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Stem Cell Biotechnology for Cell Therapy

Dong-Hee Lee and Ha Won Kim

Department of Life Sciences, University of Seoul, 90 Jeonnong-Dong, Dongdaemun-Gu, Seoul 130-743, Korea

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Abstract – Cell therapy (CT) is a group of techniques to treat human disorders by transplantation of cells which have been processed and propagated independent of the living body. Blood transfusion and bone marrow transplant have been the primary examples of cell therapy. With introduction of stem cell (SC) technologies, however, CT is perceived as the next generation of biologies to treat human diseases such as cancer, neurological diseases, and heart disease. Despite potential of cell therapy, insufficient guidelines have been implemented concerning safety test and regulation of cell therapy. This review addresses the safety issues to be resolved for the cell therapy, especially SC therapy, to be successfully utilized for clinical practice. Adequate donor cell screening must preceed to ensure safety in cell therapy. In terms of SC culture, controlled, standardized practices and procedures should be established. Further molecular studies should be done on SC development and differentiation to enhance safety level in cell therapy. Finally, animal model must be further installed to evaluate toxicity, new concepts, and proliferative potential of SC including alternative feeder layer of animal cells.

Key words □ cell therapy, stem cell, pluripotent, multipotent, differentiation

INTRODUCTION

Cells are the basic building blocks of the living body. These tiny structures differentiate into leaves and roots in plants while bones, muscles, skin, and all of the internal organs in animals. Cells serve both a structural and a functional role in the body, performing very specific functions for the tissue or organ they consist of. They also hold many of the keys to how our bodies function. Different from plants, animal cells lose a totipotency at early stages during development. Cell therapy (CT) is defined as group of techniques, or technologies, substituting damaged tissues with healthy and functional ones by the transplantation of cells which have been processed and proliferated independent of the living body. CT offers an opportunity to treat many degenerative diseases caused by the damage or malfunction of specific cell types and the failure to replace or restore them. The first evidence of the CT is traced as back to 1912 when German physicians tried to treat cases of hypothyroidism with thyroid cells. Modern cell therapy, however, was practiced in the early 1930s by Paul Niehans, a Swiss physician who became known as the father of cell therapy.

*Corresponding author
Tel: +82-2-2210-2480, Fax: +82-2-2210-2888
E-mail: hwkim@uos.ac.kr

To date, a well-established form of CT is bone marrow transplantation, by which leukemia and various genetic disorders of blood cells are treated. Bone marrow holds blood-forming SCs that develop into a variety of functional cells: oxygen-supplying red blood cells, white blood cells in charge of immune system, and platelets helping cease bleeding (Armson, 2005; Teshima and Harada, 1997). CT using bone marrow SC also helps treat cancers, such as leukemia and lymphoma, and some non-cancerous conditions such as genetic immune disorders and aplastic anemia. Currently, researchers are also investigating the potential of bone marrow transplant to treat breast and ovarian cancers.

SCs seemingly possess a cure-all capacity to form any cell type (Fig. 1). Successful treatment of previously devastating disease seems viable when SCs are in clinical use (Aslam *et al.*, 2000). Even generic types of SC can be developed to treat human disorders including many types of cancer, neurological diseases, spinal cord injuries, and heart diseases. The ability to culture human stem cells (HSCs) long term and to control how such cells specialize into the different tissues of the body offers the possibility of major advances in healthcare. The use of human pluripotent SCs is highly controversial from ethical and safety points of view (Bahadur, 2004; Caufield *et al.*, 2004)). Indeed, much of the current research is focused on producing these cells from human embryos and cadaveric fetal tissue. SCs

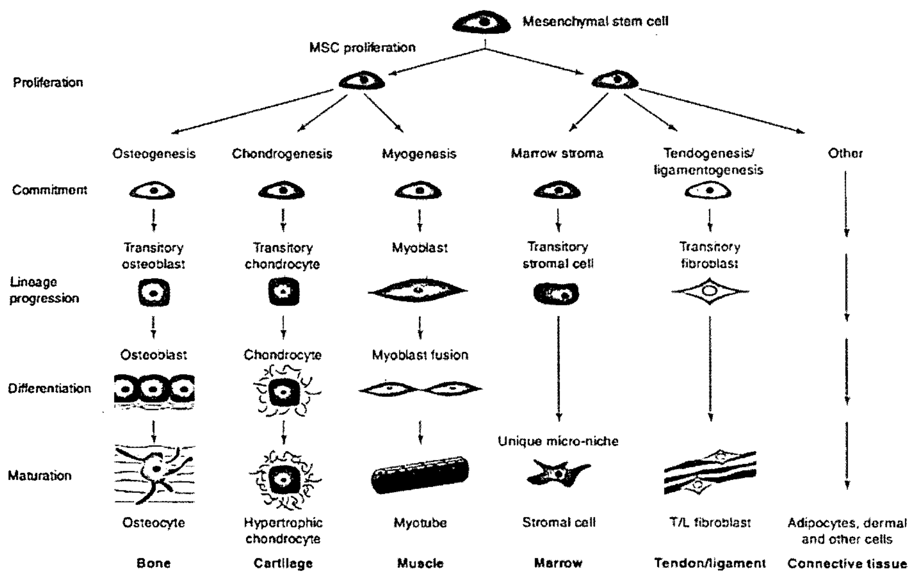

Fig. 1. Destinations of mesenchymal stem cell (Caplan and Bruder, 2001)

have been isolated and cultured in laboratories; still, a major research effort is required to develop cell lines which can generate replacement cells and tissues to treat many diseases.

With advancement of SC technology, the CT becomes very effective groundbreaking medical treatments. The medical use of SCs promises profound impact on our search for therapies to improve public health. Especially, human pluripotent SCs provide a valuable source of cells for transplantation and CT (Hingen *et al.*, 2002). This potential could allow application of SCs to treatment of a wide variety of human diseases. At present, patients with from major heart malfunction are rescued only by heart transplant. There are not enough donors to treat all patients. Often than not, treatment is performed by transplanting cells from another individual (allotransplantation) (Prosper *et al.*, 2004). Heart transplant occurs across genders (Fig. 2). As shown in the figure, the organ transplant is macro level of CT since heart massive cell replacement occurs after the heart transplant.

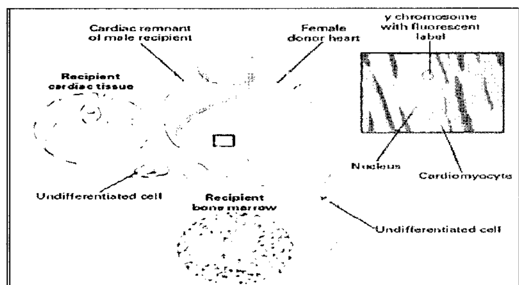

Fig. 2. Gradual cell replacement following heart transplant by endogeneous stem cells (Schwartz, 2002)

Stem cell biotechnologies to reinforce cell therapy

HSCs can give rise to many different types of cells, such as muscle cells, nerve cells, heart cells, blood cells and others. They raise the possibility, therefore, of revolutionary advances in healthcare. For example, SCs could be used to generate replacement cells and tissues to treat many diseases and conditions, including diabetes, multiple sclerosis, rheumatoid arthritis, spinal cord injury and skin conditions, including severe burns. The availability of SCs may also change the way that drugs are tested. New drugs could be tested for safety and efficacy on cultured liver or skin cells derived from SCs before being tested on humans (Cogle, 2003). Further research on SCs also promises to improve our understanding of the complexities of normal human development.

Scientists have been working on therapeutic methods to make SC treatments safe and efficient. The most revolutionary is the ability to generate tailor-made SCs from a patient's own cells, known as somatic cell nuclear transfer or as therapeutic cloning. The process of nuclear transplantation can be used to produce SCs. This method involves removing the genetic material from an unfertilized human egg and injecting new genetic materials. SCs lines derived by nuclear transplantation using specific patients' cells would also allow scientists to study the basic nature of a disease and help develop appropriate treatments. Upon a slight electric pulse following nuclear trans-

plant, the eggs began to divide as if they had been fertilized. The cells multiply and produce new stem-cell lines according the genetic information carried within the nucleus from the skin cells.

The advantage of using this technique to produce SCs is that the cells would have the same genetic makeup as the individual who donated the body cell. This would be beneficial for cellular therapies since the SCs become compatible with different types of body tissues; thus, they can used to repair damage from disorders such as spinal-cord injuries and burns.

Scientists have recently begun isolating and culturing human pluripotent SCs, which have an unlimited capacity to divide and the potential ability to develop into most of the specialized cells or tissues of the body. Working with embryonic stem cells (ESCs) faces a greater challenge. Many feel great hope and a sense of the possibilities for cures that SC research offers, while others are incensed by safety issues concerning SC preparation and actual effects on human body. Concerns that transplanted SCs may not work in conjunction with the tissue of the person receiving them also exist. Transplanted cells could "overgrow," as happens in many cancers. And a person's immune system may reject the cells as foreign, because cells derived from embryonic or fetal tissue are genetically different from mature tissue. SCs are among the most complex biologics to date. The United States Food and Drug Administration (USFDA) recommendations for SC-based product manufacture and characterization cover the experience gained in many CT clinical trials and the experience of the entire field of SC research (Deisseroth *et al.*, 1996). For SCs to evolve from basic scientific research into clinical use, it is essential to demonstrate that rigorously controlled in translating into fully differentiated cell for clinical use.

SC-based therapies will likely cover a variety of approaches including administration of undifferentiated SCs or transplantation of differentiated cells derived from SCs. There is growing interest worldwide in discovering and developing a permanent source of tissues which would be capable of generating any cell type and which would avoid the problem of transplant rejection. Indeed, there is need for increased understanding of the mechanisms regulating SC growth, cell fate determination, and differentiation. To bring stem-cell therapies into the pool of reliable medical treatment, more scientific efforts are made to eliminate the risk of inappropriate cell differentiation or transformation while ensuring that long-term benefits to patients free of adverse events (Schetten, 2002; Parens *et al.*, 2003). To this end, SCs must demonstrate integrity, uniformity, and reliability in establishing and maintaining human SC lines in culture and that standardized practices and procedures are established under scientific control.

Development of safety guidelines in research on stem cell therapy

To ensure safety in cell therapy, sources of donor cell must be carefully screened irrespective of SC origins: embryonic, fetal, or adult. The intent of donor screening is to convey the intended therapeutic effect for patients with disease-free products. Additional screening must be done on molecular genetic testing and pedigree evaluation when HSCs intended for transplantation from an allogenic donor when individuals other than the recipient, especially if the cells are obtained from a master cell bank contributing human embryonic stem or germ cells.

The purpose of pedigree analysis or genetic testing is to establish whether the HSCs in question are suitable for the upcoming clinical situation. For instance, SCs originating from a donor with a familial neural disease may be inappropriate when the CT aims at repairing damaged neural tissues. Detecting such a genetic abnormality in neuronal progenitor cells derived from an established embryonic germ cell line could block the use of those cells as a treatment for a number of neurodegenerative conditions. Similarly, the use of molecular genetic analysis could detect a mutation in the gene for diseases developing later in the life stage.

Further characterization of cell preparations intended for transplantation is critical to ensure the level of safety of HSCs for clinical use. Identifying a HSC population intended for clinical study requires sorting cells exhibiting the desired phenotype within the preparation against those that do not. This work is greatly challenging considering human embryonic stem and embryonic germ cells are capable of differentiating into all cell types, while adult HSCs, though generally more restricted in their plasticity, can generate all cell types that make up the tissue from which they originate.

Considering the complex biological properties of HSCs including their potential to differentiate along multiple lineages and give rise to a variety of cell types, the characterization of SC preparations will require a panel assessments. Mass and clonal culture assays can be utilized to determine how a single cell differentiates into neurons, cardiomyocytes, smooth muscle and cartilage. To characterize differentiation aspects of SCs, stage-specific markers are very important to distinguish stages of differentiation. Clonal cell lines that recapitulate normal development are also important to use these tools to define the

factors that regulate differentiation.

Homogeniety of stem cell

In evaluating the purity of a cellular preparation, rigorous and quantitative identification of cell types should be performed within a heterogeneous population of differentiating HSCs. This allows evaluation of the extent to which purity of a HSC preparation predicts efficacy after transplantation. In a mixed cell population following transplantation to provide dopaminergic neurons to the patients of Parkinson's Disease, tyrosine hydroxylase-expression could forbode the acquisition of relevant biologic activity in neural progenitor cells. Parameters that will prove useful in establishing identity include cell morphology (visual microscopic inspection of cells to assess their appearance), landmark genes unique to a particular cell type, and functional biochemical markers such as a tissue-specific enzymatic marker for producing neurotransmitters in nerve cells. In addition, unique cell-surface antigenic markers are very informative to identify specific differentiation such $CD34^+$ for hematopoietic SCs. Karyotyping may be a good visual marker to assess genetic stability of established human embryonic stem and embryonic germ cell lines maintained in culture for prolonged time periods. Development and standardization of DNA microarray and protein profiling analyses will significantly enhance the level of characterization for SC.

For cell therapies, homogenous populations consisting of a single cell type will be more useful than heterogeneous counterparts. Their uniformity is helpful to ensure maximum survival and functional capability. When cells that have not acquired fully differentiated functionality are to be transplanted, it may be appropriate to use substitute indicator that predict the acquisition of the intended biologic activity upon further differentiation. Differentiation of cultured SCs obtained from the brain leads to formation of all the cell types found within the nervous system (e.g., neurons, astrocytes, and oligodendrocytes). The interaction of various phenotypic cell types within a preparation of progenitor cells obtained after the controlled differentiation of cultured human ESCs is being actively investigated.

Pathogen-free bovine serum in culture medium

Controlling cultured SCs is undertaking regardless of adult or embryonic origins. In terms of adult SCs, they are not present in great numbers and they are hard to find and to extract for growth. A sufficient number of cells are hard to achieve for use in clinical studies involving transplantation. HSCs from virtually every source other than blood-derived hematopoietic SCs are maintained in tissue culture for some defined period of time. Culturing HSCs requires the use of formulated liquid media supplemented with growth factors and other chemical substances that promote cellular replication and govern the differentiation of the cultured HSCs. To cope with difficulty in grow batches of unspecialized cells, SC scientists need to better understand how these cells reproduce in the laboratory.

In culturing established HSC, one particular concern is how safe bovine serum is as an indispensable factor in culture media. Researchers are engaged in a vigorous effort to develop serum-free, chemically defined media that circumvent risks associated with the use of bovine serum. Due to the outbreak of bovine spongiform encephalopathy (BSE or mad cow disease), serum must be obtained from the cattle raised in BSE-free countries. Consumption of beef contaminated with the agent responsible for causing BSE has lead to the emergence of human Creutzfeldt-Jakob disease (hCJD). These diseases cause inevitably fatal outcomes in the persistent damage of brain tissue. Introduction of SCs infected with the BSE agent into a patient's nervous system by way of cellular-replacement therapies for neurological disorders is both negligent as the cell therapists and destructive to the patients' health.

Applications for marketing SCs will require information that demonstrates manufacturing control and product consistency as characterized by composition, size distribution, potency, and purity profiles across multiple batch of SC preparations. Slight deviation from standard protocol could even result in significant alterations in the intrinsic properties of the cells in culture. Considering dynamic nature of HSCs, altering the concentrations of supplemental growth factors and chemical substances, depending on vendors, may lead to changes in cell growth rate, expression of defining cell markers, and differentiation potential. In addition, such alterations could reflect the introduction of genetic mutations as a consequence of culture conditions used to promote expansion and to induce differentiation of the progenitor cell population. The initial seeding density of the cells, the frequency with which the culture medium is replenished, and the density cells are permitted to achieve before subdividing will all affect the characteristics of HSCs maintained. Alterations in SC properties caused by the use of non-standardized culture practices are likely to affect the nature and effectiveness of the cells once transplanted. Once the purity profile has been established for a population of HSCs generated using standardized procedures, any significant deviations should be avoided from normal biologic variations. Such deviation would deliver deleterious changes in terms of cellular properties in differentiation.

Pathogen transfer to human by mouse feeder cells

The culture of human embryonic stem and embryonic germ cells requires the use of mouse embryonic fibroblast feeder cells to keep the embryonic cells in a proliferating undifferentiated stage. Human embryonic stem and embryonic germ cells are planted directly onto a bed of irradiated mouse feeder cells (Testa, 2005). Transplanting onto HSC preparations derived from founder cells that have been in direct, intimate contact with non-human animal cells constitutes xeno transplantation. The use of organs, tissues, and cells derived from animals to treat human disease may exert a great effect to human health. The primary concern of xenotransplantation is the unintended transfer of animal viruses into humans.

To avoid potential transfer of toxic factors including virus from mice to humans, researchers are paying considerable attention to developing culture conditions that do not use mouse feeder cells. Scientists from Geron Corporation, a biotech company focusing on the development of ESC technology for treating disease, presented findings at a scientific conference demonstrating that human ESCs can be maintained without mouse feeder cells. Human ESCs planted on a commercially available basement membrane matrix in media conditioned by feeder cells show their proliferative potential and capacity to form all three embryonic germ layers (mesoderm, endoderm, and ectoderm). This strongly indicates that human ESCs can be cultured free of a mouse feeder cell layer and that many types of non-mouse feeder cells can be utilized in the near future.

Before clinical studies involving HSC transplantation, it is essential to demonstrate that HSC preparations possess relevant biological safety (Yim, 2005). A critical element of the safety is the transplantation of HSCs into animals to demonstrate that the therapy does what it is supposed to substantiate new concept and to assess toxicity. The bioassay using animal model provides a quantitative measurement of the potency of a cell preparation and ensures that cells destined for transplantation are actively growing. Differentiation into desired cells has been studied in many different species including *C. elegans*, Xenopus, and mouse using transplantation, cell culture and single cell injections. A major effort is being made to develop mice lacking expression of individual genes for differentiation and/or survival. The role of the transcription factor for SC fate determination by analyzing cells isolated from mice lacking the factor.

Application of animal model to human

Animal models of human disease are not perfect because most human diseases do not spontaneously occur in animals. HSCs must be transplanted into animal models resembling human disease. Transplantation of neural SCs should demonstrate measurable evidence of efficacy in models of neurodegenerative disease, such as diseases of Parkinson's, Huntington's, and Alzheimer's as well as spinal cord injury and stroke. In situations when focal genetic lesions are known to cause disease, the creation of transgenic mice in which the gene of interest is either eliminated or over-expressed results in disease models that are capable of faithfully reproducing human-disease-specific pathologies. Animal study should involve transplantation into immunocompromised animals of undifferentiated or partially differentiated ESCs, as well as adult SCs. Model animals organisms can be observed in real time with reasonable resolution thanks to advancements in noninvasive imaging technologies of MRI and PET. Animal studies thus do not require large numbers of individuals.

To validate SC transplantation to treat type I diabetes or heart disorder, these questions can be analyzed by taking advantage of known mutants and recently generated transgenic mice. After chemical, surgical, and immunologic methods are used to induce diabetes and simulate heart attacks, stroke, and hypertension, assays may be done to measure a biologic activity such as insulin release from pancreatic islet-like cells, glycogen storage by cells intended for regeneration of pancreatic tissue, or synchronous contraction in the case of SC-derived cardiomyocytes to be used for repairing damaged heart muscle. In an animal model of hepatic failure, improved heart function after transplantation of cardiomyocyte precursors should be observed. Normalization of blood insulin concentrations and mitigation of diabetic disease symptoms should result from the transplantation of pancreatic islet progenitors in a mouse model of diabetes.

Using animal-transplantation models, questions can be answered which is a better choice, embryonic vs. adult SCs, with respect to stability and strength. Questions at what point during differentiation does this risk become insignificant can be answered using the animal model study. The questions whether less-differentiated cells will be more effective than more-differentiated cells following transplantation could be better answered in the animal transplantation model. From the perspective of toxicology, the proliferative potential of undifferentiated human embryonic and embryonic germ cells evokes the greatest level of concern. A characteristic of human ESCs is their capacity to generate teratomas when transplanted into immunologically incompetent strains of mice. Undifferentiated ESCs are not considered as suitable for transplantation due to the risk of unregulated growth. Identifying the stage at which the risk for

tumor formation is minimized will depend on whether the process of SC differentiation occurs only in a forward direction or is reversible. Before clinical trials are begun in humans, the issue of unregulated growth potential and its relationship to SC differentiation must be evaluated in animal models. Using animal models, careful toxicology studies should be performed that are of the appropriate length of time.

Stem cell differentiation factors for clinical therapy

In order to enhance integrity, uniformity, and reliability of the SCs, mechanisms that regulate SC differentiation must be better understood at the molecular level. Plant version of SC research was done several decades ago; however, no major breakthrough has been made on how to regulate differentiation of economically important plants. This is because insufficient efforts were put to understand the basic molecular mechanism in the differentiation. Identifying factors that regulate SC differentiation is important since there are many prominent human disorders for which such knowledge may avoid unnecessary concern and risk at the stage of clinical therapy. Identifying informative and trophic molecules and their stage-specific roles in regulating normal development will provide important information on diagnosing neurological disorders as well as on suggesting possible therapeutic strategies.

To evaluate the roles of specific genes in the regulation of SC proliferation, interaction between multiple genetic factors should be studied in details. Scientists should funnel more efforts to elucidate the molecular and cellular interactions that dictate pluripotent cells to differentiate into cells restricted to a particular phenotype to trigger them to differentiate into the specific types of cells needed. Scientific efforts should be made on how the repertoire of responses of individual precursor cells becomes progressively restricted during development. These studies should cover the subjects as follow: External signals or factors that specify the phenotypic fate of neural precursor cells and scope of regulation achieved by environmental factors. Additional efforts are to be made to identify of the earliest phenotypic and antigenic changes that distinguish a restricted precursor cell from more pluripotent cells and development factors involved in known human disorders as well.

Under established culture conditions, SCs can be maintained as in an undifferentiated state or triggered to the initiation of the differentiation process by removal of the proliferation signals. In order to identify external differentiation signals that influence differentiation, joint efforts should be made focusing upon in vitro culture and clonal analysis experiments and in vivo expression studies. Evidence for anatomic and functional integration of transplanted HSCs should be assessed. HSCs destined for transplantation may be tagged with a marker, such as green fluorescent protein, that allows transplanted cells to be readily identified upon histological examination. A similar approach should be used to evaluate the migration of transplanted HSCs from the site of injection into adjacent and more distant tissues. The migration of transplanted HSCs to a nontarget site and subsequent differentiation into a tissue type that is inappropriate for that anatomic location could be problematic.

The more principles of differentiation at the molecular level are understood, the safer application of CT to other developmental stages and locations where it is better choice to treat human disorder. To identify cell-specific transcriptional factors in differentiation, degenerate PCR was performed at specific stage of development. Several novel genes are present in subsets of early precursor cells and these candidate genes are induced in and over-expressed in precursor cell lines to identify their role in development. Once established, these cell lines can be used to identify novel genes that may be involved in the process of differentiation for large-scale genomic screens. The interactions between identified factors and other transcriptional regulators can be studied to better understand SC development such as proteins with HLH, homeodomain, and zinc finger motifs

Concluding remarks

SC therapy is considered the next great frontier of medical treatment, becoming a leader at the forefront of the future of the science of medicine. SCs possess enormous potential to solve many biological and medical problems. SCs have two properties that distinguish them from other cells. When a SC divides, it creates two identical cells, the first of which is an exact replica of itself. Therefore, the populations of SCs remain intact and are considered to be immortal. SCs are initially cells of pure potential for differentiation into diverse end tissues. In other words, they have not committed to become any particular type of cell, such as a neuron or skin cell.

The science behind SC research, however, is often pushed into the back ground during these debates. This review examines safety issues raised by the potential of SCs derived from donated embryos, embryos created specifically for research purposes, fetal tissue and somatic cell nuclear transfer. This review also addresses safety issues raised by recent advances in biological and medical research on cell therapy. In order to make arrangements for examining and reporting on such issues

with regard to promoting understanding and discussion, this review deals with the potential guidelines by the appropriate regulatory systems.

ESCs are totipotent cells of the blastocyst that are capable of forming any cell type in the body. SC scientists examine ES cell cultures to determine whether normal embryonic development can be repeated in vitro. The use of SCs must meet criteria for biologic product regulation in most countries. Under circumstances, clinical trials of CT must be conducted according to good clinical practices and should be done within the context of an adequate clinical trial safety monitoring program. Clinical trial protocols should include well-defined eligibility criteria; anticipated endpoints; and a statistical plan for endpoint analysis. Current research on SC is restricted to isolating other lineages and determining if similar strategies can be used to isolate more differentiated precursors from human cell cultures.

Consistency in the SC culture practice will be important for the premarket approval. Together with Information on the organ procurement and transport, the manufacturing process should be open to public with the information on donor screening and testing issues. Thus, it has been recommended that a well-defined SC preparation method be chosen and supported by data. Adoption of a standard protocol will be necessary to allow for data collection and submission in support of FDA approval. It is unclear what impact protocol evolution will have on the FDA approval process, in terms of the need for additional data collection or reapplication for approval.

In order to develop specific safety guidance, subsequent protocol improvements must be incorporated later with regulatory review on safety issues. Specific guidelines include acceptance criteria for donor organs; SC isolation procedure standardization; pre-transplant assessment of SC function; key criteria for demonstrating allogeneic product comparability. Although startup costs are high and regulatory issues are demanding in SC industry, a substantial learning of SC safety is necessary for SC scientists to ensure consistent success. Safety requirements will play a major role in SC therapy in terms of institutional collaborations with transportation of tissues to distant preparation centers and return of SC preparations. If no safety guideline is implemented, inter-institutional collaboration is not highly encouraged; then, SC research and clinical application advance slower than general public expect.

Currently, SCs are prepared especially for treatment of type 1 diabetes and the CT is at the early phase of clinical study. In US, a recent amendment to the Medicare Prescription Drug, Improvement, and Modernization Act (MPDIM) 2003 requires

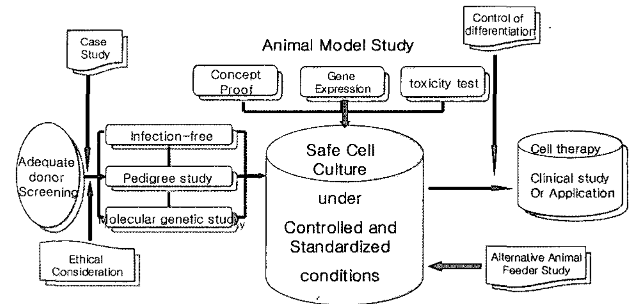

Fig. 3. Suggested pathway to enhance the level of safety in cell therapy.

that National Institute of Diabetes and Digestive and Kidney Diseases (NIDDK) conduct a clinical investigation of pancreatic islet transplantation including Medicare beneficiaries. This is a good indicator that SC therapy becomes a safe choice when treating type 1 diabetes considering Immunosuppressive drugs are already approved for other related indications in post-transplantation maintenance.

The safety guideline can evolve with advances in technology and through accumulating experience. Fig. 3 summarizes how to ensure a safe cell therapy. To formulate safety guideline regarding SC manufacturing and characterization, potential risks and benefits of each CT product and each proposed clinical trial must be assessed. This case-by-case approach, which takes into account the severity of the disease and the proposed patient population, permits flexibility in product manufacture and characterization when making its recommendations on safety issues. In terms of SC culture, controlled, standardized practices and procedures should be established. Further molecular studies should be done on SC development and differentiation. Finally, animal model must be further installed to evaluate toxicity, new concepts, and proliferative potential of SC as well as to study alternative feeding by animal cells.

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