Trends in Stem Cell Biology and Technology

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Editor

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To the memory of Dr. Saeid Kazemi Ashtiani, a wonderful colleague, a great stem cell biologist, and an inspirational advocate of stem cell research in Iran.

And

To my family:
My wife (Parvaneh), daughter (Fatemeh), father (Ali), mother (Fatemeh), brothers (Hassan, Abbas, Mohammad, Amir), and sister (Afsaneh)

Preface

Stem cells, characterized by the ability to both self-renew and to generate differentiated functional cell types, have been derived from the embryo and from various sources of the postnatal animals and human. The recent advances in stem cell research have led to a better understanding of self-renewal, maintenance, and differentiation of both embryonic and somatic stem cells. This has significantly increased our knowledge of cellular and developmental biology in general and will certainly continue to do so for a long time to come. Moreover, given their role in maintaining and replenishing tissues, stem cells represent a potential means of restoring tissue function and thereby treating the root cause of degenerative disease. Therefore, in parallel, we need to improve our cognizance of the challenges involved in applying stem cells in clinical settings. The current chapters highlight both of these aspects: that of understanding the "actual" and that of developing the "possible."

In recognition of the growing excitement and potential of stem cells as models for both the advancement of basic science and future clinical applications, I felt it timely to edit this book in which forefront investigators would provide new findings for the use of stem cells to study various lineages and tissue types and some applications. We are pleased to provide *Trends in Stem Cell Biology and Technology*, a broad-scaled series of cutting-edge chapters that have already been shown to have, or will soon have, tremendous utility with stem cells and their differentiated progeny. The authors have put together recent advances and perspectives in important fields of stem cell research: embryonic stem (ES) cells, somatic stem cells, and stem cell therapy, which deal with embryonic and somatic stem cells and their potential therapeutic applications.

Embryonic stem cells are pluripotent cells with the capacity to give rise to every somatic cell type. The nature, characteristics, and potentials of human ES cells are described in the article by Bongso and Fong. In addition, Eckardt and McLaughlin describe the generation of ES cells from gamete-derived uniparental embryos, which can be patient-derived and potentially histocompatible with the gamete donor. They also address evaluation of the integrity of the lines generated, an essential criterion in interpreting differentiation assays in vivo and in vitro. Also, Ragina and Cibelli explain the derivation of parthenogenetic embryonic stem (PGES) cells from the inner cell mass of parthenogenetic embryo at the blastocyst stage. These pluripotent stem cells offer an easily obtainable pool of stem cells that can be used as a source for derivation of autologous tissues, albeit limited to females

in reproductive age. PGES cells' derivation does not require destruction of a viable embryo and therefore bypasses the ethical debates surrounding the use of naturally fertilized embryos. Moreover, Zuccotti and coworkers summarize the advancement in nuclear reprogramming and in cell reprogramming by cell fusion, using amphibian eggs or egg extracts, with cell extracts, with synthetic molecules, or by induced expression of specific genes and production of induced pluripotent stem cells. In contrast, Mardanpour et al. describe general considerations regarding molecular and cellular aspects of reprogramming of germ cells at different developmental stages to stem cells compared with their counterpart, ES cells. Moreover, epigenetic modifications, such as covalent modifications of histones and DNA methylation, are extremely important control mechanisms for self-renewal, cell fate, and cloning which describe by Andollo et al. and Balbach et al. Production of genetically manipulated mice by genetic manipulation of mouse ES cells is one of the premier tools for the study of genetic diseases. Matthaei describes his methods to produce these animals that have proven to be highly reliable as well as give exceptionally high rates of germline transmission with all strains of ES cells that he has used. Moreover, in just the past few years amazing progress has been made in germ cells differentiation from stem cells in vitro, which is review by Marqués-Mari et al.

Several chapters summarize the current state of knowledge in the somatic stem cell field. De Rooij reviews recent developments in the field of spermatogonial stem cells (SSCs). These cells are important for male fertility and recently it has been shown that at least mouse SSC are able to transform into multipotent stem cells capable of differentiation into various other cell lineages. Moreover, Olive and coworkers describe recent experimental results, including data from their laboratory, regarding gene expression profile of the SSC population. The chapter focuses on both up- and down-regulated protein coding transcripts and several differentially expressed microRNAs, which are increasingly being implicated in stem cell functions, such as pluripotency. In their article, Abdallah et al. describe mesenchymal stem cells, which occur in bone marrow stroma and in the stroma of diverse organs. They can give rise to, for example, osteoblasts, adipocytes, and chondrocytes and are currently being introduced into the clinic for the treatment of a variety of diseases.

Stem cells and their application in therapeutic replacement strategies are described in six articles focusing on heart failure, deafness, diabetes, and corneal injury. Stamm and coworkers summarize the basic research background of cardiac regenerative medicine and give a critical appraisal of the current efforts to translate the experimental approaches into the clinical setting. Moreover, Saric et al. critically review the current literature on use of fully undifferentiated ES cells for cardiac repair, elaborate on the tumorigenic risk of ES cells and pluripotent cells in general, and summarize strategies for elimination of this threat as an important step toward translation of ES cell–based therapies to clinic. This discussion is also highly relevant for clinical applicability of newly developed autologous ES cell–like stem cells, so-called induced pluripotent stem (iPS) cells, which circumvent ethical and, to some extent, immunological concerns linked to the use of blastocyst-derived ES cells, but still possess high tumorigenic potential. Trachoo and Rivolta review several

protocols used to generate neural precursors from human ES cells, including initial attempts to establish otic placodal precursors. They discuss their potential application in the development of a new therapy for deafness.

Franceschini and coworkers describe recent experimental results, including data from their laboratory, regarding the first evidence that transplanted stem cell that migrate to the neurolfactory mucosa may contribute to neuroepithelium structure restoration with resumption of the sensorineural olfactory loss. Moreover, diabetes is a degenerative pathology that has different causes. Roche et al. summarize the key work that has been performed in the bioengineering of both ES cells and adult stem cells toward insulin-secreting cells to treat diabetes. The adult corneal epithelium is continuously regenerated from stem cells under both normal conditions as well as following injury and is located at the basal layer of the corneoscleral limbus. These stem cells simultaneously retain their capacity for self-renewal and maintain a constant cell number by giving rise to fast-dividing progenitor cells. Kolli and coworkers discuss corneal epithelial anatomy, corneal epithelial stem cell biology, and the application of this biology in the field of regenerative medicine.

Moreover, in an interesting review, Hosseinkhani and Hosseinkhani review the application of scaffolding materials together with stem cell technologies for applications in tissue regeneration. Conventional in vitro models to study differentiation of stem cells are freshly isolated cells grown in two-dimensional cultures. Clinical trails using in vitro stem cell culture can be expected only when the differentiated stem cells mimic the tissue regeneration in vivo. Therefore, the design of an in vitro three-dimensional model of biodegradable scaffolds that mimics the in vivo environment is needed to effectively study its application for regenerative medicine. Tissue engineered scaffolds have a significant effect on stem cells proliferation and differentiation. Moreover, Wolf and Mofrad describe the significance of processes that convert mechanical signals into a cascade of biochemical signals that affect the phenotype of stem cells, a process called cellular mechanotransduction. Mechanotransduction, in combination with other experimental techniques, may provide new insights into the operations that occur at the cellular level. Understanding cellular mechanotransduction can also prove useful in understanding the overall effect on biological systems resulting from a change in just a few small variables. To elucidate the particular roles that stem cells play in healing during the adult stages, a role for stem cells that is still poorly understood as compared to what is known about them in an embryonic environment, experimental approaches must combine both mechanical and biochemical observations.

Collectively, these chapters should prove a useful resource not only to those who are using or wish to use stem cells to study specific applications, but also to those interested in stem cell biology advances. We hope this book will also serve as a catalyst to spur others to use stem cells for both the fundamental understanding of stem cells and their potential utility.

I am extremely grateful to the contributors for their commitment, dedication, and promptness with submissions! I am also grateful to Dr. Hamid Gourabi, Dr. Abdolhossein Shahverdi, Dr. Ahmad Vosough Dizaj and Dr. Mohsen Gharanfoli, for having faith in and supporting me throughout this project. I wish also to

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Finally, I hope that the book will achieve the intent that I had originally imagined: that it will prove to be a book with something for both experts and novices alike, and that it will serve as a launching point for further developments in stem cells.

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Human Embryonic Stem Cells: Their Nature, Properties, and Uses

Ariff Bongso and Chui-Yee Fong

Abstract The ability to grow human embryos in vitro to the blastocyst stage via coculture or sequential culture media led to the isolation and growth of human embryonic stem cells (hESCs) from blastocysts left over from in vitro fertilization programs. These cells being pluripotent can be differentiated into almost all the tissues types of the human body and therefore offers promise in the treatment of a variety of incurable diseases by transplantation therapy. They also provide an ideal screening tool for potential drugs in the pharmaceutical industry and allow the study of early human development and infant cancers. Although all National Institutes of Health (NIH)–registered hESC lines are research grade, having been derived and propagated on xenosupports and with xenoproteins, clinical grade hESC lines derived and propagated in xenofree culture conditions and under current good manufacturing practices (cGMP) facilities are now available. hESCs have been differentiated in vitro into pancreatic islets, neurons, and cardiomyocytes, and transfer of such hESC-derived tissues into diseased animal models have shown successful engraftment. However, two hurdles are delaying hESC-derived cell therapy reaching human clinical trials: (1) possible immunorejection of hESC-derived tissues and (2) the concern of teratoma formation. To overcome immunorejection, attempts are being made to customize tissues for patients via nuclear transfer and other reprogramming methods. Recently, human skin fibroblasts were reprogrammed to the pluripotent embryonic state by transfection with four genes (induced pluripotent stem cells). This approach not only allows tissue customization but is also an embryo-free method that overcomes ethical sensitivities. The development of several hESC banks worldwide containing a diverse range of clinical grade hESC lines that could be HLA typed and tissue matched for treatment is also a practical approach to preventing immunorejection. Several approaches to eliminating teratoma formation from undifferentiated renegade hESCs residing in transferred hESC-derived tissues are in progress. It is hoped that this hurdle will be circumvented soon, then allowing the application of current successful animal validated transplantation studies in the human.

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Introduction

Certain lower animals possess remarkable regenerative qualities that have fascinated man for many years. Regeneration is a physiological process where lost body parts are replaced over time with no human intervention. The household gecko can drop its tail at will to protect itself from its predator and within days the remaining tissues at the base of the tail can organize itself to reproduce the missing body part ***(1)***. Skinks from the Outback of Australia drop their tails, and a barrage of tails are reproduced instead of one. If the head of a flatworm is removed, a completely new head will be formed, and if a flatworm is cut into ten pieces, each cut piece can produce a completely new flatworm. Interestingly, although the molecular machinery for such regenerative abilities is present in mammals, they have lost the regenerative powers in major organs except for the liver. However, nature has provided two tradeoffs for this loss of regenerative power: (1) efficient wound healing and (2) the presence of a very mysterious cell, the stem cell, which gets involved in repair during tissue injury. Stem cells behave as blank slates that can not only assist in immediate repair but can also differentiate into a variety of cell types, each with its own functions. It is known today that most tissue repair events in mammals are dedifferentiation-independent events resulting from activation of preexisting stem or progenitor cells ***(2)***.

Today the field of stem cell biology has gained tremendous importance and has drawn a lot of publicity, with several reports showing the promise of this science in the future cure of a variety of diseases by transplantation therapy. The successful translation of this science from bench to bedside will change the quality of life of millions of people worldwide who suffer from illnesses where current approaches in medicine have not been able to take full control. The field of human embryonic stem cell (hESC) biology unfortunately is fraught with many ethical controversies as human embryos need to be destroyed to derive such cells. Additionally, since the transplanted hESC-derived tissues originally come from donor embryos, there is concern of immunorejection, so customization of tissues to the sick patient by reprogramming the patient's own cells is necessary to circumvent this and this in itself involves the equally sensitive area of nuclear transfer (NT) or therapeutic cloning ***(3)***. However, rapid progress is being made in this field and hopefully such issues will be circumvented.

Definition of a Stem Cell

The term "stem cell" actually originated from botanical monographs where the word "stem" was used for cells in the apical meristem, which is responsible for the continued growth of plants ***(4)***. In mammals, given the vast variety of stem cells isolated from preimplantation embryos, the fetus, umbilical cord, and adult organs, it becomes necessary to provide a more general definition for the term "stem cell"

and a more specific definition based on the type of stem cell. In general, stem cells can be defined as specialized or undifferentiated cells that can self-renew and be differentiated into other cell types, each new cell type possessing a different function ***(5)***. The degree of differentiation of stem cells to various other tissue types varies with the different types of stem cells, and this phenomenon is referred to as plasticity. Plasticity can range from totipotency to pluripotency to multipotency to unipotency. Mammalian blastomeres from early cleaving embryos are considered totipotent as they have the potential to produce complete organisms, while embryonic stem cells are considered pluripotent as they can differentiate into almost all 210 tissue types of the mammalian body but cannot produce a whole individual. Multipotency is restricted to those mesenchymal stem cell types that can differentiate into a small variety of tissues, while unipotency is generally restricted to stem cell sources that can be differentiated only into one lineage ***(2)***.

More recently, hESCs have been defined more specifically as cells that must have the following properties: the ability to (1) self-renew, (2) differentiate into cells of all three primordial germ layers (ectoderm, mesoderm, and endoderm), and (3) pass through a full battery of stem cell characterization tests, such as (a) morphological characteristics, (b) surface marker antigens (e.g., SSEA-1, -3, -4; Tra-1-60, -80), (c) Oct-3 and -4, (d) alkaline phosphatase, (e) karyotype, (f) genomic markers for the three primordial germ layers, (f) telomerase, and (g) the production of teratomas in severely combined immunodeficient (SCID) mice. Such a definition becomes necessary for (1) registration of hESC lines on the National Institutes of Health (NIH) registry, (2) exchange of cell lines between institutions, and (3) the reliable differentiation to tissue types given the fact that several differences exist between derived hESC lines ***(6)***, which may be intrinsic, based on the quality of embryos used or due to different derivation protocols. In fact, transcriptome profiling has clearly illustrated several properties that are common to all hESCs at the molecular level, but certain gene differences do exist between some cell lines ***(7, 8)***. Such essential attributes of "stemness" have been proposed in detail by Ramalho-Santos et al. ***(9)***.

Classification of Stem Cells

Stem cells in the human can be classified into many types based on their source of origin. More recently, they have been classified based on the presence or absence of a battery of CD and embryonic stem cell (ESC) markers (Fig. 1). The male and female gonads contain stem cells referred to as spermatogonia and oogonia, respectively. Through their self-renewal and subsequent meiosis they are responsible in producing the cells of the germ line and eventually spermatozoa and oocytes. These two haploid gametes eventually fertilize to establish diploidy and produce the zygote. The zygote remains at the top of the hierarchical stem cell tree, being the most primitive cell, and the germ cells therefore possess the unique feature of developmental totipotency ***(10, 11)***. The zygote undergoes cleavage in the human through a period of 5–6 days, producing two to four blastomeres (two- to four-cell stage) on day 2, eight blastomeres (eight-cell stage) on day 3, fusing or completely fused blastomeres (compacting or compacted stage) on day 4, and blastocyst stages

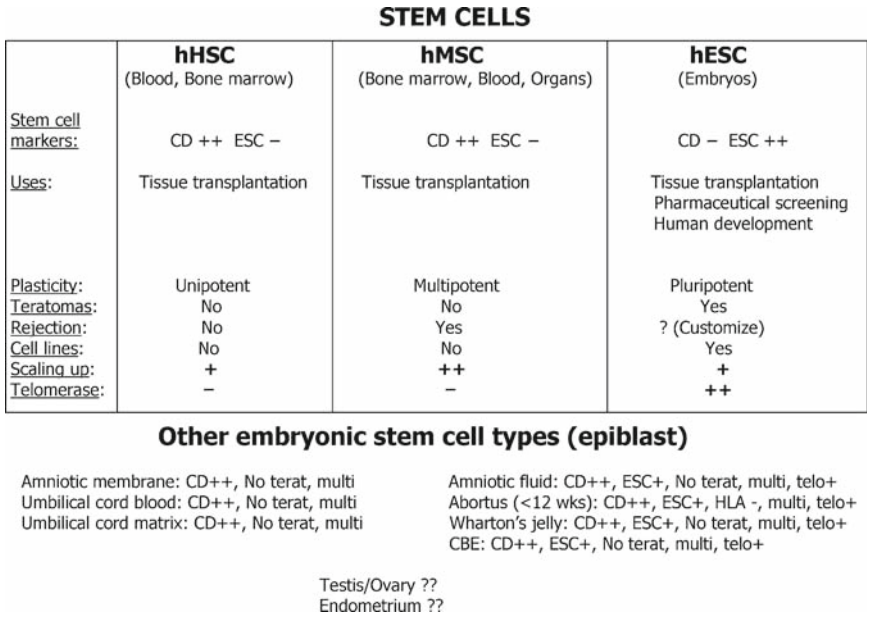

Fig. 1 Classification of stem cells according to characteristics, uses, and plasticity. Note (in *lower part* of figure) that besides embryonic stem cells several other stem cells of the reproductive system that possess characteristics in between adult and embryonic stem cells have been isolated, opening a new area of reproductive stem cell science. *Abbreviations: hHSC* human hematopoietic stem cells; *hMSC* human mesenchymal stem cells; *hESC* human embryonic stem cells

on days 5 and 6 ***(12)***. Each of the blastomeres is considered totipotent because it has the potential to produce a complete organism, as demonstrated when blastomeres are placed in the uterus of rabbits or mice. In the strictest sense of the definition of a stem cell, however, such blastomeres cannot be called stem cells because they do not self-renew.

The first bona fide stem cell to be produced in the mammal is in the inner cell mass (ICM) of the 5-day-old blastocyst. These cells self-renew and eventually produce two cell layers: the hypoblast and epiblast. The hypoblast generates the yolk sac, which degenerates in the human, and the epiblast produces the three primordial germ layers (ectoderm, mesoderm, and endoderm). These germ layers produce all the various tissues of the organism. Transmission electron microscopy studies have shown in the 9-day-old human embryo the transition of ICM to hESCs ***(13)***. Thus hESCs are considered pluripotent and not totipotent because they cannot produce complete human beings but have the potential to produce all the 210 tissues of the human body. During embryogenesis and fetal growth such embryonic stem cells that have not participated in organogenesis remain as adult stem cells in organs during adulthood. It can thus be hypothesized that the adult stem cells residing in specific organs are already differentiated cells, and their function is to be dedifferentiated and be recruited for repair of injury incurred by the specific organ. Unfortunately, such adult stem cells in the organs are few in number and inadequate

to complete repair with a subsequent breakdown of disease of that specific organ. It has been shown that fetal and adult stem cells could cross boundaries by transdifferentiating into other tissue types and are thus referred to as multipotent ***(14–17)***. Those stem cells that are unable to transdifferentiate but differentiate into one specific lineage are referred to as unipotent. An example of such unipotency is the differentiation of bone marrow hematopoietic stem cells to blood. Thus as embryogenesis shifts to organogenesis, infancy, and then adulthood, stem cell plasticity shifts from pluripotency to multipotency.

Recently there has been tremendous interest in the derivation of stem cells from other embryonic tissues that arise from the epiblast, such as the amniotic membrane, amniotic fluid, and umbilical cord ***(18, 19)***. The amniotic membrane, amniotic fluid, and some stem cell types in the umbilical cord possess both CD and some ESC markers, and although considered multipotent, some of them have certain properties in between pluripotency and multipotency and as such are useful cells for transplantation therapy ***(19)***. The umbilical cord, for example, has three types of stem cells: (1) in cord blood, (2) in the Wharton's jelly (Fig. 2), and (3) in the perivascular matrix around the umbilical blood vessels within the cord itself ***(20)***.

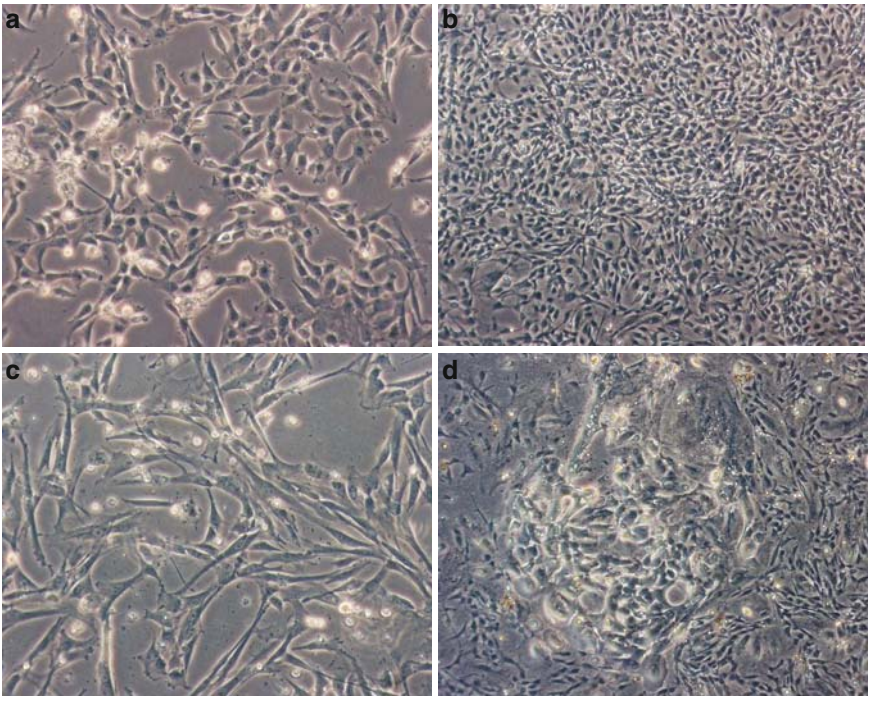

Fig. 2 Human Wharton's jelly stem cells (hWJSC). (**a**) Primary culture of hWJSC grown in the presence of human embryonic stem cell (HES) culture medium showing epitheliod-like cell growth. (**b**) Same culture maintaining epitheliod-like morphology when confluent after 7 days. (**c**) Primary culture of hWJSC grown in umbilical cord matrix stem cell (UCMSC) medium showing fibroblast-like morphology. (**d**) hWJSCs showing human embryonic stem cells (hESC)-like colony formation when grown in HES medium

Embryonic stem cells have the advantages of possessing pluripotent markers, producing increased levels of telomerase, and being coaxed into a whole battery of tissue types and thus remain as the hallmark of stem cell biology with the greatest potential for cell-based therapy. However, they have the disadvantages of potential teratoma production, their derived tissues have to be customized to patients to prevent immunorejection, and their numbers have to be scaled up in vitro for clinical application. Adult bone marrow stem cells and stem cells from the Wharton's jelly have the advantages of availability in large numbers and do not produce teratomas, but have the limitations of being multipotent or unipotent and yield low levels of telomerase.

Genuine hESCs have the following characteristics: (1) self-renewal in an undifferentiated state for very long periods of time with continued release of large amounts of telomerase, (2) maintenance of "stemness" or pluripotent markers, (3) teratoma formation in SCID mice that contains tissues from all three primordial germ layers, (4) maintenance of a normal stable karyotype, (5) clonality, (6) OCT-4, and other genomic (e.g., NANOG) expression, and (7) ability to produce chimeras when injected into blastocysts in the mouse model. Many of the multipotent stem cells from fetal, cord, and adult tissues that are usually positive for only CD markers are mesenchymal stem cells (MSCs).

Derivation and Propagation of hESCs

The ability to grow human embryos to the day-5 blastocyst stage (blastocyst culture) in in vitro fertilization (IVF) programs ***(21)*** set the stage for the first isolation of hESCs ***(22, 23)***. Thereafter, several methods of hESC derivation have been reported with success ***(24, 25)***. These include (1) the whole embryo method, (2) immunosurgery, and (3) mechanical separation of the ICM. In the whole embryo method ***(23)***, the zona pellucida of the blastocyst is first removed by enzymatic treatment with a protease (pronase). The zona-free blastocyst is then plated on mouse or human fibroblast feeder layers previously treated with mitomycin C or irradiated to stop their own growth. The culture medium (human embryonic stem cell [HES] medium) used is a mixture of Dulbecco's modified Eagle's (DMEM) medium, supplemented with fetal calf serum, human serum or a knockout (KO) serum supplemented with basic fibroblast growth factor (bFGF), mercaptoethanol and insulin–transferrin–selenium (ITS) supplement. After approximately 2 weeks, the ICM grows as a raised clump of cells on the feeder layer, while the peripheral trophectodermal (TE) cells spread out as a patch of large cells. The ICM clump is then carefully dissected out with fine needles, disaggregated into smaller clumps of cells, and the small clumps are plated on fresh feeder layers in the presence of HES medium. After about 7–8 days, each cluster forms a small colony of hESCs and each hESC has high nuclear-cytoplasmic ratios with prominent nucleoli. Once the colonies reach a reasonably sized diameter, they are exposed to a brief treatment of dispase for slight detachment from the feeder, then dissected into smaller pieces, and each

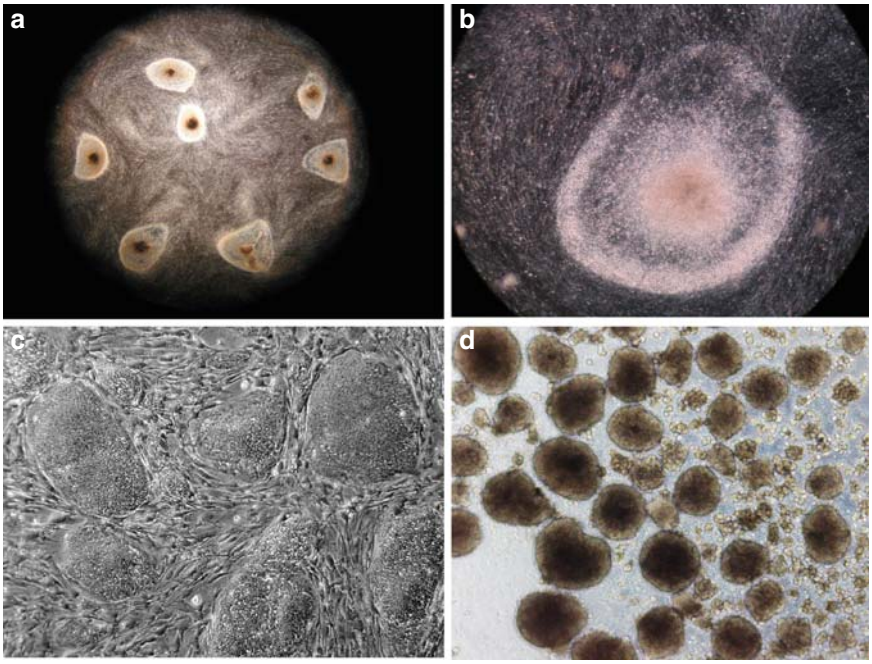

Fig. 3 (**a**) Stereo microphotograph showing hESC colonies growing on human feeder cells (fetal skin). Note *oblong thin flat* colonies with minimal differentiation. (**b**) Large human embryonic stem cells (hESC) colony growing on murine embryonic fibroblast (MEF) feeder. Note *circular* shaped colony with thick edges and slight differentiation at center and periphery of colony. (**c**) hESC colonies on MEFs grown by the enzymatic bulk culture method. Note many colonies of different sizes. (**d**) hESC embryoid bodies (hESC-EB) of different sizes

piece is then plated onto fresh feeders. Dissection is carefully carried out to avoid transfer of any differentiated hESCs present at the center and periphery of the colonies. This method is commonly referred to as the "cut and paste" method (Fig. 3a, b).

hESCs could also be derived from the zona-free blastocyst by first separating the ICM from the TE and culturing only the ICM. The ICM can be separated from the TE either mechanically with pointed needles or by immunosurgery. The immunosurgery approach is more efficient and reliable ***(24, 25)***. For immunosurgery, the zona is first removed with pronase and the zona-free blastocyst is exposed to anti-human antibodies in the presence of guinea pig complement and monitored under phase contrast optics to observe the gradual lysis of the TE cells keeping the ICM intact. The ICM is then washed in DMEM medium to remove the antibodies and complement, and then seeded onto fresh inactivated mitomycin-C murine embryonic fibroblast (MEFs) or human feeders in the presence of HES medium and incubated at 37°C in a 5% CO_2 atmosphere. The rest of the protocol is the same as the cut and paste method described above. hESC numbers can be scaled up to some extent using the enzymatic bulk culture method (Fig. 3c). In this method at the time of passaging the colonies and feeder cells are enzymatically treated and seeded

together onto fresh feeders. Several small, medium, and large colonies sprout up from clumps of hESCs that attach to the new feeder.

To avoid any risk of contamination to the hESC from the animal xenosupport system (MEFs) and xenoproteins (guinea-pig complement, porcine transferrin, bovine insulin), xenofree protocols using sterile human feeders and recombinant or human-based reagents have been reported for derivation and propagation of hESCs ***(26–28)***. Feeder-free protocols using Matrigel have recently replaced feeder cells for propagation of hESCs but not used for hESC derivation ***(29)***. Based on the xenofree protocol, the first six proprietary clinical grade hESC lines developed under current good manufacturing practices (cGMP) conditions were produced by ESI Singapore (http://www.escellinternational.com). Xenofree cryopreservation methods (vitrification or slow programmable freezing) using human-based freezing reagents have also been reported to allow storage of hESCs in closed embryo straws in the vapor phase of liquid nitrogen (−180°C) ***(30)***.

Donation of Embryos

Embryos that are used for derivation of hESCs are usually those in surplus that are donated by informed consent from IVF patients. Such patients have a choice of (1) donating their spare embryos to other childless couples, (2) disposal, or (3) donating for research with informed consent. Interestingly, some centers have also provided IVF patients a fourth choice of using their spare frozen embryos to derive and store hESC lines for their own use later on. This approach which may be ethically controversial is being practiced in some states in the USA (http://www.stemlifeline.com). It is important to note that a vast number of surplus IVF embryos are currently frozen in centers worldwide, and if consent can be sought to derive hESC lines from these embryos, repositories containing large numbers of hESC lines can be established on a diverse ethnic basis, which may be useful later for HLA tissue matching for transplantation therapy. Currently, there are only two major hESC line repositories: one based with the NIH in the United States and the Medical Research Council (MRC) bank in the United Kingdom. Tremendous differences appear to occur between the currently stored hESC lines in terms of gene differences and method of derivation ***(6)***, and this is all the more reason that many more hESC lines must be derived and stored. It has been estimated that about 150 hESC lines may give a reasonably good tissue match to avoid hESC-derived tissue rejection, although some workers claim that many more cell lines may be required ***(31)***.

Embryoid Body Formation

hESCs have the unique ability of producing embryoid bodies (EBs) (Fig. 3d). These are circular sphere-like structures that contain cells from all three primordial germ layers. EBs are usually produced by the hanging drop method, where hESC clusters

are discouraged from attaching to specially coated plastic culture plates in the presence of special ingredients in the culture medium, thus allowing them to round up to form the spherical EBs. EB production is usually the first step in attempting to differentiate hESC into desirable cell lineages for transplantation therapy. The spontaneous development of hESCs to EBs in vitro and then mechanical separation and enrichment of neuronal cells from the rest of the lineages within the EB without the need for the use of differentiating agents has been successfully carried out ***(25)***.

Applications of hESCs

hESCs have many applications in human medicine. The first is the production of hESC-derived tissues for transplantation therapy for the treatment of a variety of incurable diseases. A list of the potential diseases that may be treated with hESC-derived tissues is shown in Table 1. Future interaction of such tissues with scaffolds made from polymers may find their use in organ transplantation. After transplantation of the scaffolded tissue, the scaffold could be broken down in vivo with depolymerizing agents, allowing the tissue to take the place of the organ. This has opened a whole new field referred to as tissue engineering, involving both bioengineers and medical researchers. Second, hESCs and their derived tissues also serve as useful tools in the screening of potential drugs for the pharmaceutical industry. For example, a potentially new heart drug could be tested on a hESC-derived cardiomyocyte cell line in vitro. Currently, pharmaceutical companies use animal cell lines for drug testing, which gives tremendous variability in the results in response to different drugs. Third, hESCs can be used to study early human development (e.g., congenital anomalies) and the pathogenesis of infant cancers. Fourth, hESCs can serve as ideal vehicles for gene therapy.

Scientific Hurdles to hESC Application

Tremendous progress has been made thus far in the conversion of hESC into desirable cell lineages. Additionally, successful functional outcome has also been demonstrated when such hESC-derived tissues are transplanted into animal models.

Table 1 Some potential diseases treatable with human embryonic stem cells–derived tissues

Human embryonic stem cells–derived tissue	Disease
Cardiomyocytes	Myocardial infarction
Neuronal cells	Parkinson's, Alzheimer's, spinal cord injuries
Pancreatic islets	Diabetes
Keratinocytes	Burns, cosmetic surgery
Hepatocytes	Cirrhosis, hepatitis
Bone, cartilage	Cartilage injuries, osteoarthritis
Blood	Leukemias, thalassemias
Skeletal muscle	Muscular dystrophy
Retinal, corneal cells	Macular degeneration, corneal diseases

The transplanted tissues engraft successfully, enter the in vivo stem cell niche, integrate with the host microenvironment, and improve cell function of malformed organs or tissues ***(32–34)***. However, there are still some obstacles that are delaying taking hESC-derived tissues to human clinical trials. These obstacles are: (1) the fear that such transplanted tissues may be rejected as they originate from donor embryos, (2) the concern that any residual rogue undifferentiated pluripotent hESCs in the hESC-derived tissue (after the differentiation process) may produce teratomas at the transplanted site if the cells are injected directly into the site or in extratransplanted sites if the cells are administered systemically, and (3) the number of cells available for treatment are inadequate, and methods to scale up cell numbers rapidly are urgently required.

To overcome the issue of immunorejection several approaches are being investigated. Many laboratories are attempting to customize hESC-derived tissues to patients by NT. This involves electrofusing the somatic nucleus of the patient, requiring tissue customization with an enucleated donor human oocyte. The fused product undergoes cleavage to yield a blastocyst from which customized hESCs and hESC-derived tissues for the patient could be derived and propagated (Fig. 4). Thus far this approach has not been successful in the production of cloned human embryos but has recently

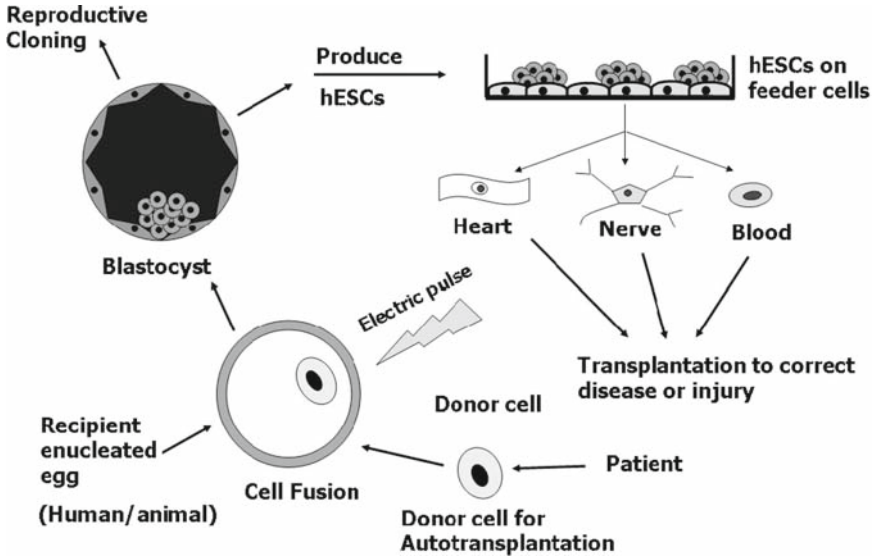

Fig. 4 Cartoon illustrating the customization of tissues by the method of nuclear transfer (NT) or therapeutic cloning. A somatic cell nucleus from the sick patient is inserted into an enucleated human or animal egg, the product then electrofused to produce a blastocyst. Such blastocysts may have two uses: (**a**) the production of a reproductive clone if placed in a surrogate mother or (**b**) the derivation of human embryonic stem cells (hESC) lines from which customized tissues for the patient could be generated because the genome of such hESC-derived tissues would be the same as the donor nucleus of the same patient that generated the blastocyst. Theoretically, when such customized tissues are transplanted back to the patient, immunorejection could be avoided

been successful for the nonhuman primate. Cattle, sheep, and other domestic animal embryos are routinely produced by the NT method, and the birth of live offspring from such NT embryos has helped increase the genetic merit of livestock industries. There have been recent claims that the use of rabbit or bovine oocytes to reprogram human somatic cells by the NT method may be more practical, given the paucity of human oocytes. Although ethically sensitive, the United Kingdom recently approved research on human–animal chimeras for this purpose. Several scientific hurdles need to be overcome before NT becomes a routine useful approach to customize hESC-derived tissues for sick patients (Table 2).

Other embryo-free and reprogramming methods have also been successful (Table 3). Recently, fetal and adult somatic skin fibroblasts were reprogrammed to the embryonic state by transfection with four pluripotent genes (induced pluripotent stem cells [iPSCs]) ***(35–37)***. The ensuing cell lines were confirmed as pluripotent and were similar to hESC lines derived from surplus embryos. In one of these reports, an adult patient's skin fibroblasts were reprogrammed to produce iPSCs ***(35)***, demonstrating that iPSC-derived tissues could thus be customized to a specific patient, preventing immunorejection. The iPSC approach has the added advantage of not requiring embryos to derive hESC lines, bypassing the ethical sensitivities of using surplus IVF embryos and creating embryos by NT ***(38)***.

It has also been shown that lymphocytes and other somatic cell types can be reprogrammed by whole intact hESCs, hESC karyoplasts, and hESC cytoplasts by chemical fusion. It was claimed that the hESC cytoplast has powerful reprogramming powers similar to the ooplasm of oocytes ("stembrids") ***(39–41)***. The production of parthenogenetic hESC lines that are pluripotent has also been successful for

Table 2 Problems with nuclear transfer

Parthenogenesis?
Efficiency: 0.57–6%
Faulty faithful epigenetic reprogramming
Eggs needed for each patient to customize human embryonic stem cells (hESCs)
Availability of human eggs?
Animal eggs useful, but unethical?
Disharmony between nuclear and mitochondrial genes
Meiotic spindle retention after egg enucleation: Implications!
Disarrayed mitotic spindles after nuclear transfer
Misaligned chromosomes after nuclear transfer
Aneuploid embryos after nuclear transfer
Stricter molecular requirements for mitotic spindle assembly in primate nuclear transfer

Table 3 Methods to derive human embryonic stem cells (hESC) without destroying embryos

From single blastomeres via blastomere biopsy for preimplantation genetic diagnosis (PGD)
Reprogramming adult fibroblasts to embryonic state by ectopic expression of transcription factors (POU5F1, SOX2, c-MYC, KLF4, LIN28)
Reprogramming adult fibroblasts with hESC karyoplast and cytoplasts using cell fusion
hESC chromosome transfer into arrested zygotes

Table 4 Alternative approaches to preventing rejection

Producing panels of xeno-free human embryonic stem cells (hESC) lines from surplus IVF embryos for tissue matching (~450 hESC lines)
Modifying the histocompatability locus: universal donor hESC lines
Encapsulating hESC derived cells with immunoprivileged membranes
hESCs being embryonic: will they be immunoprivileged?

Table 5 Prevention of teratoma formation

Purification and lineage selection
FACS (flow cytometric fluorescence-activated cell sorting)
MACS (magnetic-activated cell sorting)
Directed expression of suicide or apoptosis-controlling genes in graft tissues (ceramide analogues prevent hESC-induced teratoma formation)
Alginate encapsulated cell delivery
Selection against undifferentiated hESCs by cytotoxic antibodies
Separation of undifferentiated hESCs from hESC-derived cells using discontinuous density gradients

customizing hESC-derived tissues for the females only ***(42)***. However, the most logical and easiest approach to preventing immunorejection may be the development of several stem cell banks worldwide containing large numbers of fully characterized diverse clinical grade hESC lines derived from surplus IVF embryos that are HLA typed that can then be tissue matched for treatment (Table 4).

Several approaches are being attempted to eliminate the concern of teratoma production in transplanted hESC-derived tissues ***(43)*** (Table 5). These include methods to separate residual rogue undifferentiated hESCs present in differentiated tissues by flow cytometric fluorescence-activated cell sorting or magnetic-activated cell sorting (FACS/MACS), the use of density gradients, the selective induction of apoptosis in such residual hESCs, the encapsulation of hESC-derived tissues with immunoprivileged membranes at the time of delivery ***(44)***, and the administration of hESC conditioned medium (hESC-CM) or hESC extracts (membrane disrupted cells) rather than administration of whole intact hESC-derived cells ***(45)***. It is also definitely not known whether the injection of differentiated tissues containing some renegade undifferentiated hESCs will actually induce teratomas in specific transplanted sites besides the hind limb and kidney capsule where such teratomas have been demonstrated in animal models after injection of clusters of hESCs only.

Currently, hESC can be grown in bulk by the enzymatic culture method. However, cell numbers are still inadequate to provide for patient treatment as it is estimated that at least one to five million hESC-derived cells need to be administered at each site for successful functional outcome. Also, repeated injections may be necessary. As such, methods are being investigated (e.g., the use of bioreactors) in attempting to scale up numbers for future treatment. An alternate approach to cell-based therapy would be to only prime differentiation of hESCs in vitro along a specific lineage for about 36–48 h and then inject the primed hESC-derived cells rather than inject terminally differentiated tissues. It is hoped that the host's damaged organ itself or

its stem cell niche will trigger the continuation of the differentiation process of the transplanted hESC-derived cells in vivo along the injured organ's lineage, thus increasing cell numbers to help in repair. Additionally, it is also possible that the mere presence of the transplanted primed hESC-derived cells in the diseased organ will also help to mobilize important growth factors from the transplanted or extratransplanted sites to the injured site to assist in repair via a paracrine effect. The delivery of hESC-CM or hESC extracts may also provide improved functional outcome via similar mechanisms.

Strategies for Differentiation of hESCs Along Desirable Cell Pathways

Differentiation is a biological phenomenon where an unspecialized cell acquires the properties of a specialized cell. For example, in vivo, bone marrow stem cells differentiate into blood. Differentiation in vitro can either be spontaneous or controlled. In high density culture, hESCs differentiate spontaneously into cells of the three primordial germ layers with preference via a default pathway into neuronal cells ***(25)***. The desired differentiated cell type can be mechanically separated, enriched, and a pure culture of that specific cell type obtained. Neuronal cells secreting dopamine and serotonin in vitro have been produced in this way ***(32, 46)***. Controlled differentiation can be achieved in three ways. The first way is the coculture of hESCs with companion cells (preferably fetal). The companion cells release certain factors that entice the hESCs to differentiate along a desired lineage. For example, hESCs have been cocultured in direct contact with visceral endodermal cells in vitro, and within 10–14 days the hESCs were differentiated into beating cardiomyocytes ***(33)***. Second, certain growth factors and biochemical agents can be added into the culture medium that bathes the hESCs, helping them to differentiate. For example, retinoic acid is well known to differentiate hESCs into neurons, and dimethylsulfoxide (DMSO) differentiates hESCs into bone ***(47)***. The third way is transfection of the hESCs with specific gene constructs that can induce differentiation along a desired lineage. The cardiomyosin gene, when transfected into murine embryonic stem cells (mESCs), can convert mESCs into functional cardiomyocytes ***(48)***. Either undifferentiated hESCs or hESC-derived EBs could be used for differentiation by the various methods outlined above.

Current State of the Art with Respect to Clinical Application of hESC-Derived Tissues

Tremendous progress has been made in the field of hESC biology, although the tissues produced by these cells have not been used to date clinically in patients. hESCs have been successful differentiated into cardiomyocytes in vitro using a