe combined immunodeficiency (ADA-SCID), Shwachman-Bodian-Diamond syndrome (SBDS), Gaucher disease (GD) type III,

Duchenne (DMD) and Becker muscular dystrophy (BMD), Parkinson disease (PD), Huntington disease (HD), juvenile-onset, type 1 diabetes mellitus (JDM), Down syndrome (DS)/trisomy 21, and the carrier state of Lesch-Nyhan syndrome. Such disease-specific stem cells offer an unprecedented opportunity to recapitulate both normal and pathologic human tissue formation *in vitro*, thereby enabling disease investigation and drug development (28).

Neurodegenerative disorders

Patient-specific fibroblasts offers a unique opportunity for studying and modeling the effects of specific gene defects on human neuronal development *in vitro* and for testing small molecules or other potential therapies for the relevant neurogenetic disorders. Parkinsons Disease (PD) is the second most common chronic progressive neurodegenerative disorder and is characterized primarily by major loss of nigrostriatal dopaminergic neurons. The majority of cases are sporadic, not linked to a known genetic mutation, and likely the result of complex interactions between genetic and environmental factors[9]. One of the major reasons for the lack of understanding of the underlying pathophysiology of PD is the paucity of reliable experimental models that recapitulate all features of the human disease. The derivation of PD patient-specific iPS cells and subsequent differentiation into dopaminergic neurons would provide patient-specific *in vitro* models that are otherwise experimentally not accessible. Recently it has been shown that fibroblasts from five patients with sporadic PD could be efficiently reprogrammed and demonstrated that these patient-derived iPS cells could be subsequently differentiated *in vitro* into dopaminergic neurons[36]. Alzheimer's disease is characterized by degeneration and dysfunction of synapses and neurons in brain regions critical for learning and memory functions. The endogenous generation of new neurons in certain regions of the mature brain, derived from primitive cells termed neural stem cells, has raised hope that neural stem cells may be recruited for structural brain repair. Stem cell therapy has been suggested as a possible strategy for replacing damaged circuitry and restoring learning and memory abilities in patients with Alzheimer's disease [46].

Degenerative Cardiac diseases

Cardiovascular disease is the number one cause of death globally and is projected to remain the leading cause of death. Since adult cardiomyocytes have a very limited regenerative capacity, their loss permanently compromises myocardial contractile function. Heart failure is characterized by the loss of functional cardiomyocytes and thereby its inability to pump enough blood to maintain physiological functions. Heart transplantation is currently the last resort for end-stage heart failure, but is hampered by a severe shortage of donor organs and immune rejection. Cell replacement therapy is emerging as an innovative approach for the treatment of degenerative cardiac diseases, and pluripotent stem cells appear to be an ideal source of cells in this approach. In particular, human iPS cell-derived cardiomyocytes

theoretically fulfill most, if not all, of the properties of an ideal donor cell, but several critical obstacles need to be overcome [11,12] iPS cell based cell replacement therapy is currently generating a great deal of interest in the treatment of ischemic heart diseases since iPS cells are capable of differentiating into patient-specific functional cardiomyocytes. The replacement of akinetic scar tissue by viable myocardium should improve cardiac function, impede progressive left ventricular remodeling, and revascularize ischemic areas[19]. But still, much has to be studied about the formation of electrical coupling between endogenous and transplanted cardiomyocytes.

Diabetic diseases

Type 1 diabetes is characterized by the selective destruction of pancreatic beta-cells caused by an autoimmune attack. Type 2 diabetes is a more complex pathology which, in addition to beta-cell loss caused by apoptotic programs, includes beta-cell dedifferentiation and peripheric insulin resistance. beta-Cells are responsible for insulin production, storage and secretion in accordance to the demanding concentrations of glucose and fatty acids. The absence of insulin results in death and therefore diabetic patients require daily injections of the hormone for survival. However, they cannot avoid the appearance of secondary complications affecting the peripheral nerves as well as the eyes, kidneys and cardiovascular system. These afflictions are caused by the fact that external insulin injection does not mimic the tight control that pancreatic-derived insulin secretion exerts on the body's glycemia. Restoration of damaged beta-cells by transplantation from exogenous sources or by endocrine pancreas regeneration would be ideal therapeutic options[32]. Like the human ES (hES) cells, iPS cells are also shown to differentiate into mature insulin-producing cells in a chemical-defined culture system[45].

1.3.2 Human Pluripotent Stem Cells in pharmaceutical Industry

Both human ES cells and iPS cells augment pharmaceutical industry by enabling the high throughput assays such as automated robots controlled cell culture processing systems and large scale microarray profiling making an insurmountable progress in pharmaceutical arena.

1. Accelerated Novel Drug discovery

iPS cells could also boost the efficiency of drug discovery efforts if iPS technology can help researchers to identify drugs that are only effective against diseased cells with particular genetic profiles. iPS cells enable the creation of “human disease models” since iPS cells can be derived from the afflicted patient directly with relative easiness. Also, iPS cells provide added advantage that high throughput screening of drugs is possible. Part of the challenge in using iPS cells for drug screens is producing large numbers of identical cells that behave in a consistent way.

2. Toxicological Testing

A major bottleneck in the drug development process is toxicological testing. In recent years, stem cells have generated much interest as a potential tool for pharmacological and toxicology screening, due to various shortcomings of currently utilized assay models based on established cell lines, primary explanted somatic cells and laboratory animals. The most commonly utilized established cell lines for toxicological testing are of cancerous/tumorigenic origin, that are highly adapted to *in vitro* culture conditions after countless passages, and which contain chromosomal and genetic aberrations that render them immortal. Such inherent deficiencies make them non-representative of how a normal cell behaves physiologically *in vivo*. The primary explanted cultures of somatic cells used for toxicology screening are heterogeneous cultures that display a high degree of inter-batch variability, making it challenging to obtain consistent and reproducible results in toxicology screening. Live animal models used for toxicity assays have a number of inherent flaws. 1) An animal model may not compare well with human physiology. 2) The use of live animals in routine toxicology screening of biomedical and cosmetic products may be ethically contentious, and can possibly affect consumer confidence. 3) Live animals are expensive to purchase and maintain compared to *in vitro* cultured cells. Traditional *in vivo* tests performed in animals are difficult to automate**.** Of greater promise in the drug discovery field is the use of iPS cells for toxicology testing. Since iPS cell lines can be isolated from a diverse range of human individuals with well-characterized adult phenotypes and can be used on high throughput assay setups, these can potentially provide a valuable tool for characterizing how variation in toxic susceptibility displayed by different individuals correlates to their genetics, disease state and other observable phenotype [15] .

1.4 Potential Problems associated with clinical applicability of iPS cells

The iPS cell technology potentially could overcome two important obstacles associated with hES cells: immune rejection after transplantation and ethical concerns regarding the use of human embryos. However, the clinical application of iPS cells also faces many obstacles, some shared with ES cells and others that are unique. The first common obstacle is teratoma formation.

1.4.1 Transgene free iPS cells

Reprogramming of both mouse and human somatic cells into iPS cells has been achieved by expressing combinations of factors such as *OCT4, SOX2,c-Myc, KLF4, NANOG, and LIN28.* Initial methods used to derive human iPS cells used viral vectors, in which both the vector backbone and transgenes are permanently integrated into the genome. Such vectors can produce insertional mutations that interfere with the normal function of iPS cell derivatives, and residual transgene expression can influence differentiation into specific lineages or even

result in tumorigenesis[25,28,43]. Vector integration–free mouse iPS cells have been derived from liver cells with adenoviral vectors and from embryonic fibroblasts with repeated plasmid transfections but the low frequencies obtained make it unclear how practical these approaches will be for human cells, which generally require longer exposure to reprogramming factors [26,37]. To this end, recently two alternative approaches were described to remove transgenes from mouse or human iPS cells. In one approach, Cre/LoxP recombination was used to excise integrated transgenes[17,36]. This approach successfully removes transgene sequences, but leaves behind residual vector sequences, which can still create insertional mutations. A second approach used seamless excision of piggyBac transposons to produce vector- and transgene-free mouse iPS cells [40]. Although a promising approach, vector removal from human iPS cells produced by this method has not yet been reported, and removing multiple transposons is labor intensive. More recently, Thomson group has reported another approach where human iPS cells completely free of vector and transgene sequences can be derived from fibroblasts by a single transfection with oriP/EBNA1 (Epstein-Barr nuclear antigen-1)–based episomal vectors [43]. The oriP/EBNA1 vectors are well suited for introducing reprogramming factors into human somatic cells, as these plasmids can be transfected without the need for viral packaging and can be subsequently removed from cells by culturing in the absence of drug selection. However, it will be essential to determine which of these methods most consistently produces iPS cells with the fewest genetic or epigenetic abnormalities, because any abnormalities would affect the application of these cells in basic research, drug development, and transplantation therapies much more than the initial reprogramming frequencies. Substantial challenges also remain in cell-specific differentiation and delivery, but the derivation of vector- and transgene-free human iPS cells is nonetheless an important advance toward the clinical application of these cells[43]. Also, there is a possibility that chemically induced pluripotent stem cells will be ideally the last resort in the near future.

1.4.2 Tumorigenicity

The links between pluripotency and tumorigenicity are exemplified by the fact that many of the genes used to produce iPS cells are either outright established oncogenes such as Myc and KLF4[11,18,42] or are in various ways linked to tumorigenesis such as Sox2 [7], Nanog [8] and Oct3/4[27]. It is remarkable that, to date, one of the most common assays for demonstrating and studying the pluripotency of stem cells, including iPS cells, is the teratoma assay. Often this is referred to as a pluripotency assay, but of course it is also a tumor assay. The fact that a key assay of "stemness" is also a tumor assay illustrates the strong link between stem and tumor cells, a reality too rarely discussed in the field when interpreting results from teratoma assays. Even ignoring for the moment the ability of ES cells and iPS cells to produce malignant tumors in some cases [25,34], the production of benign teratoma as a side effect in humans given a hypothetical regenerative medicine therapy in the future, would be

unacceptable. Such tumors could be numerous and would prove highly destructive to surrounding normal or regenerating tissue. Thus, a key concept is that stem cells, even those with potent self-renewal and pluripotency, will almost certainly never be directly used in regenerative medicine if they cannot be proven to lack the ability to cause teratoma in mice [18]. Teratoma is not the only concern as hES cells can also form malignant tumors. A recent study found robust malignant tumor-inducing capacity of hES cell lines H1 and HSF-6 (34). iPS cells can also form both teratoma as well as malignant tumors such as neuroblastoma and follicular carcinoma[25]. Thus, the potential risk to human patients from both teratoma and malignant tumors is quite real, yet remains difficult to estimate as no human trials of hES cells or iPS cells have been conducted at this time. The stable genetic introduction of a suicide gene such as thymidine kinase (tk) into stem cells has been reported to be effective in combination with Ganciclovir (*Gan*) treatment [33]. However, in this study, the treatment was not stem cell specific and would have also killed any differentiated progeny from those stem cells in a hypothetical treatment situation causing it to fail. Differentiated teratoma cells were also readily killed by *Gan* treatment. Nevertheless, relatively simple modifications, such as using *oct4* or *nanog* promoter driven expression of tk, would make the system more stem cell specific to ideally kill only those iPS cells that have escaped differentiation. Of concern is the fact that it remains unknown if all iPS cells express what are thought of as the key stem cell factors such as *oct4* and *nanog*. Although populations of iPS cells do express these seemingly without exception, it is unclear whether small, but functionally relevant subpopulations may not [18]. Also, the major concern with the suicide gene approach is its requirement for genetically modifying the stem cells, which could raise the risk of tumorigenicity from the beginning. The simplest way to slow or even eliminate the tumorigenicity of normal stem cells prior to transplantation may be to take advantage of their natural "brakes" or pluripotency by partially differentiating them into progenitors. Therefore, a promising proposed method for making stem cell-based regenerative medicine therapies safer may seem paradoxical: to not transplant stem cells at all into patients. This avenue has gained wide acceptance as the most promising approach to regenerative medicine. The idea is to use the stem cells to produce progenitor or precursor cells of the desired lineage and then transplant progenitors purified by sorting or by positive selection with antibiotics[18] .

1.4.3 Chromosome abnormality

Also, the hES cells during long time cultures *in vitro* develop chromosomal abnormalities. It is anticipated for the same thing to happen with iPS cells during long time culturing. Safety measures need to be formulated to avoid this situation and to abolish the chromosomally aberrant cells, if any, upon transplantation [12].

1.4.4 Need for Xenofree culture conditions

The major concern about the use of iPS derived clinically relevant cell phenotypes is the contamination by xenogens. The human iPS cells need be cultivated on mouse/ human embryonic fibroblasts and with sera obtained from animals like calf and bovine. This poses a major health threat to the human host due the possible exposure to mouse retroviruses and other harmful substances. So, culture of human iPS cells under serum free and feeder (xeno) free conditions is inevitable.

In recent years, extensive investigation into improving culture systems for hES cells has yielded three main advances: 1) the ability to grow cells under serum-free conditions; 2) the maintenance of the cells in an undifferentiated state on Matrigel matrix with 100% mouse embryonic fibroblast (MEF)-conditioned medium; and 3) the use of either human embryonic fibroblasts, adult Fallopian tube epithelium, or foreskin fibroblasts as feeder layers[52]. In spite of this progress, several significant disadvantages still exist. Exposure to animal pathogens through MEF-conditioned medium or Matrigel matrix is still a possibility; human feeder layer-based culture systems still require the simultaneous growth of both feeder layers and hES cells; and the culture system cannot be accurately defined due to differences between the various feeder layer lines or the use of conditioned medium.

1.4.5 Heterogenous population

The ESC /iPS cells give rise almost all types of tissue specific cells and their subtypes. Refining the protocols to obtain the cell phenotype(s) of interest for cell replacement therapy in purified form will dictate the clinical applicability of iPS cells. This entirely depends on the optimized cell culture conditions for the selective differentiation of the phenotyes of interest and the availability of cell phenotype specific cell surface antigens for FACSorting or magnetic sorting with fluorescent or magnet labeled antibodies.

2. Objective

Human pluripotent stem cells (hESC and hiPSC) are excellent candidates for cell replacement therapies. However, currently used culture conditions contain animal-derived components that bear a risk of transmitting animal pathogens and incorporation of non-human immunogenic molecules to hESC. Thus, the use of xeno-derived components in the culture of hESC essentially limits the future clinical use of hESC-based therapies, and finding alternatives to replace these xeno-derived components has been one of the major focuses of hESC research during the past few years. Also, the improving the yield of clinically relevant phenotypic cells such as cardiomyocytes under xenofree culture conditions has also been of major importance for the translational clinical studies for treating several degenerative diseases such as heart failure by cell based regeneration therapies.

Accordingly, the objective of my project is to establish and optimize xenofree single cell expansion and differentiation of human pluripotent stem cells (both human embryonic stem cells and induced pluripotent stem cells) *in vitro* to enable their clinical applicability towards cell based regeneration therapy.

The above objective is divided into the following 3 specific aims:

1. To establish xenofree protocols for single cell expansion of human pluripotent stem cells (both hESC and hiPSC).

2. To establish Xenofree protocols for cardiac differentiation of human pluripotent stem cells (both hESC and hiPSC).

3. To screen cardiogenic small molecules which enhance cardiac differentiation of human pluripotent stem cells (both hESC and hiPSC) under xenofree conditions.

3. Materials and Methods

3.1 Materials:

3.1.1 Human Pluripotent Stem Cell Lines used:

H9 and Hes2 Cell lines were used. hIPS cell line used was **ES4SKIN.**

3.1.2 Consumables:

S.No	Consumables	Manufacturer
1	T25- tissue culture flask	Becton Dickinson
2	10 cm petri dishes (bacteriological)	Greiner
3	6 cm petri dishes (bacteriological)	Greiner,
4	6-well cell culture plates	Becton Dickinson
5	24-well cell culture plates	Becton Dickinson
6	5 ml pipettes	Sarstedt
7	10 ml pipettes	Sarstedt
8	15 ml tubes	Falcon
9	50 ml tubes	Falcon
10	cryotubes	Nunc
11	cell counting chamber	Neubauer/ Merck
12	pasteur pipettes	6127-1722
1	Multipipette	Eppendorf
14	1000 µL micro molecular-pipette	Gilson (Amersham)
15	blue tips	Biolabs
16	Sterile Gene pulser Cuvette	Bio-Rad
17	Trypan Blue 0.4%	Gibco BRL
18	Neubauer Counting Chamber	Merck
19	Hoechst dye	Molecular Probes
20	ProLong Gold Mounting medium	Molecular Probes

3.1.3 Primers included in our study

NCBI accession	gene name	Forward	Reverse primer	amplicon size
NM_002046.	GAPDH	CTCTCTGCTCCTCCTGTTCG	GGCAACAATATCCACTTTACCAG	171
NM_024865.	Nanog	TGAGTGTGGATCCAGCTTGT	CAGATCCATGGAGGAAGGAA	179
NM_002701.	4-Oct	GTGAAGCTGGAGAAGGAGAAGC	CAGCTTACACATGTTCTTGAAGC	237
NM_000280.	Pax6	AACGATAACATACCAAGCGTGTC	TGGAACTGATGGAGTTGGTATTC	238
NM_022454.	Sox17	GCTAAGGACGAGCGCAAG	CGGCCGGTACTTGTAGTTG	183
NM_003181.	Tbrachyury	ATCACAAAGAGATGATGGAGGAA	GGTGAGTTGTCAGAATAGGTTGG	250
NM_002471.	aMHC	AAGCTGTACGACAACCACCTG	AGGTTGGTCATTAGCTTGTTGAG	338
NM_153675.	FoxA2	GAGCGGTGAAGATGGAAGG	ATGGAGTTCATGTTGGCGTAG	391
NM_004387.	Nkx2.5	TCAAAGACATCCTAAACCTGGAA	CTCTGTCTTCTCCAGCTCCAC	338
NM_006172	ANF	ATGCCTTTAGAAGATGAGGTCGT	TCTTCAGTACCGGAAGCTGTTAC	309
NM_000364.	Troponin T	GACATAGAAGAGGTGGTGGAAGA	GTCTCTCGATCCTGTCTTTGAGA	397

3.1.4 Cell culture Reagents

S.No	Medium	Catalogue Number	Manufacturer
1	Knock out DMEM	10829-018	Gibco BRL
2	Fetal bovine serum (FBS)	10500064	Invitrogen
3	Phosphate buffered saline (PBS) (w/o Ca^{2+}/Mg^{2+}	. 14190-094	Invitrogen
4	Phosphate buffered saline* (PBS*) (w Ca^{2+}/Mg^{2+})	14040-091	Invitrogen
5	Trypsin/EDTA solution (Ca^{2+}/Mg^{2+}-free) (	25300-054	Invitrogen,
6	Pencillin/Streptomycin	15140122	Gibco BRL
7	ß-2-mercaptoethanol (ß-ME)	31350-010	Invitrogen
8	Gelatine Type II	G-2500	
9	Di-methyl sulfoxide (DMSO)	D-8418	Sigma Alldrich
10	bFGF	100-18B	Peprotech
11	mTeSR media	05850	Stem Cell technologies
12	BD Matrigel	354277	Becton Dickinson GmbH
13	ROCKi (Y-27632)	688000	Calbiochem
14	Interleukin 6	206-IL/CF	R&D systems
15	IGF1	CTK216	Abazyme
16	Human plasma Vitronectin purified protein	CC080	Chemicon
17	Activin A	338-AC-005	R&D Systems
18	BMP4	314-BP-010	R&D Systems
19	DMEM/F12	31330-038	Gibco BRL
20	0.5mg/ml BSA (fraction V)	30060-727	Gibco BRL
21	(±)-Methoxyverapamil, Hydrochloride	454551	Calbiochem
22	Cyclosporin A, Tolypocladium inflatum	239835	Calbiochem
23	TRIzol® LS Reagent	10296-010	Invitrogen GmbH
24	2-Log DNA Ladder(0.1-10.0 Kb)	N3200S	New England Biolabs
25	Gel Loading Dye,Blue (6X)	B7021S	New England Biolabs
26	Dispase(1mg/ml)	07923	Stem Cell Technology
27	Accutase	07920	Stem cell Technology
28	6-Well ultra low adherence plate	27145	Stem Cell Technology

Activin A
Added 9.87ml PBS (with Ca^{2+} and Mg^{2+}) with 0.13ml 75mg/ml BSA (fraction V). Mixed gently. Filter sterilized with 0.22um filter. Activin A was added to 500µl PBS with BSA for the concentration of 10µg/ml. Made 10µl aliquot and stored at -80 degree celcius. 10µl in every aliquot is to be combined with 2ml media for the final concentration of 50ng/ml.

BMP4
Added 0.98 ml HCL to 9.02 ml milliQ H_2O in a 100ml beaker for 1M HCL. Mixed well. Added 40µl of 1M HCL to 9.83ml miliQ H_2O. Added 0.13ml 75mg/ml BSA (fraction V). Filter sterilized with 0.22µm filter. 4mM HCl + 1.3ml 75mg/ml BSA (fraction V). Added 1000µl

4mmHCl + 1.3ml 75mg/ml BSA (fraction V) for the concentration of 10ug/ml. Aliquoted 20µl aliquot and stored at -80 degree celcius. 20µl in every aliquot is to be combined with 4ml media for the final concentration of 50ng/ml.

3.1.5 Instruments Used

Zeiss Axiovert 200 fluorescence microscope
FACScan (BD, Pharmingen)

3.1.6 Antibodies Used

S.No	Antibodies	Catalogue Number	Manufacturer
1	Anti-human Oct4	SC-5279	Santa Cruz Biotechnology,Inc
2	Anti-Tra-1-60	SC-21705	Santa Cruz Biotechnology,Inc
3	Anti-Tra-1-81	SC-21706	Santa Cruz Biotechnology,Inc
4	Mouse IgM PE	Cat No. sc2870 Alexa Fluor 555 dye	Santa Cruz Biotechnology,Inc
5	MouseIgG	Molecular probes A21929	Invitrogen

3.1.7 Molecular Biology Kits/Reagents:

S.No	Kit	Catalogue number	Company
1	DNAase I,Amplification Grade	18068-015	Invitrogen
2	SuperScript III Reverse Transcriptase	18080-044	Invitrogen
3	RNAse Inhibitor	N8080119	Invitrogen
4	10mM dNTP Mix	18427-013(100µl)	Invitrogen
5	JumpStart REDTaq ReadyMix Reaction Mix(For PCR)	P0982-100 RXN	SIGMA-ALDRICH
6	Random Primer	48190-011	Invitrogen

3.1.8 Culture Media

MEF Media:

DMEM	450ml
Heat-inactivated FBS	50ml
Non-essential amino acids	5ml
L-Glutamine	5ml

hESC/hiPSC Media:

DMEM/F12	400 ml
Serum Replacer	100 ml
Non-essential aminoacids	5 ml
L-Glutamine	5ml
β-mercapto ethanol	100µM (final concentration)

3.2 Methods

3.2.1 Viability Cell Count by Trypan blue exclusion method

Gently swirled a suspension culture to distribute the cells evenly. Aseptically removed a small sample (10µl) of cells from the cultures. Placed the sample in a separate test tube (it need not be sterile). Added 10µl of the 0.4% dye to your sample. Mixed gently. Set up a hemocytometer and cover slip. Immediately placed a drop of the stain/culture combination on the hemocytometer and waited one minute. Observed the cells with low power microscopy. Counted the total number of cells, and the number of stained cells. Computed the concentration of viable cells per ml of culture.

3.2.2 Crystal Violet Staining (An Assay for for hESC Morphology)

Placed plates with cells on ice. Washed two times with ice-cold 1X PBS. Fixed cells with ice-cold methanol (stored at –20oC) for 10 minutes. Aspirated methanol from plates, moved off ice and add enough 0.5% crystal violet solution (made in 25% methanol and stored at room temperature) to cover bottom of plate. Incubated at room temperature for 10 minutes. Poured off crystal violet solution into a beaker. Carefully rinsed plates in ddH20 until color no longer coming off in rinse. Allowed plates to dry at room temperature (may take overnight).

3.2.3 Flow Cytometry (FACS analysis)

Harvested and washed the cells. Determined the total cell number. Resuspended the cells to approximately 1-5x10^6 cells/ml in ice cold PBS, 20%FBS. Added 100 µl of cell suspension to each tube. Added 0.1-10 µg/ml of the primary antibody. Dilutions, if necessary, should be made in 3% BSA/PBS. Incubated for at least 30 min at 4^{o}C in the dark. Washed the cells 3-times by centrifugation at 400 g for 5 min and resuspend them in ice cold PBS. Diluted the fluorochrome-labeled secondary antibody in 3% BSA/PBS at the optimal dilution (according to the manufacturer's instructions) and then resuspend the cells in this solution. Incubated for at least 20/30 minutes at room temperature of 4^{o}C. This incubation must be done in the dark. Washed the cells 3-times by centrifugation at 400 g for 5 min and resuspend them in ice cold PBS, 3% BSA, 1% sodium azide. Stored the cell suspension immediately at 4°C in the dark. Added Propidium Iodide (PI) for exclusion of dead cells. For best results, analyzed the cells on the flow cytometer as soon as possible. Cell clumps were removed by passing through cell strainer cap of round bottom tube from Falcon® (BD, Germany). Acquisition of 10,000 live (PI negative) cells was made with FACScan (BD Biosciences) and the data analysis was done with CellQuest software (Becton Dickinson).

3.2.4 Protocol for maintenance of hESC and hIPSC on feeders and with serum

3.2.4.1 Plating

Mouse embryonic fibroblasts (CF1 strain), cultured in MEF media were mitotically inactivated by treatment with 10µg/ml mitomycin C (Roche Cat #107 409) for 2 to 3 hours at 37°C. Cells were washed three to four times with PBS, trypsinized (Invitrogen Cat #25300-054), and plated at a density of 0.75 x 10^5/ml with 2.5ml per well of a gelatin-coated 6-well dish. Immediately before plating hESC, MEFs were rinsed once or twice with PBS. hESC were plated onto MEFs as small clumps in 2.5ml per well of hESC media containing 4ng/ml bFGF (R&D Systems Cat #233-FB). Cells were fed every day until ready to passage which is determined by the size of colonies, the age of MEFs (should not be older than 2 weeks) or differentiation status of the cells. Colonies which appear to be differentiating, were manually removed before passaging.

3.2.4.2 Passaging

To passage hESC, cells were washed once or twice with PBS and incubated with filter-sterilized 1mg/ml collagenase IV (Invitrogen Cat #17104-019) in DMEM/F12 for 10 to 30 minutes. Plates were agitated every 10 minutes until colonies begin to detach. When moderate tapping of the plate caused the colonies to dislodge, they were collected and the wells washed with hESC medium to collect any remaining hESC. Colonies were allowed to sediment for 5 to 10 minutes. The supernatant, containing residual MEFs, was aspirated, and the colonies were washed with 5ml hESC medium and allowed to sediment again. This was repeated once more. After the final sedimentation, the colonies were resuspended in 1ml of hES medium and triturated gently to break up the colonies to approximately 100-cell size. Generally, cell lines were passaged at a ratio of between 1:3 and 1:6 every four to seven days.

3.2.5 Optimised Protocol For Serum Free, Feeder Free Cultivation Of hESC

3.2.5.1 Preparation of BD Matrigel

Checked the lot specific dilution from the accompanying data sheet.

The lot number is	**: 89373**
Catalog number is	**: 354277**
Volume	**: 5ml**
Dilution to be made	**: 320 µl to make 25ml ie., 1:78.125**
Dilution medium	**: DMEM/F-12**
Storage	**: 320 µl aliquots at –70^{o}C**

Calculated the aliquot volume and the number of vials to be prepared. Chilled sterile 1.5 ml vials on ice for 10 minutes. Once chilled, dispensed 80µl matrigel (kept on ice) into the vials and stored them at –80^{o}C until required for coating plates. Shelf life is 6 months. Upon thawing, diluted the 80µl matrigel to 6.25 ml (80µl+6.170ml)

3.2.5.2 Coating plates with BD Matrigel

Thawed 80µl Matigel aliquot at 4^{o}C overnight and in the next day it was diluted to 6.25 ml with ice cold DMEM/F12 medium (ie., 80µl matrigel+ 6.17ml DMEM/F12 medium). Added 1ml diluted matrigel per well and left in the sterile bench for more than 1 hour at room temperature for the matrigel to gellify. After 1 hour, just before plating the cells, the excess matrigel was removed and immediately the medium with cell clumps (2ml) was added. Care was taken not to allow the matrigel in the wells to dry out. After plating, the plates were transferred to the incubator.

3.2.5.3 mTeSR medium preparation

Ensured that the lot numbers of 5x supplement and Basal medium end with the same letter. In our case, it is **"A".** 5x Supplement was thawed overnight at 4^{o}C. Since 5x supplement aliquot can be stored for only 3 months, storing aliquots of diluted supplement in Basal medium was preferred, since the latter can be stored for 6 months time. The thawed 100 ml 5x supplement was added to 400 ml Basal Medium to get 500 ml CompletemTeSR1 medium Made aliquots of 25 ml (because it takes 5-7 days with a mean of 6 days before splitting if the culture is under optimum conditions as per the stem cell technologies protocol. That means, 2 ml for every well of a 6 well plate for 6 days = 2ml x 6 days= 12 ml. It is better to have in duplicates. So, that means 12ml x 2 times = 24 ml. Let 1 ml be extra for the pipetting errors. So, making 25 ml aliquot is better and more practical). All aliquots except 1 (20-1=19 aliquots) were frozen at –20^{o}C for their storage. The company says that the aliquots will be safe till 6 months at –20^{o}C.

3.2.5.5 Dispase treatment

Pre-warmed the media and the diluted dispase solution at 37^{o}C. Removed the media. Washed the wells with 2 ml PBS (Ca^{2+}, Mg^{2+} free). Added the diluted dispase 125µl per well. Incubated for 5 minutes in the incubator. Monitored the detachment under the microscope. if necessary, incubated for additional 5 minutes, 10 minutes or 15 minutes. Transfered the clumps to culture medium. Centrifuged. Resuspended the cells in fresh culture medium. Counted the clumps. Plated the cells as usual on Matrigel coated dishes.

3.2.6 Transition of H9 cells from feeder+serum conditions to serum free and feeder free conditions

Prepared the BD Matrigel as per the protocol column 3.2.4.2. The H9 undifferentiated clumps were cut and lifted as per our routine protocol mechanically. Counted the clumps. Added 300 clumps each in 2 wells of a 6 well plate which were precoated with matrigel (added 1 ml diluted matrigel per well of a 6 well plate and left it in the hood covered for 1-2 hours prior to plating) in 2ml complete mTeSR medium. Changed medium everyday. Checked the growth of the cells and when they are 70% confluent, passage them with dispase as outlined in section 3.2.4.5 and 1.5. It takes 5-7 days.

3.2.7 Subsequent routine maintenance of H9 cells in serum free and feeder free condition

Once 70% confluent, harvested the clumps by gentle dispase treatment. Seeded 1:5 to 1:10 per well, ie., 10% to 20% of the clumps harvested from a single well of a 6 well plate in 2 ml of the complete mTeSR medium in matrigel coated dishes. Seeded only 2 wells. Normally it takes 5 days for the subsequent splitting if seeded 1:5. Always tried to have smaller clumps. Otherwise, the cells in the innerportion of the bigger clumps undergo spontaneous differentiation and they die since they don”t have space to spread.

3.2.8 Freezing of the H9 cells in serum free conditions:

We used mFreSR™ Defined Cryopreservation Medium for hESCs (Stem Cell Technologies). Harvested the ES clumps from a single well of 48 well plate by dispase treatment and added with 0.5 ml of freshly thawed, prewarmed (15-25 degree C) 1 ml aliquot of mFreSR™ Defined Cryopreservation Medium. Gently mixed suspension and mFreSR™ and transfer 0.5 mL at a time into labelled cryovials using a 2 ml pipette. Placed vials into an isopropanol cryovessel and placed the vessel at -80°C overnight. Transferred to the liquid nitrogen vapor tank after freezing overnight.

3.2.9 Thawing hESCs cryopreserved in mFreSR™

hESCs cryopreserved from 1 well of a 48-well plate can be thawed successfully into 1 well of a 12-well plate. Have all tubes, warmed medium and plates ready before starting the protocol to ensure that the thawing procedure is done as quickly as possible. Prepare the Matrigel coated dishes. Remove frozen vial of cells from the liquid nitrogen vapor tank and immediately dip the tube into waterbath at 37°C. Agitate the tube in the water continuously until only a small ice chunk remains. Spray the tube with ethanol or isopropanol to sterilize. Transfer the contents of the tube to a 15 ml conical tube using a 2 ml pipette to minimize breaking of any clumps. Dropwise, add 3 - 5 ml of warm mTeSR™1 to the tube, mixing as the medium is added. Centrifuge cells at 300 x g for 5 minutes at room temperature (15 - 25°C). Aspirate medium leaving the pellet intact. Using a 2 ml pipette, gently pipette the cell

pellet in 1 - 2 ml of mTeSR™1 taking care to maintain the clumps as large as possible. Gently tilt the plates onto one corner and allow the excess Matrigel solution to collect in that corner. Remove the Matrigel solution using a serological pipette or by aspiration. Ensure that the tip of the pipette does not scratch the coated surface. If plates have been stored at 2 - 8°C, allow the plates to come to room temperature (15 - 25°C) for 30 minutes and keep in incubator for atleast 30mins-1hr before removing the Matrigel solution. Transfer 0.5 mL of medium plus clumps per well to a Matrigel coated 12-well plate. Ensure that clumps are evenly distributed between wells. Place plate at 37°C and move the plate in quick side to side, forward to back motions to evenly distribute the clumps within the wells. Change medium daily. Check for undifferentiated colonies that are ready to passage (dense centered) approximately 5 - 7 days after thawing. If only a few undifferentiated colonies are observed after thawing, it may be necessary to select only these colonies for passaging and replate them in the same size well on a new Matrigel coated plate.

3.2.10 Optimised Protocol For Xeno Free Cultivation Of hESC /hiPSC

3.2.10.1 Preparation of Vitronectin

Checked the lot specific dilution from the accompanying data sheet.

The lot number is	**:LV1422328**
Catalog number is	**: CC080**
Volume	**: 100µl**
Quantity	**:100µg**
Coating Concentration	**:10ng/µl**
Dilution medium	**: DPBS**
Storage	**: aliquots at –70^{o}C**

Calculated the aliquot volume and the number of vials to be prepared. Chilled sterile 0.5 ml vials on ice for 10 minutes. Once chilled, dispensed vitronectin (kept on ice) into the chilled vials and stored them at –80^{o}C until required for coating plates. Shelf life is 12 months.Upon thawing, diluted vitronectin to 10ng/ µl before coating.

3.2.10.2 Coating plates with Vitronectin

Thawed Vitronectin aliquot **on ice** and diluted to **10ng/ µl** with DPBS. Added 125µl diluted Vitronectin per well of a 5 well plate and left in the Incubator for more than 1 hour for the Vitronectin to gellify. After 1 hour, just before plating the cells, the excess matrigel was removed and immediately the medium with cells was added. Care was taken not to allow the vitronectin in the wells to dry out. After plating, the plates were transferred to the incubator. In

case, if you want to use the Vitronectin coated dish at a later time point, then seal the dish with parafilm and store it at 4^{o}C.

3.2.10.3 mTeSR medium preparation

Ensured that the lot numbers of 5x supplement and Basal medium end with the same letter. 5x Supplement was thawed overnight at 4^{o}C. The thawed 100 ml 5x supplement was added to 400 ml Basal Medium to get 500 ml CompletemTeSR-1 medium. Made aliquots and all aliquots were frozen at –20^{o}C for their storage. The company says that the aliquots will be safe till 6 months at –20^{o}C. Thawed the aliquot at 4^{o}C overnight, whenever needed

3.2.10.4 Dispase preparation

100ml of ase (Stem cell Technologies) solution was prepared at 1mg/ml and aliquots were frozen at -20^{0}C.Each aliquot to be thawed at 4^{o}C overnight

3.2.10.5 Dispase treatment

Pre-warmed the media and the diluted dispase solution at 37^{o}C. Removed the media. Washed the wells with 2 ml PBS (Ca^{2+}, Mg^{2+} free). Added the diluted dispase 125µl per well. Incubated for 5 minutes in the incubator. Monitored the detachment under the microscope. if necessary, incubated for additional 5 minutes-10 minutes or 15 minutes. Transfered the clumps to culture medium. Centrifuged. Resuspended the cells in fresh culture medium. Counted the clumps. Plated the cells as usual on Vitronectin coated dishes.

3.2.11 Transition of H9 cells from feeder+serum conditions to Xeno-free conditions

Prepared the Vitronectin as per the protocol column 1.2 of " Optimised protocol hESC cultivation under Xenofree conditions". The H9 undifferentiated clumps were cut and lifted as per our routine protocol mechanically. Counted the clumps. Added 75 clumps each in 4 wells of a 48 well plate which were already precoated with Vitronectin (added 125 µl diluted vitronectin per well of a 48 well plate and left it in the incubator for 1-2 hours prior to plating) in complete mTeSR medium. Changed medium everyday. Checked the growth of the cells and when they are 70% confluent, passage them with dispase as outlined in section 1.4 and 1.5 of " Optimised protocol hESC cultivation under Xenofree conditions". It takes normally 5-7 days.

3.2.12 Subsequent routine maintenance of H9 cells in Xeno free condition

Once 70% confluent, harvested the clumps by gentle dispase treatment. Seeded 1:5 to 1:10 per well, ie., 10% to 20% of the clumps harvested from a single well of a 48 well plate in complete mTeSR medium in Vitronectin coated dishes (see Vitronectin coating in section 1.2 of " Optimised protocol hESC cultivation under Xenofree conditions"). Seeded only 4 wells since we will have enough cells for the assay. If you need more cells, increase

proportionately. Normally it takes 5 days for the subsequent splitting if seeded 1:5. Always tried to have smaller clumps. If there were bigger ones, then the cells in the innerportion of the clumps undergo spontaneious differentiation and they die since they don"t have space to spread.

3.2.13 Protocol For Xeno -Free Single Cell Cultivation Of Human Embryonic Stem Cells

In this protocol, the ROCKi treatment includes pretreatment 1 hour before trypsinisation and the treatment during plating the cells. This protocol is intended for 6 well plate. If required in another formats , say 6 well culture dish, increase linearly all the reagents as follows.

Dish formats	Diluted BD matrigel/well	mTESR medium complete/well	ROCKi (5mM)stock/ well (final conc. 10μM)	Trypsin/well
6 well plate	1.0 ml	2ml	4 µl	1ml
12 well plate	0.5 ml	1ml	2 µl	0.5ml
24 well plate	0.25 ml	0.5ml	1 µl	250 µl
48 well plate	0.125ml	0.25ml	0.5 µl	125 µl
96 well plate	62.5 ul	0.125ml	0.25 µl	62.5 µl

This protocol consists of the following steps:

3.2.13.1 Before Passaging:

3.2.13.1.1 Preparation of Vitronectin Coated Plates

Thawed Vitronectin aliquot **on ice** and diluted to **10ng/µl** with DPBS. Added 125µl diluted Vitronectin per well of a 5 well plate and left in the Incubator for more than 1 hour for the Vitronectin to gellify. After 1 hour, just before plating the cells, the excess matrigel was removed and immediately the medium with cells was added. Care was taken not to allow the vitronectin in the wells to dry out. After plating, the plates were transferred to the incubator.

3.2.13.1.2 Pretreatment with ROCKi for 1 hour

Product : Y-27632
Purchased from : Calbiochem
Product number : 688000
Stock Concentration : 5mM
Final Concentration : 10μM

Added the inhibitor 1 hour before trypisinisation. The medium in a well of a 6 well plate was 2ml.

3.2.13.2 Passaging

After 1 hour pretreatment with ROCKi, removed the medium. Washed with PBS (2ml/well in a 6 well dish). Added trypsin (1mlper well of a 6 well plate) and incubate at 37°C for exactly 10 minutes. Harvested by pipetting up and down to ensure the single cell suspension. Taken an

aliquot for counting and Count the cells with trypan blue. Centrifuged and removed the supernatant. Made single cell suspension of titre $1x10^5$ cells/ml in mTeSR medium

3.2.13.3 Plating

Plated 200,000 cells in 2ml mTeSR medium per well of a 6 well plate. Added the ROCKi inhibitor at a final concentration of 10μM concentrations. Incubated the cells at 37°C and 5% CO_2.

3.2.13.4 Post-Passaging

Added additionally medium without ROCKi every day for the first 4 days. Thereafter, changed medium completely with fresh mTeSR medium without ROCKi.

3.2.14 Protocol for Cryo-Preservation of hES Cells Under Xeno-free Conditions

3.2.14.1 Freezing of the H9 cells in serum free conditions:

We used mFreSR™ Defined Cryopreservation Medium for hESCs (Stem Cell Technologies). Harvested the ES clumps from a single well of 48 well plate by dispase treatment and added with 0.5 ml of freshly thawed, prewarmed (15-25^0 C) 1 ml aliquot of mFreSR™ Defined Cryopreservation Medium. Gently mixed suspension and mFreSR™ and transfer 0.5 mL at a time into labelled cryovials using a 2 ml pipette. Placed vials into an isopropanol cryovessel and placed the vessel at -80°C overnight. Transferred to the liquid nitrogen vapor tank after freezing overnight.

3.2.14.2 Thawing hESCs cryopreserved in mFreSR™

hESCs cryopreserved from 1 well of a 48-well plate can be thawed successfully into 1 well of a 12-well plate. Have all tubes, warmed medium and plates ready before starting the protocol to ensure that the thawing procedure is done as quickly as possible. Prepare the Vitronectin coated dishes. Remove frozen vial of cells from the liquid nitrogen vapor tank and immediately dip the tube into waterbath at 37°C. Agitate the tube in the water continuously until only a small ice chunk remains. Spray the tube with ethanol or isopropanol to sterilize. Transfer the contents of the tube to a 15 ml conical tube using a 2 ml pipette to minimize breaking of any clumps. Dropwise, add 3 - 5 ml of warm mTeSR™1 to the tube, mixing as the medium is added. Centrifuge cells at 300 x g for 5 minutes at room temperature (15 - 25°C). Aspirate medium leaving the pellet intact. Using a 2 ml pipette, gently pipette the cell pellet in 1 - 2 ml of mTeSR™1 taking care to maintain the clumps as large as possible. Gently tilt the plates onto one corner and allow the excess Vitronectin solution to collect in that corner. Remove the Vitronectin solution using a serological pipette or by aspiration. Ensure that the tip of the pipette does not scratch the coated surface. If plates have been stored at 2 - 8°C, allow the plates to come to room temperature (15 - 25°C) for 30 minutes and keep in incubator for atleast 30mins-1hr before removing the Vitronectin solution.

Transfer 0.5 ml of medium plus clumps per well to a Vitronectin coated 12-well plate. Ensure that clumps are evenly distributed between wells. Place plate at 37°C and move the plate in quick side to side, forward to back motions to evenly distribute the clumps within the wells. Change medium daily. Check for undifferentiated colonies that are ready to passage (dense centered) approximately 5 - 7 days after thawing. If only a few undifferentiated colonies are observed after thawing, it may be necessary to select only these colonies for passaging and replate them in the same size well on a new Vitronectin coated plate.

3.2.15 Immunohistochemistry

One day prior to the sample processing, 50,000 cells per each well of Lab-Tek Permanox slide Chambers (Nalge Nunc International, USA) were seeded and cultured . After 24 hours, the samples were fixed with 4% Paraformaldehyde or with –20 ^{0}C cooled methanol-acetone (1:1) solution, permeabilised with 0.1%Triton X-100, and labeled with the appropriate primary antibody in the optimal dilutions followed by labeling with the respective secondary antibodies. The specificity of the antibodies has been tested using the appropriate tissues and isotype control antibodies.

3.2.16 Semi-quantitative RT-PCR

3.2.16.1 Design of the RT-PCR primers

For the design of the primers, the online software “Primer3” was used. (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi). Care is taken to choose the exon spanning primers to avoid the genomic DNA amplification. Primers with 23 bases and melting temperature around 60^0 C which give rise to amplicon sizes from 150-400 base pairs were preferred. Most of the Oligos were ordered from Metabion (Martinsried, Germany) and the rest from MWG (Ebersberg, Germany).

3.2.16.2 cDNA synthesis and PCR

Total RNA was extracted using RNeasy Mini Kit (Qiagen,Hilden,Germany) with on-Column DNase I (Qiagen) digestion according to the manufacturer’s instructions. 2µg total RNA was reverse transcribed using SuperScript II Reverse transcriptase (Invitrogen) with random primers according to the manufacturer’s recommended protocol. PCR amplification was done with REDTaq ReadyMix (Sigma) with 5µM each primer. GAPDH was used as an internal control. The following conditions were used. An initial denaturation at 95^{0}C for 2 minutes, followed by 22-35 cycles of 30 sec denaturation at 95^{0}C, 30 sec annealing at 60^{0}C and 60 seconds of elongation at 72^{0}C. A final extension at 72^{0}C for 5 minutes was included. Electrophorectic separation of PCR products was carried out on 2% agarose gels with 0.001% Ethidium Bromide.

3.2.17 Quantitative Real time PCR

Quantitative real time PCR analysis was perfomed with the ABI Prism 7000HT Sequence Detection System (Applied Biosystems, Foster City, CA). 100ng RNA was reverse transcribed with ThermoScript™ Reverse Transcriptase (Invitrogen). Then Real time PCR was perfomed in triplicates for every sample using SYBR green with the primers listed above with annealing temperature of 60^0C. Averaged *C*t values of each qRT-PCR reaction from the target gene were normalised with the average *C*t values of the housekeeping gene, *GAPDH,* that ran in the same reaction plate to get Δ*C*t value . The fold change was calculated by using the formula, fold-change= 2-(*C*t gene1 - *C*t gene2). Δ*C*t of the gene in the sample in which it is expressed lowest is taken as *C*t gene2 to calculate the fold change using the above formula. The resulting fold change is expressed as percentage of the maximum.

4. Results and Discussion

To achieve the ultimate goal of my project to establish xenofree single cell expansion and differentiation of human pluripotent stem cells (both hESC and hiPSC), the following steps have been designed as shown schematically in Figure 5.

4. 1 Experimental Plan

As the first step, serum free, feeder free conditions were optimized using the commercially available mTeSR media and BD Matrigel™. As a second step, using this feeder free, serum free condition, several anti-apoptotic factors were screened and tried to enable human pluripotent stem cells to expand from single cells instead of clumps. So far, the human pluripotent stem cells are propagated as clumps. When single cells were made from hESC or hiPSC, these cells undergo massive cell death mainly due to the absence of cell-cell communications. This poses a major problem in obtaining reproducible experimental results since the cells from clumps are in different state of differentiation, the inner core of the clump being more differentiated and the cells at the periphery in the undifferentiated state. The differentiated cells from the inner core of the clump greatly influence the differentiation of undifferentiated pluripotent stem cells by their cell-cell contacts (e.g. Notch signaling pathways) and paracrine actions (e.g., signaling pathways associated with TGF β, nodal etc.). To circumvent these problems associated with cell clumps, cultivation of single cells is inevitable to obtain highly reproducible results and to obtain clinically relevant phenotypic cells such as cardiomyocytes and neurons in sufficient quantities.

Once the single cell cultivation was achieved, Xenofree protocols were designed, established and optimized by replacing the BD Matrigel which is a xenogen, the gelatinous protein mixture secreted by Engelbreth-Holm-Swarm (EHS) mouse sarcoma cells with human vitronectin from human plasma to serve as substratum for the human pluripotent stem cells to attach and grow on the tissue culture dishes. Also, protocols were further improved to enable single cell expansion of human pluripotent stem cells (both hESC and hiPSC) to obtain highly reproducible results.

Then, for cardiac differentiation of human pluripotent stem cells, xenofree differentiation protocols were optimized and validated.

As the final step, using the above optimized and validated protocols, screening of cardiogeneic small molecules was performed.

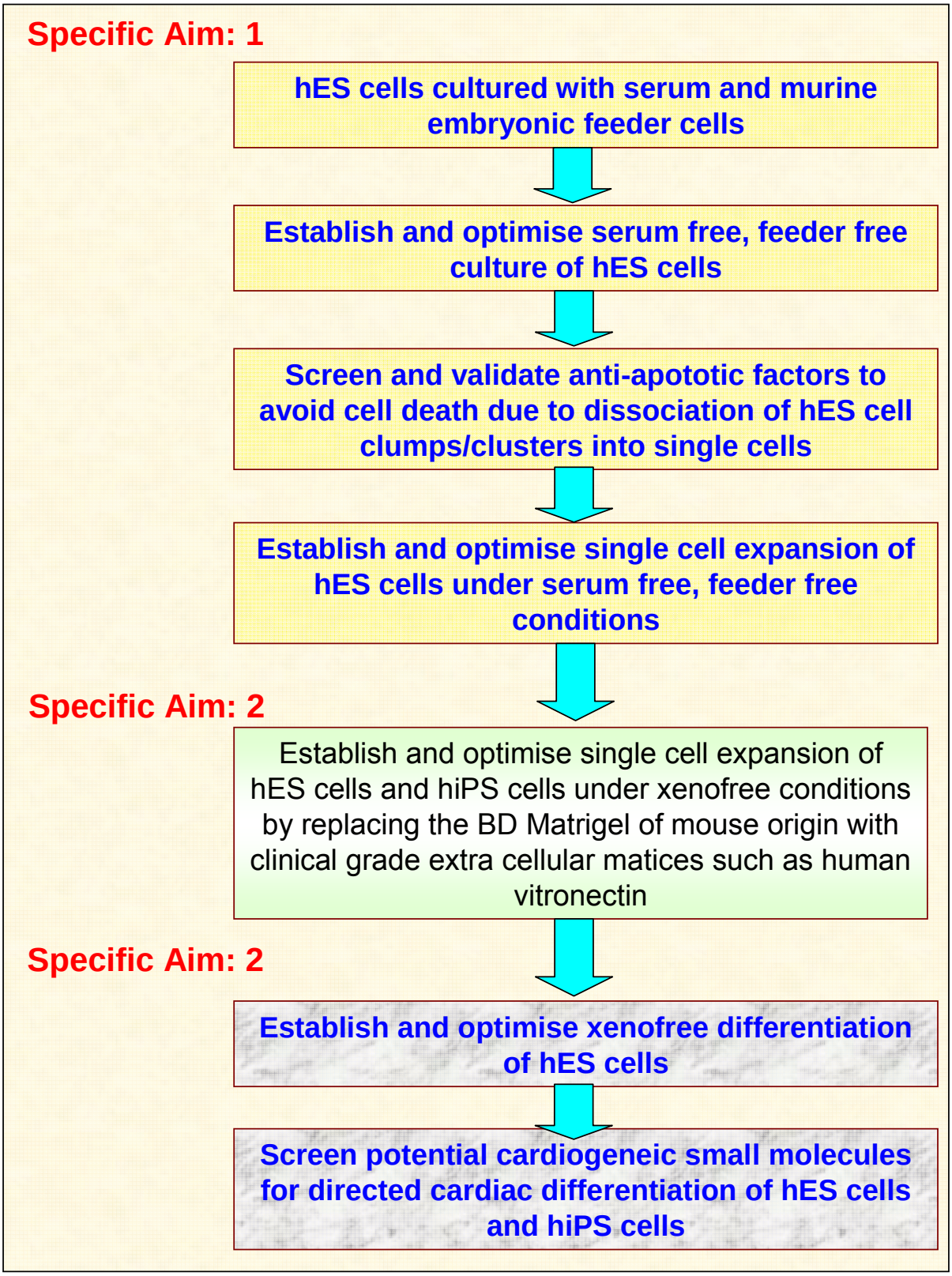


Figure 5. Schematic overview of the objective of this thesis work

4. 2 Establishment and optimization of serum free, feeder free conditions for hESC

Human embryonic stem cell line, H9 cells which were cultured on mouse embryonic feeder cells with serum containing media were transferred into BD Matrigel coated 6 well dishes with mTeSR media to establish serum free, feeder free culture of hESC. The hESC were initially

passaged as clumps. The morphology of the hESC cultured under this condition is shown in Figure 6A resemble that of typical ES cell colonies reported elsewhere, each cell with prominent nuclei and transparent cytoplasm, the true characteristics of human embryonic cells. Number of colonies contained per well of a 6 well plate was visually determined by crystal violet staining as shown in Figure 6B. There were roughly 300-400 colonies per well of a 6 well plate in an average. Most of the clumps attach preferentially in the centre of the well due to centrifugal effect. The colony size was always variable due to difference in the initial clumps at the time of seeding. After a minimum of 4 passages in this special serum free, feeder free condition, the expanded cells were subjected to FACS analysis and immunohistochemistry to determine the percentage of pluripotent cells as quality check by assaying the relative expression of pluripotent markers Tra 160, Tra 180 and Oct4 as shown in figure 6C and 7 in comparison to hES cells cultured with serum and mouse feeder cells. More than 76% of cells cultured under serum free, feeder free conditions expressed Tra-160 and Tra-180 as analyzed by FACS. A vast majority of hESC is Oct4, Tra160 and Tra180 positive (Figure 7) implying the hES cells cultured in serum free, feeder free conditions are undifferentiated. So, the cultivation of hES cells on matirgel coated dishes and in mTeSR

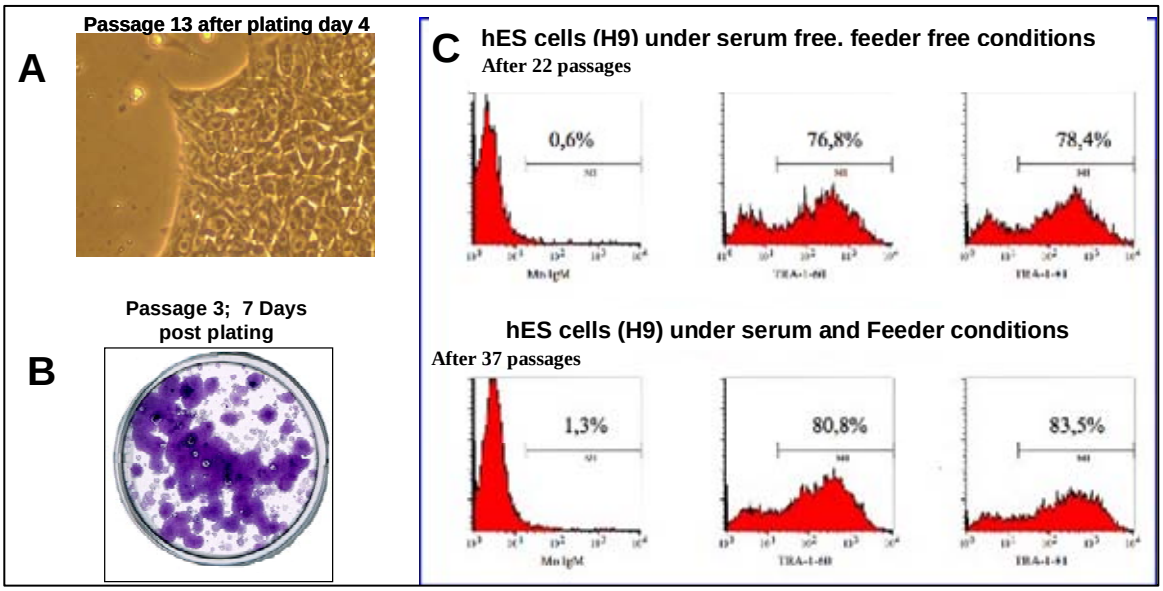


Figure 6 H9 ES cells cultured under feeder free, serum free conditions: A: morphology after 13 passages in these conditions, B: enumeration of colonies by crystal violet staining, C: quantification of pluripotent hES cells expressing Tra 160 and Tra 180 as analysed by Flow cytometry.

media enables the cultivation of undifferentiated hES cells in serum free, feeder free conditions(in a well defined media). hES cells cultured under serum free and feeder free conditions maintained pluripotent state even after 22 passages in the same way as the serum , feeder conditions.

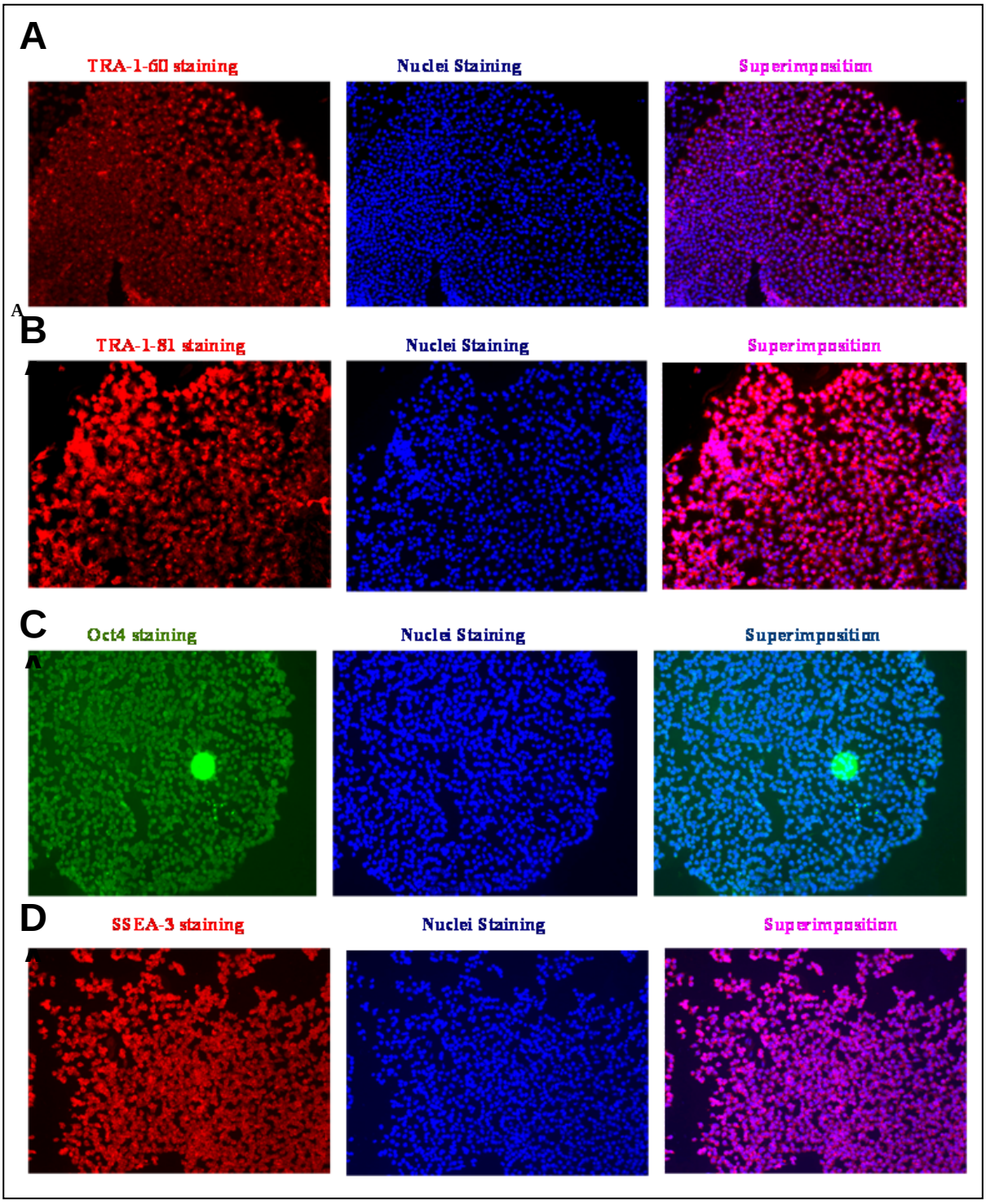


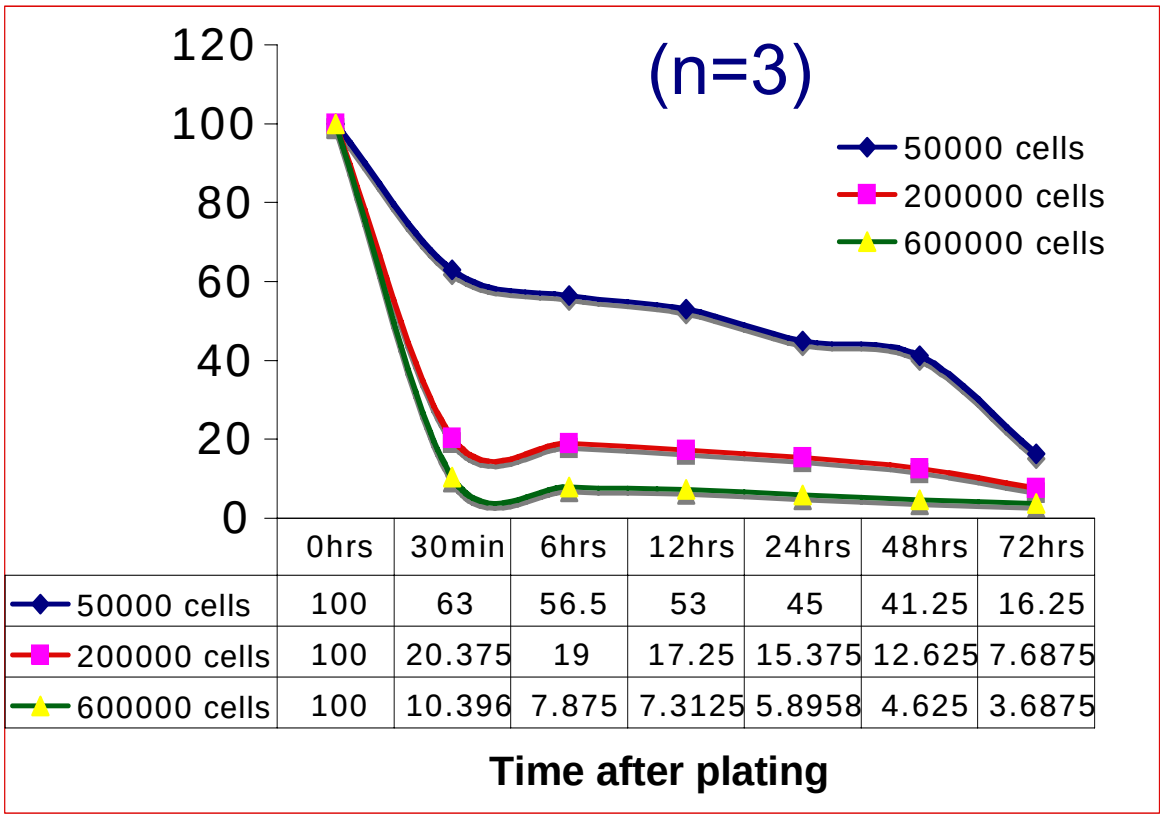
Figure 7. Immunohistochemical analysis of pluripotent markers' expression by the hES cells culture under serum free, feeder free for 12 passages. Immunohistochemical analyses of hESC expressing Tra-160 (A), Tra-180 (B), Oct4 (C) and SSEA-3 (D).

4.3 Single cell expansion of hES cells under serum free, feeder free conditions

As mentioned earlier, passaging of the human pluripotent stem cells clumps poses a major problem in obtaining reproducible experimental results since the cells from clumps are in

different state of differentiation, the inner core of the clump being more differentiated and the cells at the periphery in the undifferentiated state. When single cells were made from hESC or hiPSC, these cells undergo massive cell death mainly due to the absence of cell-cell communications. The differentiated cells from the inner core of the clump greatly influence the differentiation of undifferentiated pluripotent stem cells by their cell-cell contacts (e.g. Notch signaling pathways) and paracrine actions (e.g., signaling pathways associated with TGF β, nodal etc.,). To circumvent these problems associated with cell clumps, cultivation of single cells is inevitable to obtain highly reproducible results and to obtain clinically relevant phenotypic cells such as cardiomyocytes and neurons in sufficient quantities.

To achieve culturing hES cells as single cells, massive cell death has to be addressed. The



	0hrs	30min	6hrs	12hrs	24hrs	48hrs	72hrs
50000 cells	100	63	56.5	53	45	41.25	16.25
200000 cells	100	20.375	19	17.25	15.375	12.625	7.6875
600000 cells	100	10.396	7.875	7.3125	5.8958	4.625	3.6875

Figure 8. Survival rate of dissociated cells plated with different seeding densities. Plated 50,000, 200,000 and 600,000 cells as the starting number of cells per well in a Matrigel coated 24 well plate in 500µl serum free feeder free media (mTeSR media). At each time point, the spent medium with floating cells was collected and also the cells were collected by trypsinisation. Then both are mixed and then viable cell counting was made with trypan blue at the stipulated time points.

massive cell death is mainly due to apoptosis, since the same dissociation procedure used for other cells such as mouse embryonic stem cells and other immortalized human cell lines was employed ruling out the possible cell death by necrosis. As a first step, the survival rate of dissociated hESC with different seeding density at the time of plating was determined as shown in Figure 8 using the serum free, feeder free conditions. It was observed that

apparently, more the cells, more the single cell death. So, 50,000/ml cell titer was taken for further studies with the anti-apoptotic factors.

4.3.1 Selection of Antiapoptotic factors

A list of 17 anti-apoptotic factors were made by carefully reviewing the available scientific literatures (Table 1). From this list, ROCKi, IL6 and IGF1 were selected to proceed first with.

S.No	Anti-aoptotic factor	References
1	Rho-associated kinase (ROCK) inhibitor	(39)
2	Survivin	(29)
3	humanin	(41)
4	Interleukin-6	(20)
5	Che-1/AATF	(30)
6	Bcl-2 Activating factor (BAFF)	(23)
7	Sonic hedgehog protein	(6)
8	Insulin Like Growth Factor 1	(31)
9	Suramin	(44)
10	TIMP-1,	(2)
11	Akt/Rac-α,	(2)
12	IRAK,	(2)
13	TIMP-2,	(2)
14	RICK,	(2)
15	hVH5,	(2)
16	CHK1,	(2)
17	CK-I α	(2)

Table 1. List of anti-apoptotic factors

1. Rho-associated kinase (ROCK) inhibitor:

Application of a selective Rho-associated kinase (ROCK) inhibitor, Y-27632, to hESC markedly diminishes dissociation-induced apoptosis, increases cloning efficiency. Furthermore, dissociated hES cells treated with Y-27632 are protected from apoptosis even in serum-free suspension (SFEB) culture and form floating aggregates [39].

2. Survivin

Survivin, an inhibitor of apoptotic protein containing a single baculoviral inhibit apoptotic protein repeat domain, is a bifunctional protein that suppresses apoptosis and regulates cell division [29].

3. Humanin

Humanin (HN) is a 24 amino acids peptide with potent neuro-survival properties that protects against damage associated with Alzheimer's disease [41].

4. Interleukin-6

The production of interleukin-6 (IL-6) has been discovered in a variety of human tumors. It has been shown that both STAT and MAP kinase pathways are responsible for the IL-6-delivered survival signal in human esophageal carcinoma cells [20].

5. Che-1/AATF

Che-1 is a RNA polymerase II binding protein involved in the transcriptional regulation of E2F target genes and in cell proliferation. Recent findings identify Che-1 as a novel cytoprotective factor against apoptotic insults and suggest that Che-1 may represent a potential target for therapeutic application [30].

6. Bcl-2 Activating factor (BAFF)

The TNF family ligand B cell-activating factor (BAFF, BLyS, TALL-1) is an essential factor for B cell development. BAFF binds to three receptors, BAFF-R, transmembrane activator and CAML interactor (TACI), and B cell maturation antigen (BCMA), but only BAFF-R is required for successful survival and maturation of splenic B cells [32].

7. Sonic hedgehog protein

In vertebrates the neural tube, like most of the embryonic organs, shows discreet areas of programmed cell death at several stages during development. It has been that SHH can counteract a built-in cell death program in the developing nervous system and other tissues and thereby contribute to organ morphogenesis, in particular in the central nervous system [6].

8. Insulin Like Growth Factor 1

From the point of view of cell growth, the IGF-IR activated by its ligands has three important functions: (a) it is required for optimal growth both in vivo and in vitro, although some growth occurs even in its absence; (b) it is obligatory for the establishment and maintenance of the transformed phenotype and for tumorigenesis for several types of cells; and (c) it protects cells from apoptosis, both in vivo and in vitro. The IGF-I receptor does seem to occupy a central role in these processes. Whereas an overexpressed IGF-I receptor is mitogenic for IGF-I alone and is fully transforming and protects cells from apoptosis, the same cannot be said for overexpressed EGF and PDGF receptors[31].

9. Suramin

It has been previously shown that Chinese hamster ovary (CHO) cells capable of growing in medium free of exogenous proteins die by apoptosis during all stages of a batch culture. On the basis of the hypothesis that extracellular death factors might be important in apoptosis under these conditions, the effect of the growth factor inhibitor and antitumor agent suramin on CHO cell growth and apoptosis in serum-free culture was examined. Suramin protected against apoptosis during exponential growth, as indicated by the absence of DNA laddering

and an increase in cell viability from roughly 70% to above 95%. Suramin also effectively dispersed cell aggregates so that single-cell suspension culture was possible. However, suramin did not protect against apoptosis during the death phase, in contrast to serum, suggesting that antiapoptotic factors in the serum remain to be discovered. The increased viable cell yield following suramin supplementation resulted in a 40% increase in product yield, based on results with cells expressing recombinant secreted alkaline phosphatase. Polysulfated compounds dextran sulfate and polyvinyl sulfate worked nearly as well as suramin in dispersing cell clumps and increasing viable cell yield, which implies that suramin's high sulfate group density may be responsible for its effects in cell culture. In addition, suramin was beneficial for long-term adaptation of CHO cells to protein-free media suspension culture, and the compound was synergistic with insulin in accelerating this adaptation time [44].

Also factors such as TIMP-1, Akt/Rac-α, IRAK, TIMP-2,RICK, hVH5, CHK1, and CK-I α were chosen from literatures. (see reference [2] for more details).

4.3.2 Screening and validation of anti-apoptotic factors for their efficacy in enabling single cell expansion of hESC and hiPSC

To screen potential anti- apoptotic factor which would enable single cell expansion of hESC, 50,000 cells were plated as the starting number of cells per well in a Matrigel coated 24 well plate in 500ul serum free feeder free media (mTeSR media) with or without the addition of anti-apoptotic factors (ROCKi, IL6 and IGF) at the concentrations (10μM, 50ng/ml and 100ng/ml respectively) as used in previous work with human cells that have been shown to have profound effect under normal physiological conditions [16, 39]. At each time point, the spent medium with floating cells was collected and also the cells were collected by trypsinisation. Then both are mixed and then viable cell counting was made with trypan blue at the stipulated time points.

Apparently, IL6 at 50ng/ml and IGF1 at 100ng/ml didn't have any effect on the survival rate as shown in Figure 9, where as ROCKi at 10μM showed profound effect on the survival rate even after 72 hours. In addition, all the 3 factors investigated were added together to the same cell preparation. Again, the ROCK inhibitor in the cocktail caused significant positive influence on the survival of single cells with nearly the same magnitude as that of when applied alone. But a synergism among these anti-apoptotic factors was not observed as per the expectation.

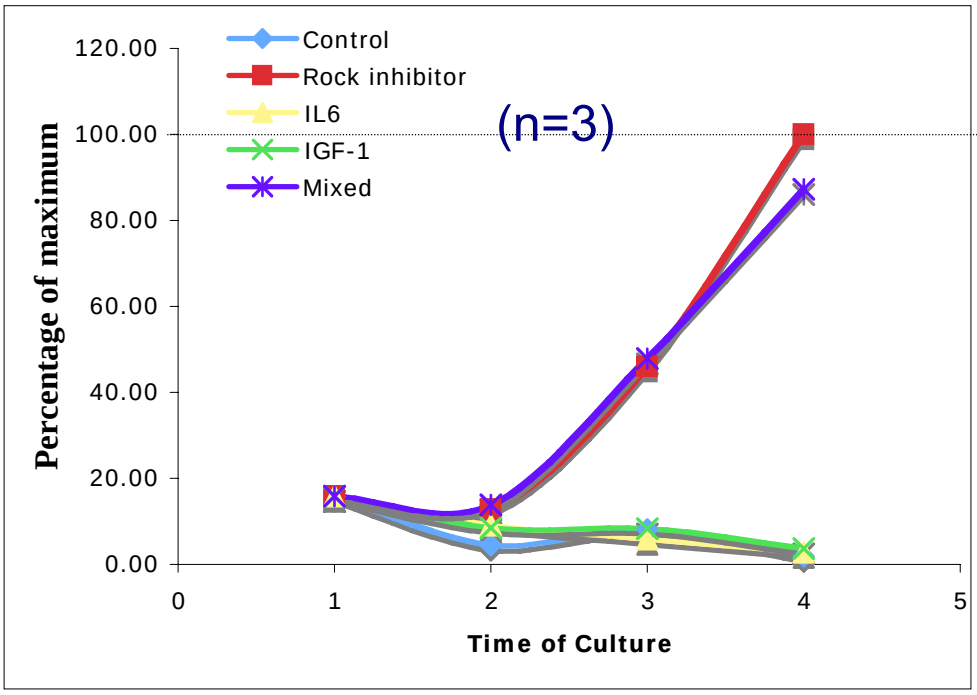


Figure 9. Comparison of 3 anti-apoptotic factors investigated on the survival rate of dissociated single cells of hES cells. The maximum number of cells survived with ROCKi was normalized to 100% and the numbers of cells in rest were expressed relative to that in terms of percentage of maximum.

4.4 Xenofree single cell expansion of hESC

This was done with ROCKi treatment just before and after Single cell plating on vitronectin (Chemicon) coated tissue culture dishes and mTeSR media. The percentage of pluripotent hESC cultured under Xenofree condition was assessed relative to cells cultured under serum free, feeder free conditions and hESC grown with CF-1 feeder and serum as used routinely in our lab by FACS analysis. The markers used were Oct ¾, Tra 160 and 180. The cells cultured under Xenofree conditions showed 67 % of cells expressing Tra 1-60 and 82.8 % of cells expressing Tra 1-80.

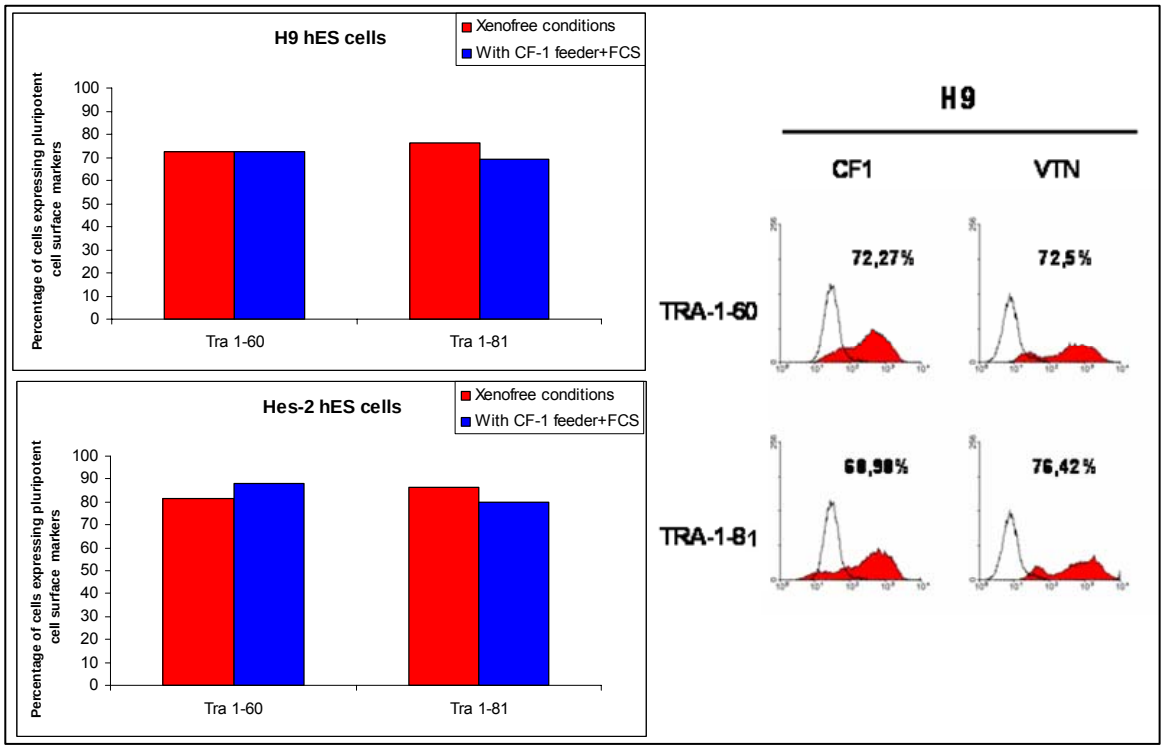


Figure 10. FACS analyses of the relative expression of Tra 160 and Tra 180 by the hES cells under Xenofree conditions, serum free and feeder condition and conditions with serum and CF1 feeders.

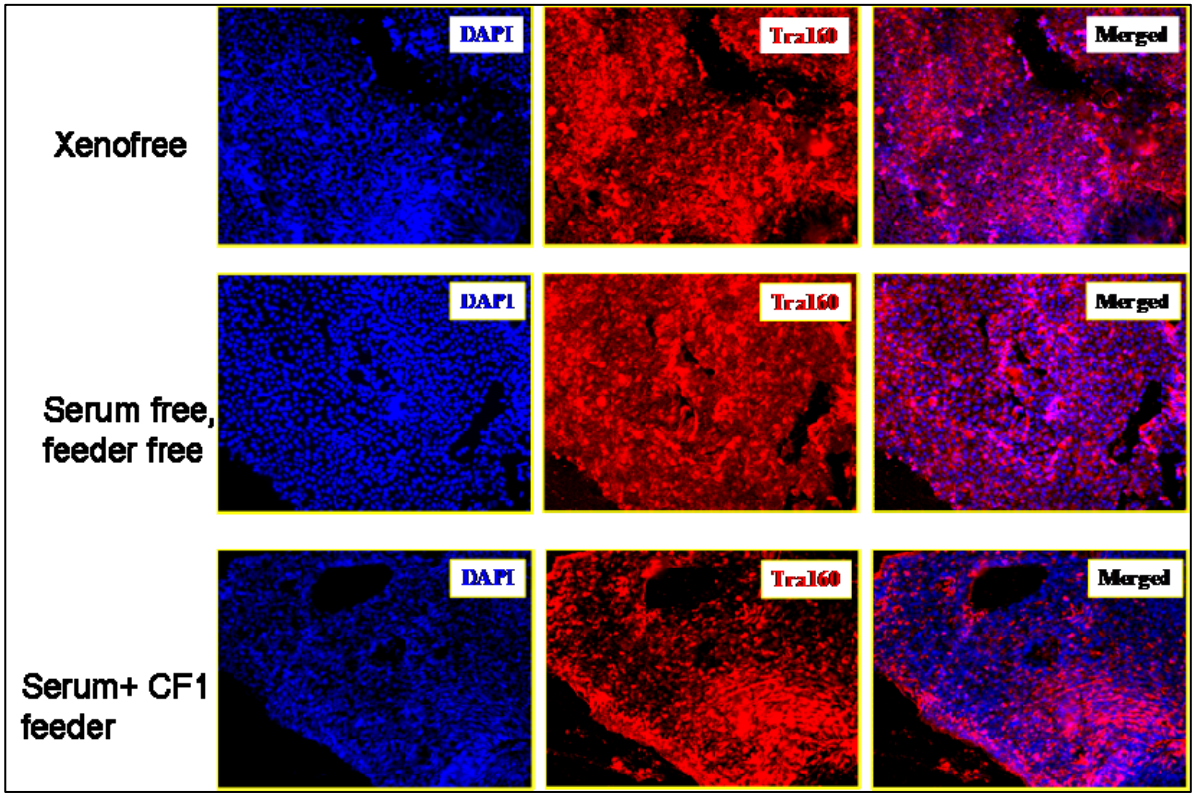


Figure 11. Immunohistochemical analyses of TRA-1-60 expression by the hES cells under Xenofree conditions, serum free and feeder condition and conditions with serum and CF1 feeders.

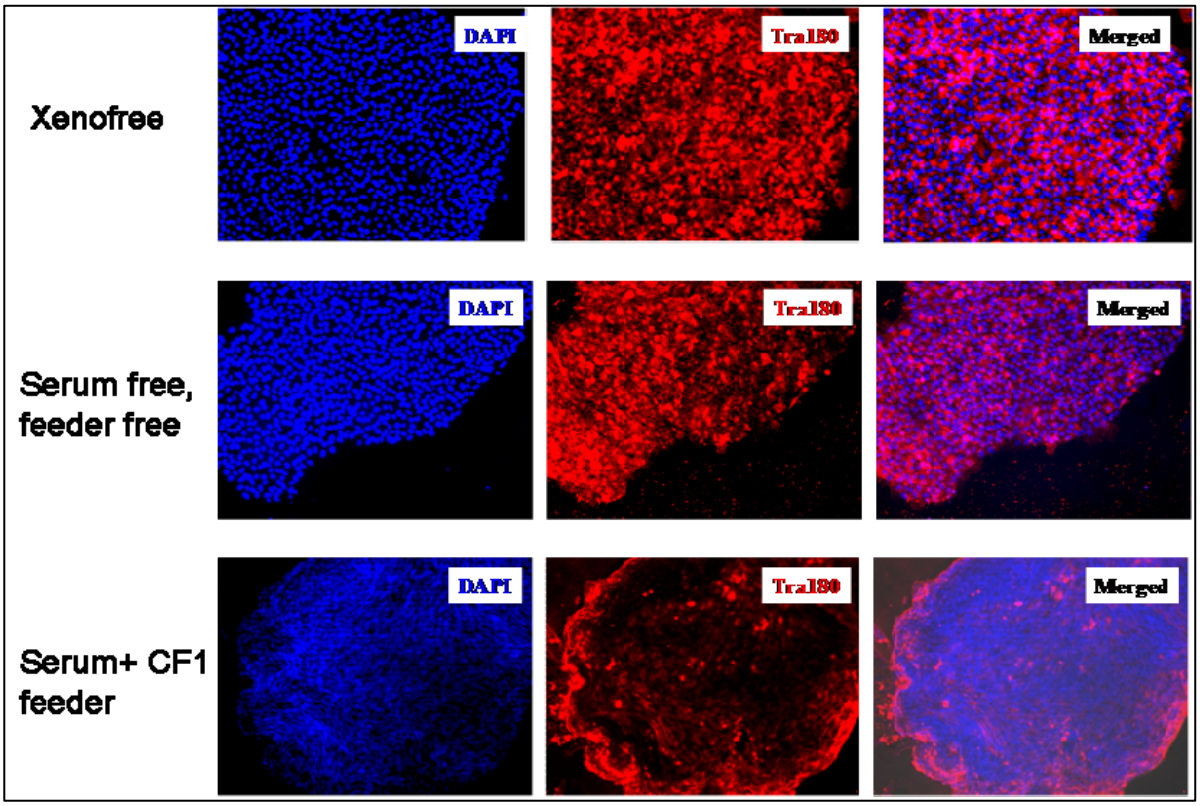


Figure 12 . Immunohostochemical analyses of TRA-1-80 expression by the hESC under Xenofree conditions, serum free and feeder condition and conditions with serum and CF1 feeders.

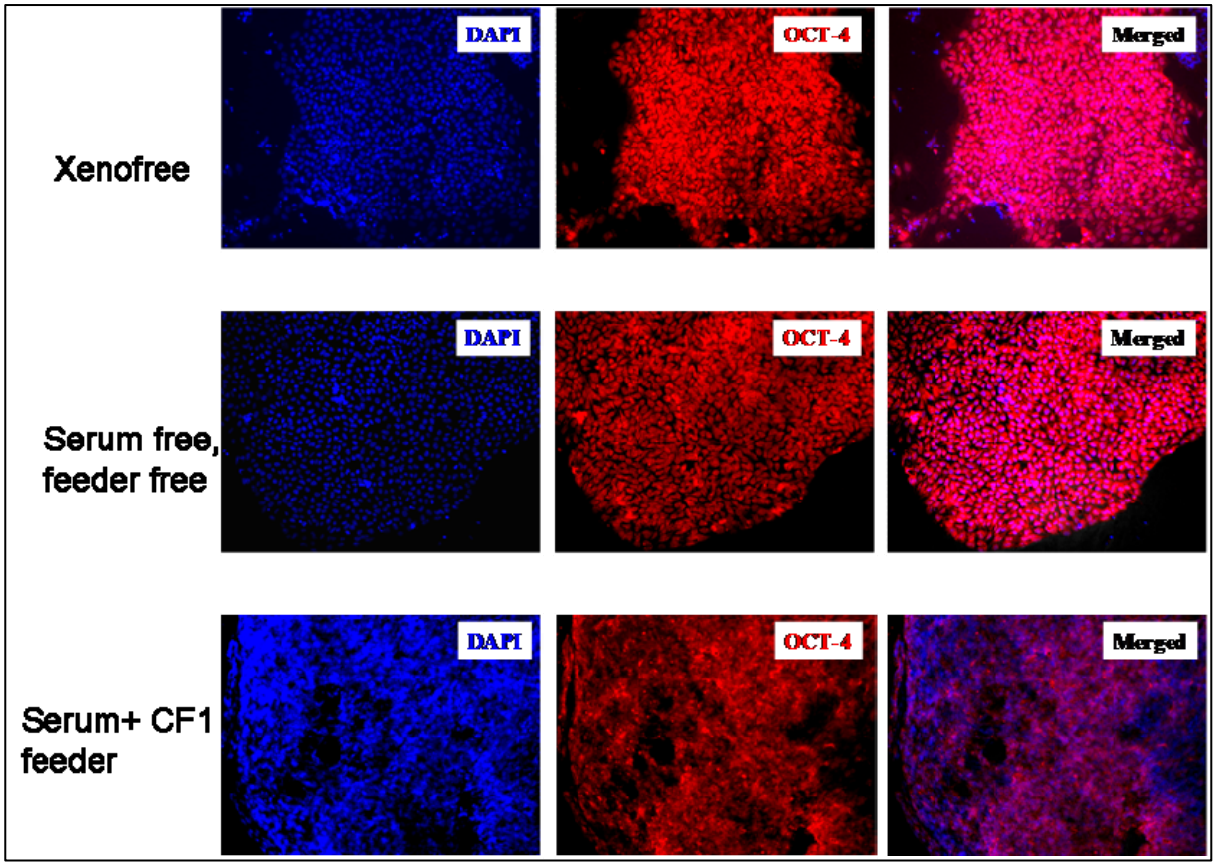


Figure 13. Immunohistochemical analyses of OCT4 expression by the hESC under Xenofree conditions, serum free and feeder condition and conditions with serum and CF1 feeders.

Nearly same percentage of hESC cultured under xenofree conditions expressed Tra 1-60 and 1-81 in nearly equal levels to the cells cultured with CF-1 feeder cells. There are no significant differences between these conditions: xenofree versus feeder+serum conditions. Also there have been no difference in rate of proliferation as shown in Figure 14 and the morphology of hESC cultured under xenofree conditons showed typical characteristic morphology of pluripotent hESC.

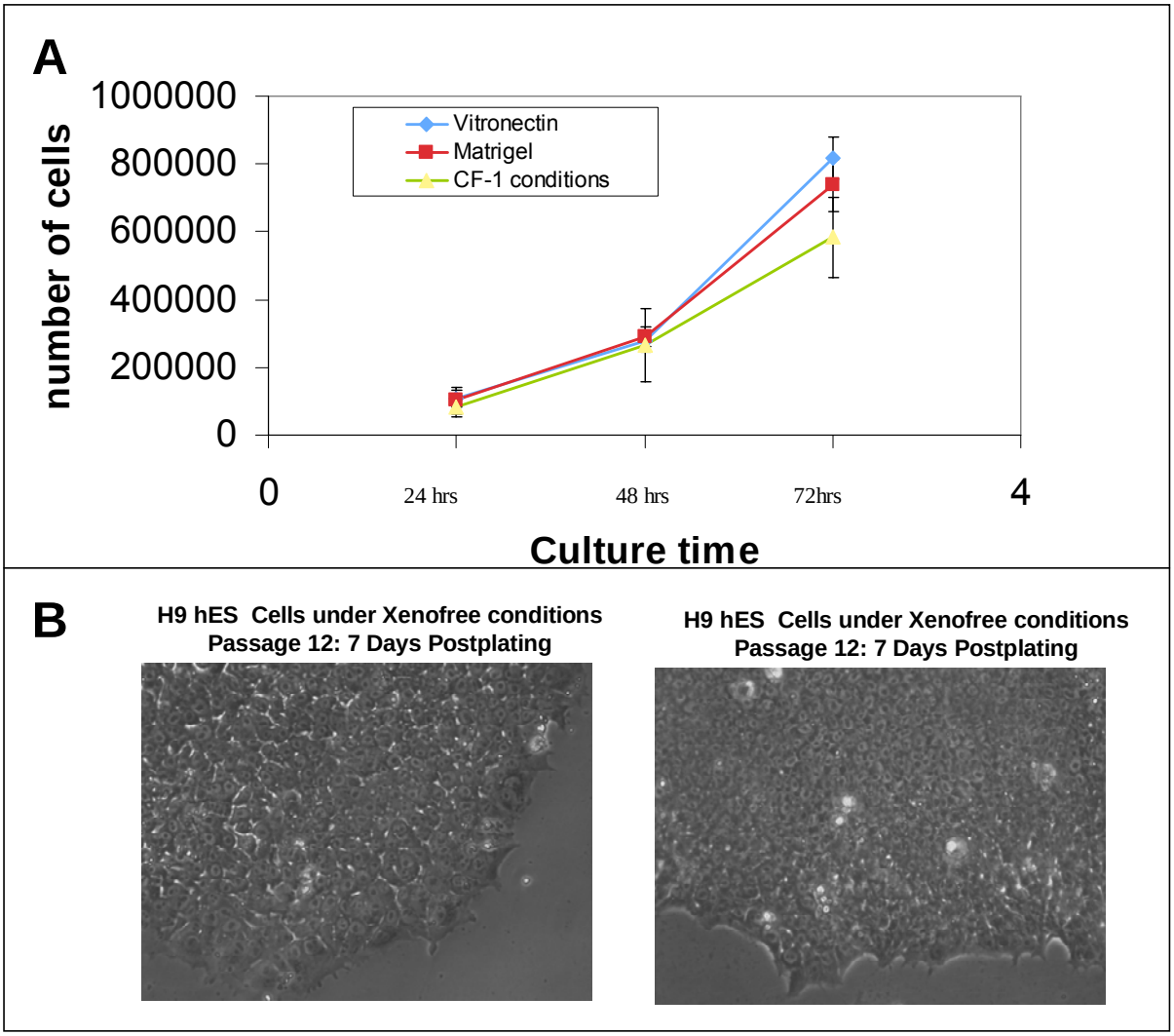


Figure 14. hESC cultured under xenofree conditions. A; growth rate as measured by viral staining by trypan blue and B: morphology of hESC cultured for 12 passages under xenofree conditions

4.5 Xenofree single cell expansion of hiPS cells

The above xenofree protocol optimized with hES cells were further evaluated for its applicability in xenofree single cell expansion of hiPS cells. As shown in Figure 15, the hiPS cells cultured with this xenofree protocol showed typical ES cell morphology and also over 87% cells expressed the pluripotent markers Tra 160 and Tra 180 in the same way as the hiPS cells cultured with feeder cells and serum thereby implying the successful application of this protocol for Xenofree expansion of human iPS cells.

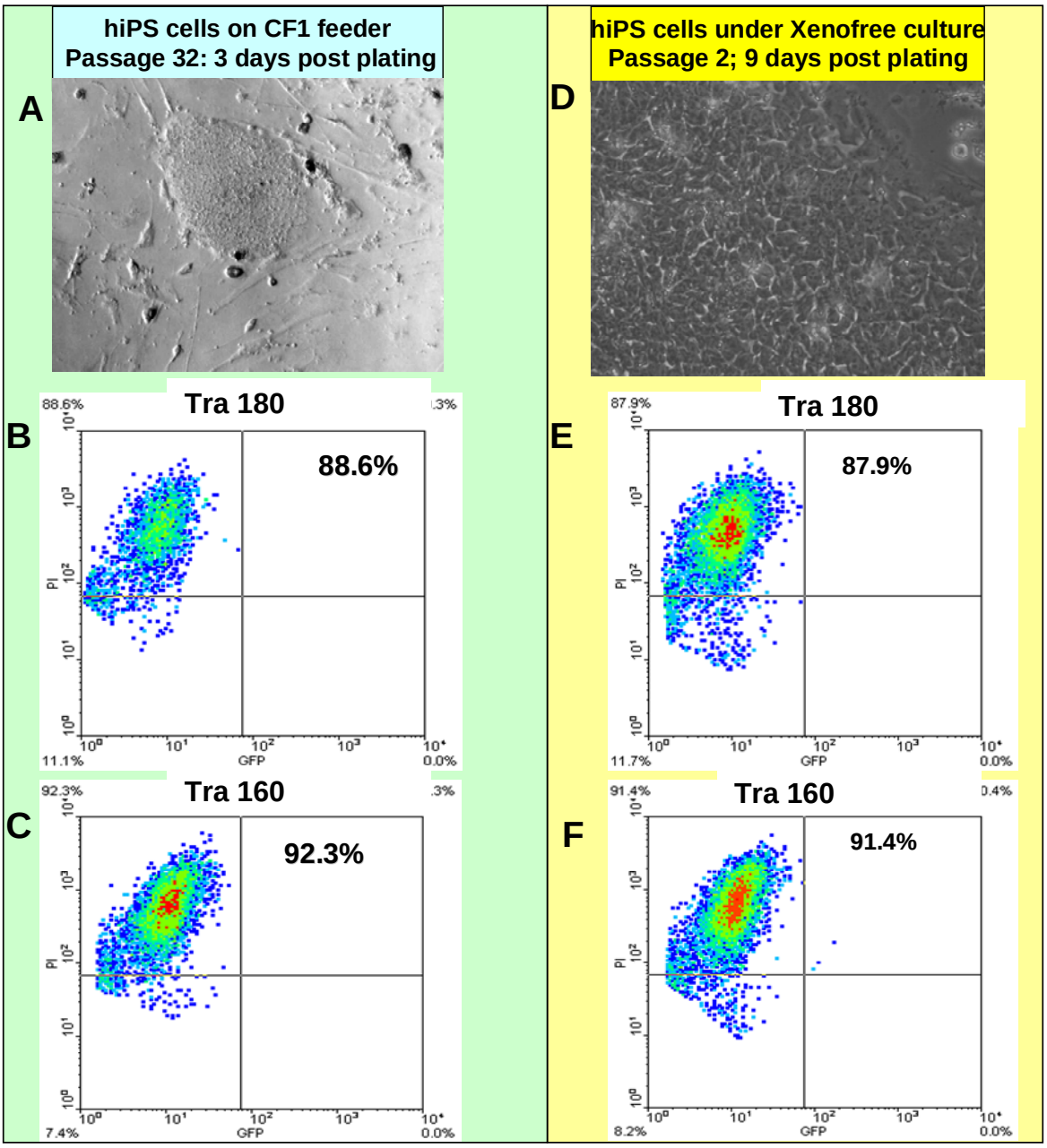


Figure 15. hiPSC cultured under xenofree conditions. A; morphology of hiPSC cultured under, B: FACS analysis of hiPSC expressing TRA 180, cultured under serum+ feeder conditions, C: FACS analysis of hiPSC expressing TRA 160, cultured under serum+ feeder conditions, D; morphology of hiPSC cultured under xenofree conditions, E: FACS analysis of hiPSC expressing TRA 180, cultured under xenofree conditions, F: FACS analysis of hiPSC expressing TRA 180, cultured under xenofree conditions.

4.6 Xenofree differentiation of hESC

Xenofree differentiation protocol was established with knockout DMEM medium supplemented with minimum essential aminoacids, β-mercaptoethanol and penicillin and streptomycin. This protocol was executed in parallel with 2 other experimental protocols., one being the protocols using END2 cell line on gelatin coated dishes and other with matrigel coated dishes. The same media (knock out media) was used for all the 3 protocols. Dissociated single cells with pre- and post-treaement with ROCKi (Y-276372) were plated either on vitronectin coated dish for Xenofree conditions, or on matrigel coated dishes for serum free conditions, or direcly on END2 cells. RNA was extracted on samples at time points on days 0, 3, 5, 8, 10 , 12 and 14 and reverse transcriped for cDNA synthesis. Both semiquantitative PCR (Figure) and quantitative PCR was performed on these samples to obtain the transcript expression profiles of the canonical germ layer markers, pluripotent marker and lineage specific markers as shown in Table 2.

GAPDH	Endogenous loading control
Nanog	Pluripotent marker
Oct3/4	Pluripotent marker
Pax6	Neuronal marker
Sox17	Endoderma maker
FoxA2	endodermal marker
Tbrachyury	mesoderma marke
aMHC	cardiac marker
Troponin T	cardiac marker
ANF	cardiac marker
Nkx2.5	cardiac progenitor marker

Tabel 2. Canonical pluripotent, germ layer- and lineage specific markers

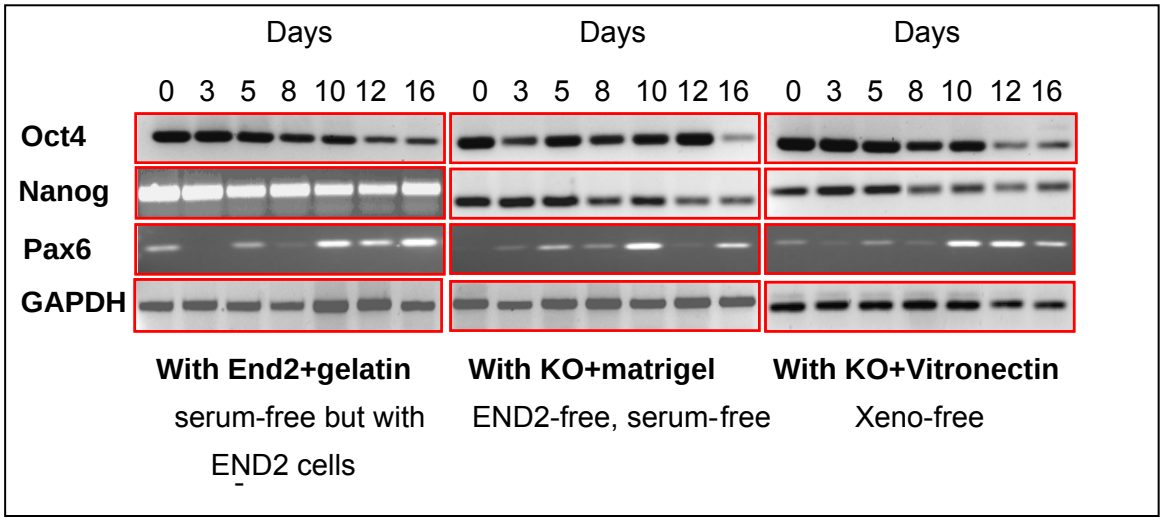
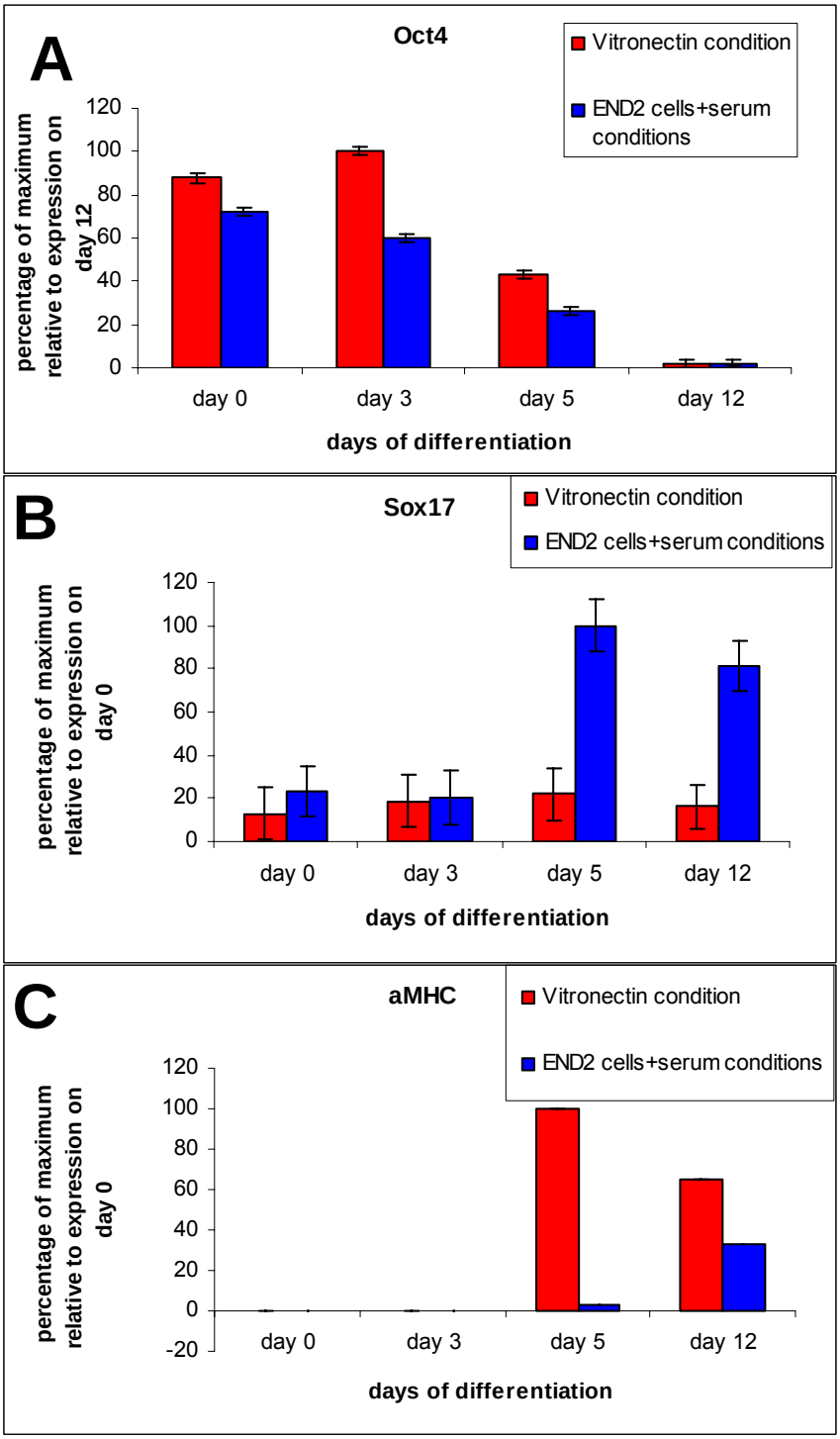


Figure 16. Semi-quantitative PCR analyses of expressions of canonical markers

As shown in Figure 16, the expressions of the representative transcripts are more or less in the same way in all the 3 conditions. When the same RNA samples were subjected to quantitative PCR analyses, it was found that Oct4 expression level was more sustained in

xenofree conditions during differentiation in comparison to conditions with END2 cells. Sox17 expression levels are increased at late stages in conditions with END2. The increased levels may be due to enhanced endodermal differentiation due to END2 cells. END2 cells are visceral endodermal like cells. Notably, αMHC expression is more enhanced in xenofree conditions in comparison to END2+serum conditions thereby proving xenofree conditions to be optimal for screening cardiogenic small molecules.

Figure 17.



Comparison of canonical lineage specific marker expression between Xenofree conditions and conditions with END2 cells+ serum.

4.7 Screening of cardiogenic small molecules for directed cardiac differentiation of hES cells and hiPS cells

As schematically shown in Figure 18, single cells (100,000 cells) were plated with ROCKi on vitronectin coated wells in a 24 well plate in mTeSR media and cultured for 48 hours. After 48 hours, the cells were washed and added with K/O differentiation media with small molecules at the approporate concentrations. 3 different concentrations were used for each of the small molecules, 1μM, 5 μM and 10 μM. The treatment was continued only for the first 5 days (from day 2 to day 7 of differentiation). On day 8, Only 2 wells with 10μM Cyclosporine and 1 well with 10μM Verapamil showed beating clusters. Nifdipine failed to show any cardiogenic potential even with 10uM concentrations. So, we used only the cyclosporine and Verapamil for our later experiments. Nifdipine was omitted. Beating frequency was determined on a regular basis from day 8 to day 56 after which there was no beating observed (Figure 19). Even though initially, Verapamil treated clusters showed more beating frequency, their beating stopped by day 41 whereas cyclosporine treated cultures showed sustained beating frequency even after 41 days and stayed till 56 days.

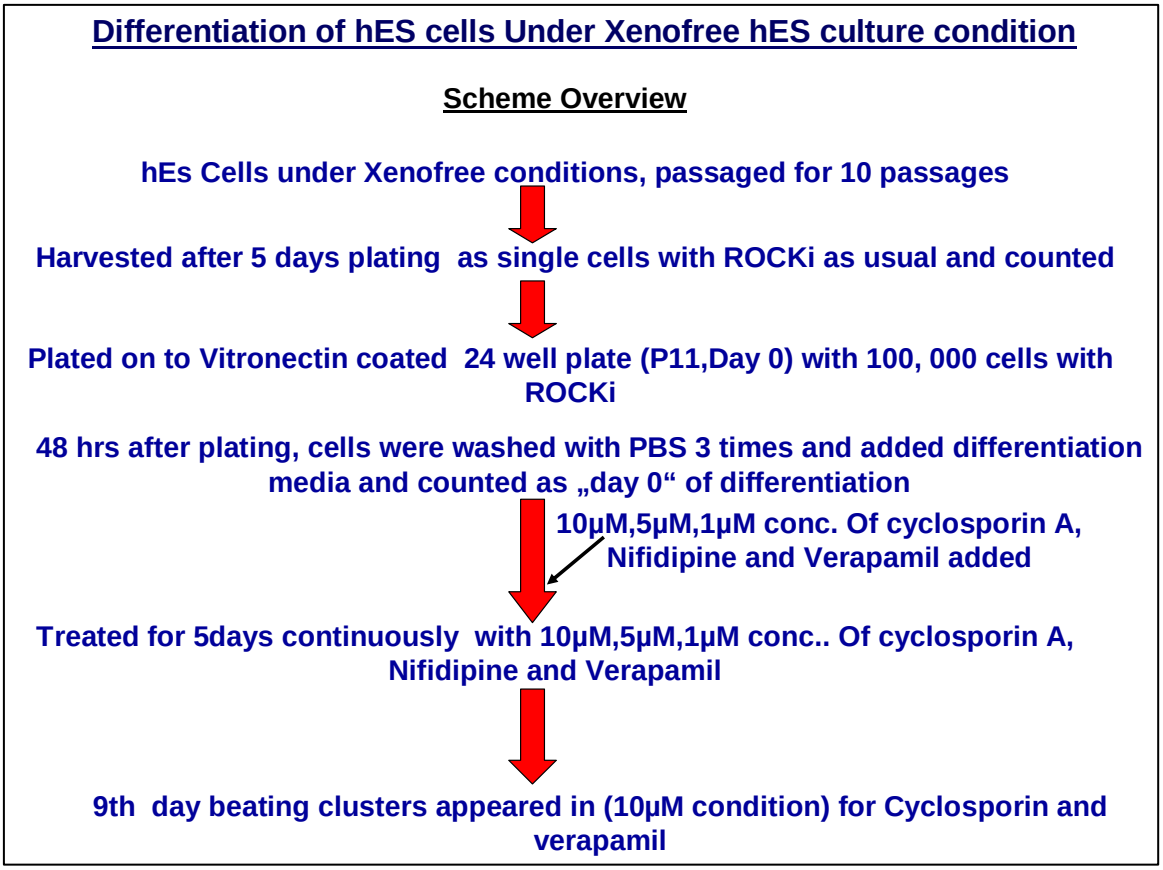


Figure 18. Schematic overview of the experimental protocol used for screening small molecules under xenofree conditions

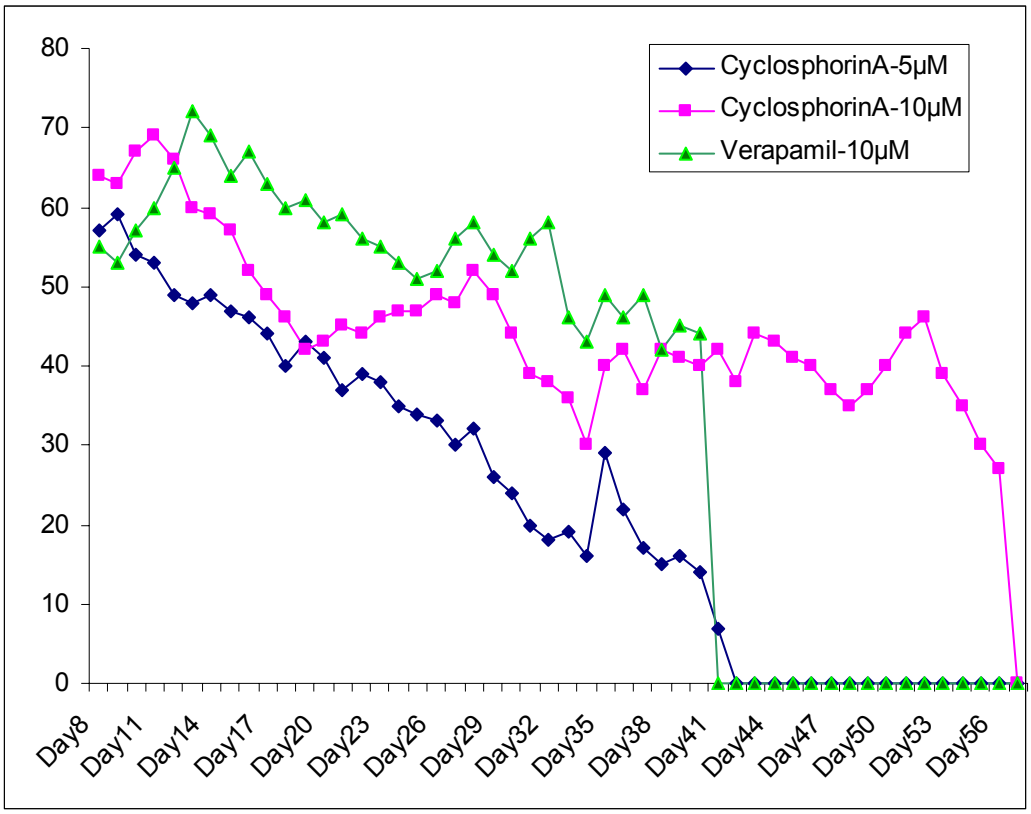


Figure 19. Beating frequency of clusters differentiated under Xenofree conditions with different small molecules with different concentrations

Quantitative PCR analyses of the samples taken at different periodic intervals of differentiation showed profound enhancement of cardiomyogenesis on treatment with both cyclosporine and verapamil (Figure 20). As we can see, the enhanced cardiomyogenesis by both verapamil and cyclosporine was coupled with enhanced mesoderm formation as evident from the upregulated expression of T brachyury (a mesodermal marker) at the end of treatment with cyclosporine and verapamil for first 5 days, implying the enhanced cardiomygenesis is more likely due to effect of cyclosporine and verapamil directly on the formation of mesodermal cells rather than on cardiogenic progenitors or their proliferation in hESC context. Also, the enhanced mesoderm formation by ethanol used as vehicle control (days 5, 8,12 and 16 compared to untreated control) also weakens the conclusion on the efficacy of cyclosporine on mesoderm formation, although eventually, cyclosporine treated cultures showed significant Troponin T expression in an earlier time compared to controls (both control and ethanol treated) and even to verapamil treated cultures. However, these results show that these cardiogenic drugs are effective in accelerating the cardiac differentiation but not in improving the yield of cardiomyocytes at the end of the protocol, since Troponin T expression in the cell population treated with these drugs didn't show sustained upregulated levels after 16 days after which expression of Troponin T levels were well below that of the experimental control and vehicle control. Interestingly, ethanol used as vehicle control for cyclosporine treatment increased Pax6 level on day 5 (the time point

immediately after the 5 days treatment window), implying that the immediate effect of ethanol treatment is on the neural differentiation. Still, the possible positive effects of cyclosporine and Verapamil on cardiac progenitors need to be explored.

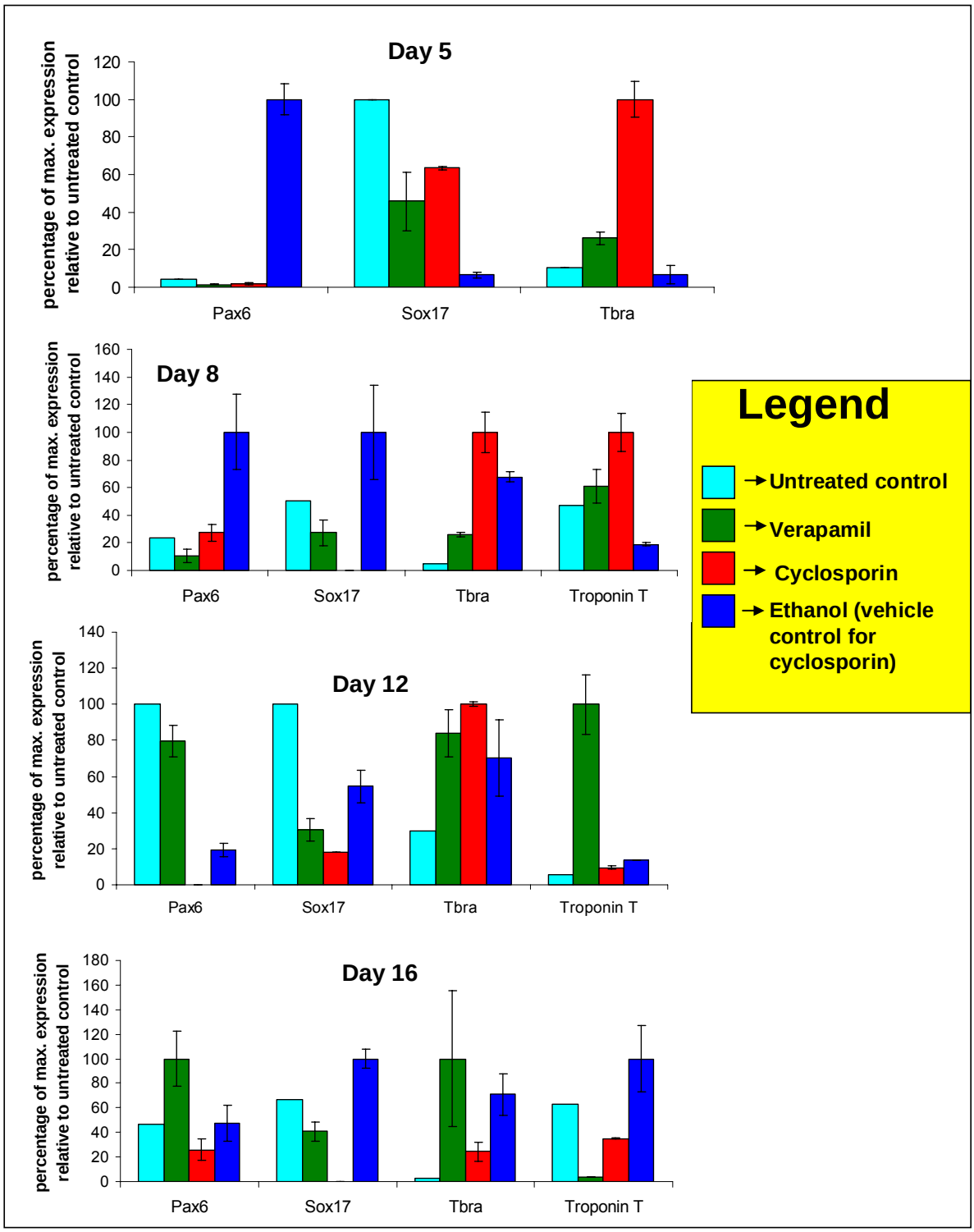


Figure 20. Screening and validation of possible cardiogenic effects of small molecules by real time PCR on differentiated cells from hESC cultured under xenofree conditions. . The expression level for each of the transcripts in cells untreated at the respective time points under xenofree cultured conditions was normalized to 1 and the relative expression levels were expressed as percentage of maximum, with +/- the standard deviation as determined from the biological replicates (n=3).

4.8 Xenofree differentiation of hiPS cells and small molecule screening

Xenofree differentiation protocol as validated with hESC were used for the differentiation of hiPSC with knock out DMEM medium supplemented with minimum essential aminoacids, beta mercapto ethanol and pencillin and streptomycin. RNA was extracted on samples at time points on days 0, 5, 8, 12 and 16 and reverse transcriped for cDNA synthesis. Quantitative PCR was performed on these samples to obtain the transcript expression profiles of the canonical germ layer markers, pluripotent marker and lineage specific markers as shown in Table 2. The expression level for each of the transcripts in cells untreated at the respective time points under xenofree cultured conditions was normalized to 1 and the relative expression levels were expressed as percentage of maximum (Figure 21).

As shown in Figure 21, the upregulation of Troponin T by verapamil and cyclosporin has been always accompanied with the increased expression of T brachyury as evident on day 8 cyclosporine treated cultures, on day 12 both Verapamil and cyclosporine treated cultures, and very significantly on day 16 with ethanol (vehicle control for cyclosporine treated conditions). Thus, the increased cardiomyogenesis with Verapamil, Cyclosporin and ethanol are effected at the mesoderm formation level, boosting formation of more mesodermal cells and thus proportionally more cardiomyocytes. Interestingly, the cyclosporine treated cultures show increased expression levels of Sox17 on day 8, the endodermal lineage marker and pax6, the maker for neural lineages where as verapamil show increased levels of T bra, the mesodermal marker, at this time point.

Apparently, although treatment with verapamil and cyclosporine enhances early cardiac differentiation as seen on days 8 and 12, the yield of cardiomyocytes on day 16 as analysed by Troponin T mRNA expression levels remains the same as that of untreated cultures. Thus Cyclosporine and Verapamil enhance early cardiac differentiation (facilitating the early onset of cardiomyogenesis)m but don’t increase the cardiomyocyte yield at the end of the protocol. Both Verapamil and cyclosporine were found to be more significantly effective to induce early onset of cardiomyogenesis.

Interestingly, the enhancement of cardiac differentiation by ethanol was observed at later points on day 16 and is accompanied by T brachyury upregulation, the same trend observed for verapamil and cyclosporine.

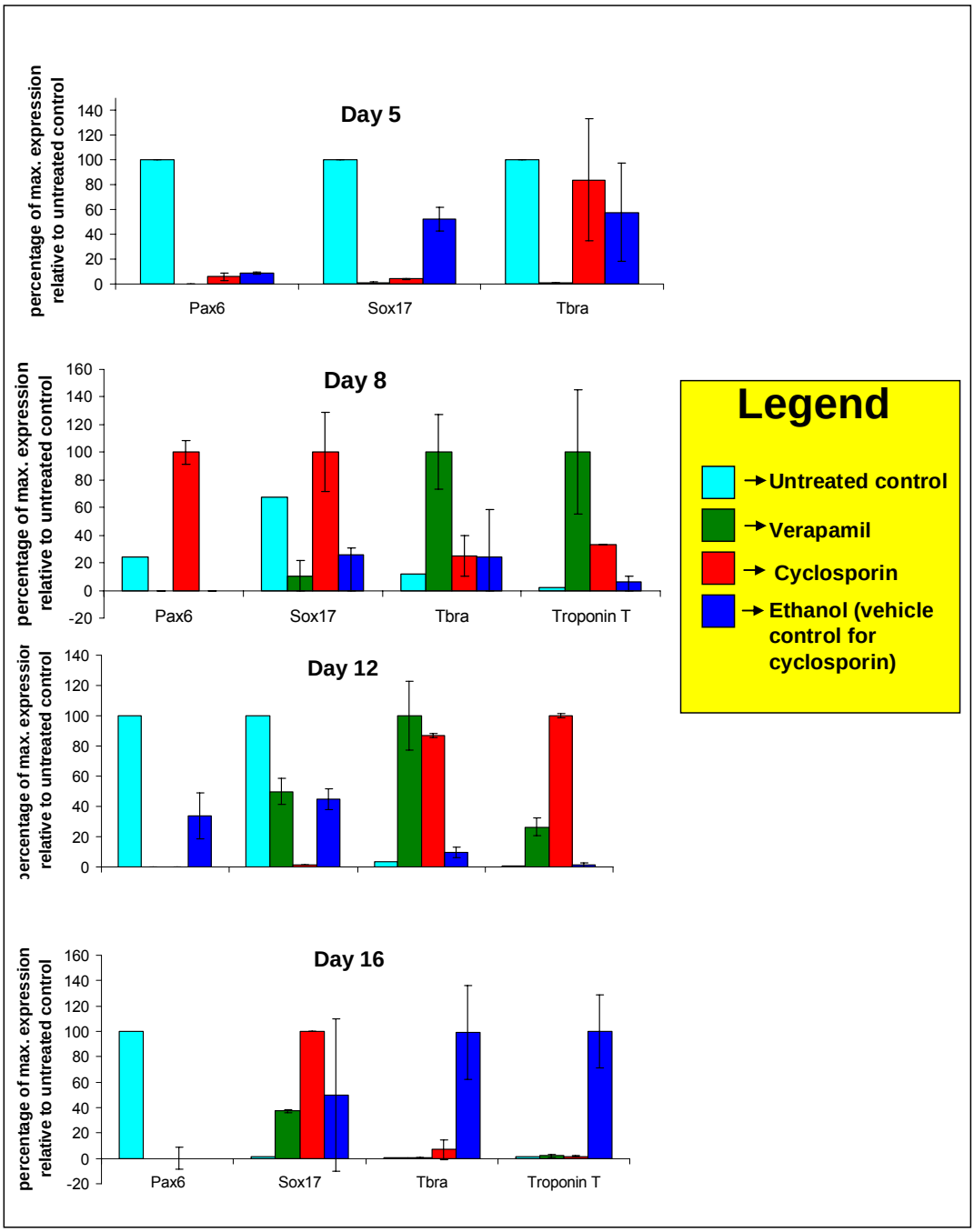


Figure 21. Quantitative RT-PCR analyses of the germ layer and cardiomyocyte specific markers on hiPS derived differentiated cells cultured under xenofree conditions. . The expression level for each of the transcripts in cells untreated at the respective time points under xenofree cultured conditions was normalized to 1 and the relative expression levels were expressed as percentage of maximum, with +/- the standard deviation as determined from the biological replicates (n=3).

5. Conclusion and Outlook

The established and optimized xenofree expansion and differentiation protocols are equally valid as the protocols with Matrigel and feeder cells and serum conditions. These protocols could be used to obtain the highly reproducible results. This differentiation protocol does not need the use of END2 cells and hence it is completely Xenofree. This protocol will serve as relatively optimal one for high throughput screening of small molecules for their cardiogenic and other celllogenic potentials. Using these protocols, the cardiogenic potential of Verapamil and Cyclosporin has been revealed. The cardiogenic effects of cyclosporine and verapamil have to be further investigated to determine whether their effects are either at the level of mesoderm formation (i.e., they enhance formation of mesoderm and hence more mesodermal derivatives) and/or at the level of mesoderm specification (i.e., mesodermal patterning into cardiomyocytes is specifically enhanced).

6. Summary / Zusammenfassung

Summary

Till date, the majority of currently available human embryonic stem (hES) cell lines have been directly or indirectly exposed to materials containing animal-derived components during their derivation, propagation, and cryopreservation. The routine Long-term in vitro cultures of human embryonic stem (hES) cells require mouse embryonic fibroblast (MEF) feeders and animal component-containing media like fetal bovine serum (FBS) or serum replacement for maintaining cells in an undifferentiated state and to increase karyotype stability. Therefore, the use of animal materials increases the risk of transmitting pathogens to hES cells, graft rejection and is not optimal for use in cultures intended for human transplantation. There are also other limitations with conventional feeder cells, such as MEFs, which have a short lifespan and can only be propagated five to six passages before senescing. This necessitates for a xenofree culturing conditions in obtaining clinical grade cell derivatives for cell replacement therapy. Also, passaging of the hES cells in the form of clumps causes greater variability and hence for obtaining consistent data, one needs to use single cells. Accordingly, the objective of the present project is divided into the following 3 specific aims: First to establish xenofree protocols for single cell expansion of human pluripotent stem cells (both hESC and hiPSC), secondly to establish Xenofree protocols for cardiac differentiation of human pluripotent stem cells (both hESC and hiPSC) and thirdly to screen cardiogenic small molecules which enhance cardiac differentiation of human pluripotent stem cells (both hESC and hiPSC) under xenofree conditions. During my thesis work, xenofree single cell expansion and differentiation protocols were established and optimized. These protocols are equally valid as the protocols using Matrigel and feeder cells and serum conditions. These protocols could be used to obtain highly reproducible results. The established differentiation protocol does not need the use of END2 cells for cardiac differentiation and hence it is completely Xenofree. This protocol will serve as relatively an ideal one for high throughput screening of small molecules for their cardiogenic and other celllogenic potentials. Using these protocols, the cardiogenic potential of Verapamil and Cyclosporin has been revealed. The cardiogenic effects of cyclosporine and verapamil have to be further investigated to determine whether their effects are either at the level of mesoderm formation (i.e., they enhance formation of mesoderm and hence more mesodermal derivatives) and/or at the level of mesoderm specification (i.e., mesodermal patterning into cardiomyocytes is specifically enhanced).

Zusammenfassung

Bisher werden humane embryonale Stammzellen (hES) Zellen während ihrer Gewinnung, Vermehrung und Kryopreservation Materialien ausgesetzt, die tierischen Ursprungs sind. Die üblichen Langzeitkulturen von hES-Zellen in vitro benötigen embryonale Mausfibroblasten (MEF, auch als Feederzellen bezeichnet) und Medien mit tierischen Bestandteilen, wie z. B. fötales Rinderserum (FBS) oder Serumersatz, um die Zellen in einem undifferenzierten Zustand zu erhalten und um die Karyotypstabilität zu steigern. Der Einsatz von tierischen Materialien erhöht das Risiko der Übertragung von Pathogenen auf die hES-Zellen und das Risiko einer Transplantatabstoßung von somatischen Zellen, die aus den hES Zellen gewonnen werden. Ebenso gibt es zusätzliche Einschränkungen bei konventionellen Feederzellen, wie z. B. ihre kurze Lebensdauer. Diese Probleme machen es erforderlich, xeno-freie Kultivierungsbedingungen zu etablieren, um die klinische Qualität von somatischen Zellen für eine mögliche Zelltherapie zu gewährleisten. Ebenso bewirkt das Passagieren der hES Zellen in Form von Clustern eine größere inkonsistent der Zellkulturbedingungen und infolgedessen ist für den Gewinn konsistenter Daten die Verwendung von Einzellen erforderlich. Um diese Probleme zu vermeiden, wurde im Rahmen dieser Arbeit als Hauptziel gesetzt, die Expansion und Differenzierung xeno-freier Einzelzellen, sowohl hES-Zellen als auch induziert pluripotenter Stammzellen (hiPSC), *in vitro* zu etablieren und zu optimieren, um ihre potentielle klinische Anwendung in Richtung Zelltherapie zu ermöglichen. Die oben erwähnte Hauptzielsetzung ist in die folgenden drei spezifischen Ziele unterteilt: 1) Xeno-freie Protokolle für die Einzelzellexpansion von hES-Zellen und hiPSC zu etablieren, 2) xeno-freie Protokolle für die kardiale Differenzierung von hES Zellen und hiPSC zu etablieren und 3) kardiogene kleine Moleküle zu identifizieren, welche die Differenzierung von hES Zellen und hiPSCs unter xeno-freien Bedingungen zu Kardiomyozyten fördern. Im Rahmen dieser Doktorarbeit wurden Protokolle für die xeno-freie Einzelzellexpansion und Differenzierung etabliert und optimiert.

Diese xeno-freien Kultivierungsprotokolle sind gleichwertig mit den Protokollen, die tierisches Matrigel und Feederzellen bzw. tierisches Serum verwenden. Diese Protokolle können verwendet werden, um in hohem Maße reproduzierbare Ergebnisse zu erzielen. Das etablierte Differenzierungsprotokoll benötigt keine Verwendung von tierischen END2 Zellen für die kardiale Differenzierung und ist infolgedessen vollkommen xeno-frei. Dieses Protokoll ist für eine Hochdurchsatz-Identifizierung von kardiogenen kleinen synthetischen Molekülen optimal geeignet. In diesem Zusammenhang wurde durch die Verwendung dieser Protokolle das kardiogene Potential von Verapamil und Cyclosporin nachgewiesen. Die molekulare Mechanismen der kardiogenen Effekte von Cyclosporin und Verapamil müssen noch weiter untersucht werden in Hinblick darauf, ob diese entweder auf der Ebene einer vermehrten Mesodermbildung und/oder auf der Ebene einer vermehrten Differenzierung von mesodermalen Zellen zu Kardiomyozyten stattfinden.

7. References

1. Aasen T, Raya A, Barrero MJ, Garreta E, Consiglio A, Gonzalez F, Vassena R, Bilic J,Pekarik V, Tiscornia G, Edel M, Boue S, Belmonte JC (2008). Efficient and rapid generation of induced pluripotent stem cells from human keratinocytes. Nat Biotechnol. 26(11): 1276-84

2. Abraham R, Schafer J, Rothe M, Bange J, Knyazev P, Ullrich A (2005). Identification of MMP-15 as an anti-apoptotic factor in cancer cells. J Biol Chem. 280(40): 34123-32

3. Amabile G, Meissner A (2009). Induced pluripotent stem cells: current progress and potential for regenerative medicine. Trends Mol Med. 15(2): 59-68

4. Brambrink T, Foreman R, Welstead GG, Lengner CJ, Wernig M, Suh H, Jaenisch R (2008). Sequential expression of pluripotency markers during direct reprogramming of mouse somatic cells. Cell Stem Cell. 2(2): 151-9

5. Brook FA, Gardner RL (1997). The origin and efficient derivation of embryonic stem cells in the mouse. Proc Natl Acad Sci U S A. 94(11): 5709-12

6. Charrier JB, Lapointe F, Le Douarin NM, Teillet MA (2001). Anti-apoptotic role of Sonic hedgehog protein at the early stages of nervous system organogenesis. Development. 128(20): 4011-20

7. Chen Y, Shi L, Zhang L, Li R, Liang J, Yu W, Sun L, Yang X, Wang Y, Zhang Y, Shang Y (2008). The molecular mechanism governing the oncogenic potential of SOX2 in breast cancer. J Biol Chem. 283(26): 17969-78

8. Chiou SH, Yu CC, Huang CY, Lin SC, Liu CJ, Tsai TH, Chou SH, Chien CS, Ku HH, Lo JF (2008). Positive correlations of Oct-4 and Nanog in oral cancer stem-like cells and high-grade oral squamous cell carcinoma. Clin Cancer Res. 14(13): 4085-95

9. de Lau LM, Breteler MM (2006). Epidemiology of Parkinson's disease. Lancet Neurol. 5(6): 525-35

10. Dimos JT, Rodolfa KT, Niakan KK, Weisenthal LM, Mitsumoto H, Chung W, Croft GF, Saphier G, Leibel R, Goland R, Wichterle H, Henderson CE, Eggan K (2008). Induced pluripotent stem cells generated from patients with ALS can be differentiated into motor neurons. Science. 321(5893): 1218-21

11. Doss MX, Koehler CI, Gissel C, Hescheler J, Sachinidis A (2004). Embryonic stem cells: a promising tool for cell replacement therapy. J Cell Mol Med. 8(4): 465-73

12. Doss MX, Sachinidis A, Hescheler J (2008). Human ES cell derived cardiomyocytes for cell replacement therapy: a current update. Chin J Physiol. 51(4): 226-9

13. Gardner RL (1999). Polarity in early mammalian development. Curr Opin Genet Dev. 9(4): 417-21

14. Hanna J, Wernig M, Markoulaki S, Sun CW, Meissner A, Cassady JP, Beard C, Brambrink T, Wu LC, Townes TM, Jaenisch R (2007). Treatment of sickle cell anemia mouse model with iPS cells generated from autologous skin. Science. 318(5858): 1920-3

15. Heng BC, Richards M, Shu Y, Gribbon P (2009). Induced pluripotent stem cells: a new tool for toxicology screening? Arch Toxicol.

16. Humphrey RK, Beattie GM, Lopez AD, Bucay N, King CC, Firpo MT, Rose-John S, Hayek A (2004). Maintenance of pluripotency in human embryonic stem cells is STAT3 independent. Stem Cells. 22(4): 522-30

17. Kaji K, Norrby K, Paca A, Mileikovsky M, Mohseni P, Woltjen K (2009). Virus-free induction of pluripotency and subsequent excision of reprogramming factors. Nature. 458(7239): 771-5

18. Knoepfler PS (2008). Why myc? An unexpected ingredient in the stem cell cocktail. Cell Stem Cell. 2(1): 18-21

19. Lapar DJ, Kron IL, Yang Z (2009). Stem cell therapy for ischemic heart disease: where are we? Curr Opin Organ Transplant. 14(1): 79-84

20. Leu CM, Wong FH, Chang C, Huang SF, Hu CP (2003). Interleukin-6 acts as an antiapoptotic factor in human esophageal carcinoma cells through the activation of both STAT3 and mitogen-activated protein kinase pathways. Oncogene. 22(49): 7809-18

21. Loh YH, Agarwal S, Park IH, Urbach A, Huo H, Heffner GC, Kim K, Miller JD, Ng K, Daley GQ (2009). Generation of induced pluripotent stem cells from human blood. Blood. 113(22): 5476-9

22. Mauritz C, Schwanke K, Reppel M, Neef S, Katsirntaki K, Maier LS, Nguemo F, Menke S, Haustein M, Hescheler J, Hasenfuss G, Martin U (2008). Generation of functional murine cardiac myocytes from induced pluripotent stem cells. Circulation. 118(5): 507-17

23. Ng LG, Mackay CR, Mackay F (2005). The BAFF/APRIL system: life beyond B lymphocytes. Mol Immunol. 42(7): 763-72

24. Okita K, Hong H, Takahashi K, Yamanaka S (2010). Generation of mouse-induced pluripotent stem cells with plasmid vectors. Nat Protoc. 5(3): 418-28

25. Okita K, Ichisaka T, Yamanaka S (2007). Generation of germline-competent induced pluripotent stem cells. Nature. 448(7151): 313-7

26. Okita K, Nakagawa M, Hyenjong H, Ichisaka T, Yamanaka S (2008). Generation of mouse induced pluripotent stem cells without viral vectors. Science. 322(5903): 949-53

27. Palma I, Pena RY, Contreras A, Ceballos-Reyes G, Coyote N, Erana L, Kofman-Alfaro S, Queipo G (2008). Participation of OCT3/4 and beta-catenin during dysgenetic gonadal malignant transformation. Cancer Lett. 263(2): 204-11

28. Park IH, Arora N, Huo H, Maherali N, Ahfeldt T, Shimamura A, Lensch MW, Cowan C, Hochedlinger K, Daley GQ (2008). Disease-specific induced pluripotent stem cells. Cell. 134(5): 877-86

29. Park SY, Kim EY, Jeon K, Cui XS, Lee WD, Kim NH, Park SP, Lim JH (2007). Survivin acts as anti-apoptotic factor during the development of bovine pre-implantation embryos. Mol Reprod Dev. 74(5): 582-90

30. Passananti C, Floridi A, Fanciulli M (2007). Che-1/AATF, a multivalent adaptor connecting transcriptional regulation, checkpoint control, and apoptosis. Biochem Cell Biol. 85(4): 477-83

31. Rubin R, Baserga R (1995). Insulin-like growth factor-I receptor. Its role in cell

proliferation, apoptosis, and tumorigenicity. Lab Invest. 73(3): 311-31

32. Santana A, Ensenat-Waser R, Arribas MI, Reig JA, Roche E (2006). Insulin-producing cells derived from stem cells: recent progress and future directions. J Cell Mol Med. 10(4): 866-83

33. Schuldiner M, Itskovitz-Eldor J, Benvenisty N (2003). Selective ablation of human embryonic stem cells expressing a "suicide" gene. Stem Cells. 21(3): 257-65

34. Shih CC, Forman SJ, Chu P, Slovak M (2007). Human embryonic stem cells are prone to generate primitive, undifferentiated tumors in engrafted human fetal tissues in severe combined immunodeficient mice. Stem Cells Dev. 16(6): 893-902

35. Smith AG (2001). Embryo-derived stem cells: of mice and men. Annu Rev Cell Dev Biol. 17(435-62

36. Soldner F, Hockemeyer D, Beard C, Gao Q, Bell GW, Cook EG, Hargus G, Blak A, Cooper O, Mitalipova M, Isacson O, Jaenisch R (2009). Parkinson's disease patient-derived induced pluripotent stem cells free of viral reprogramming factors. Cell. 136(5): 964-77

37. Stadtfeld M, Nagaya M, Utikal J, Weir G, Hochedlinger K (2008). Induced pluripotent stem cells generated without viral integration. Science. 322(5903): 945-9

38. Takahashi K, Yamanaka S (2006). Induction of pluripotent stem cells from mouse embryonic and adult fibroblast cultures by defined factors. Cell. 126(4): 663-76

39. Watabe T, Miyazono K (2009). Roles of TGF-beta family signaling in stem cell renewal and differentiation. Cell Res. 19(1): 103-15

40. Woltjen K, Michael IP, Mohseni P, Desai R, Mileikovsky M, Hamalainen R, Cowling R, Wang W, Liu P, Gertsenstein M, Kaji K, Sung HK, Nagy A (2009). piggyBac transposition reprograms fibroblasts to induced pluripotent stem cells. Nature. 458(7239): 766-70

41. Xu X, Chua CC, Gao J, Hamdy RC, Chua BH (2006). Humanin is a novel neuroprotective agent against stroke. Stroke. 37(10): 2613-9

42. Yamanaka S (2007). Strategies and new developments in the generation of patient-specific pluripotent stem cells. Cell Stem Cell. 1(1): 39-49

43. Yu J, Hu K, Smuga-Otto K, Tian S, Stewart R, Slukvin II, Thomson JA (2009). Human induced pluripotent stem cells free of vector and transgene sequences. Science. 324(5928): 797-801

44. Zanghi JA, Renner WA, Bailey JE, Fussenegger M (2000). The growth factor inhibitor suramin reduces apoptosis and cell aggregation in protein-free CHO cell batch cultures. Biotechnol Prog. 16(3): 319-25

45. Zhang D, Jiang W, Liu M, Sui X, Yin X, Chen S, Shi Y, Deng H (2009). Highly efficient differentiation of human ES cells and iPS cells into mature pancreatic insulin-producing cells. Cell Res. 19(4): 429-38

46. Zhongling F, Gang Z, Lei Y (2009). Neural stem cells and Alzheimer's disease: challenges and hope. Am J Alzheimers Dis Other Demen. 24(1): 52-7

8. Preliminary Publications

Gunaseeli I, Doss MX, Antzelevitch C, Hescheler J, Sachinidis A. Induced pluripotent stem cells as a model for accelerated patient-and disease-specific drug discovery. Curr Med Chem, 17:759-766, 2010.

Jesudoss I, Chen SH, Spitkovsky D, Winkler J, Pfannkuche K, Hescheler J, Sachinidis A. Combined Meeting of the 17th European-Society-of-Gene-and-Cell-Therapy/16th German-Society-for-Gene-Therapy/4th German-Society-for-Stem-Cell-Research, Date: NOV 21-25, 2009 Hannover GERMANY. HUMAN GENE THERAPY Volume: 20 Issue: 11 Pages: 1388-1388.

9. LEBENSLAUF

Name : Ignatius Gunaseeli Jesudoss

Addresse : AG. Prof. Agapios Sachinidis
Institute of Neurophysiology,
Robert Kock Strasse 39
50931 Cologne
Germany.

Heimataddresse : 3/373 Nehru Nager 2nd Street
Mani Nagar
T. Nallur-Post
Paramakudi-623707.
Tamil Nadu.
Indien.
Email: iseeli7677@yahoo.com

Geburtsdatum : 7. Juni 1982.

Staatsangehörigkeit : Indisch

Schulausbildung

1987-1992	Grundschule	Paramakudi, Indien
1992-1999	Gymnasium und Abitur	Paramakudi, Indien

Universität Studium

1999-2002	Bachelor of Science in Biochemie	Madurai Kamaraj Universität, Indien
2002-2004	Diplomarbeit in Biotechnologie	Bharadhidasan Universität, Indien.
2004-2006	PostGraduate Diplomin molekularer Zellbiologie und Stammzellentechnologie	Universität Madras, Indien.
2006-2007	Master der Philosophie in Biotechnologie	Universität Bharadhidasan,Indien

Forschung

2007-2010 Doktorand am Institut für Neurophysiologie, Universität Köln, Deutschland.

Köln, 01.04.2010. Unterschrift