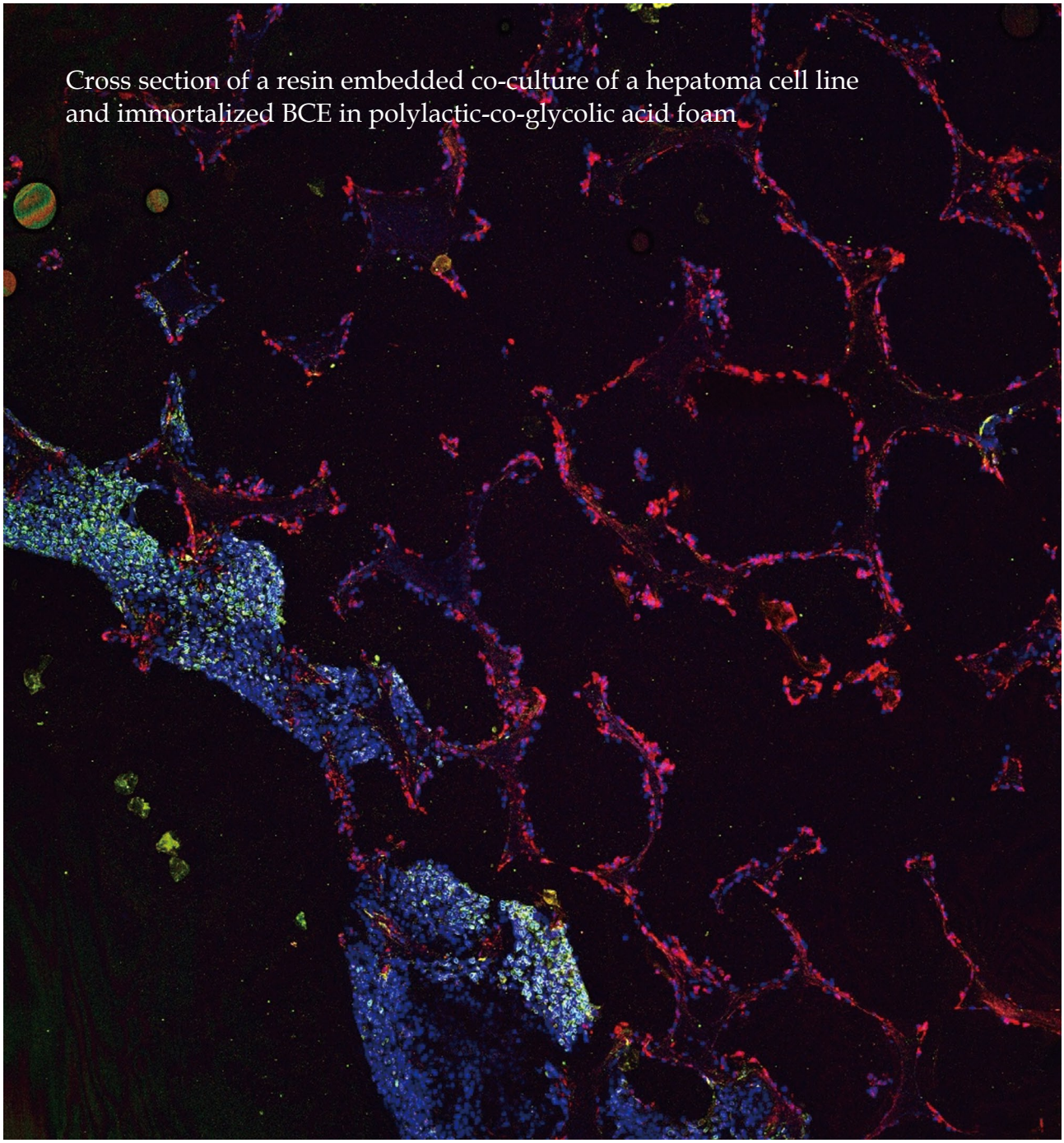
ISSN 1948-0210 (online)

World Journal of Stem Cells

World J Stem Cells 2009 December 31; 1(1): 1-66

A peer-reviewed, online, open-access journal of stem cells



Cross section of a resin embedded co-culture of a hepatoma cell line and immortalized BCE in polylactic-co-glycolic acid foam

WJSC

World Journal of Stem Cells

A peer-reviewed, online, open-access journal of stem cells

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2009-2013

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WJSC

World Journal of Stem Cells

Contents

Monthly Volume 1 Number 1 December 31, 2009

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World Journal of Stem Cells
Volume 1 **Number** 1 **December 31**, 2009

AIM AND SCOPE

World Journal of Stem Cells (*WJSC*, online ISSN 1948-0210, DOI: 10.4252), renamed from Stem Cells Review Letters, is a monthly open-access (OA) peer-reviewed journal supported by an editorial board consisting of 281 experts in stem cell research from 28 countries.

The major task of *WJSC* is to rapidly report original articles and comprehensive reviews on basic laboratory investigations of stem cells and their application in clinical care and treatment of patients. *WJSC* is designed to cover all aspects of stem cells, including embryonic stem cells, neural stem cells, hematopoietic stem cells, mesenchymal stem cells, tissue-specific stem cells, cancer stem cells, the stem cell niche, stem cell genomics and proteomics, and translational and clinical research. In a word, papers published in *WJSC* will cover the biology, culture, and differentiation of stem cells from all stages of development from germ cell to embryo and adult.

NAME OF JOURNAL
World Journal of Stem Cells

LAUNCH DATE
December 31, 2009

SPONSOR
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Room 903, Building D, Ocean International Center,
No. 62 Dongsihuan Zhonglu, Chaoyang District,
Beijing 100025, China
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Fax: 0086-10-8538-1893
E-mail: baishideng@wjgnet.com
http://www.wjgnet.com

ONLINE SUBSCRIPTION
One-Year Price 216.00 USD

PUBLICATION DATE
December 31, 2009

CSSN
ISSN 1948-0210 (online)

EDITORIAL OFFICE
Lai-Fu Li, Director
World Journal of Stem Cells
Room 903, Building D, Ocean International Center,
No. 62 Dongsihuan Zhonglu, Chaoyang District,
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Full instructions are available online at http://www.wjgnet.com/1948-0210/index.htm. If you do not have web access please contact the editorial office.

ONLINE SUBMISSION
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World Journal of Stem Cells

Online Submissions: http://www.wjgnet.com/1948-0210office
wjsc@wjgnet.com
doi:10.4252/wjsc.v1.i1.1

World J Stem Cells 2009 December 31; 1(1): 1-2
ISSN 1948-0210 (online)

EDITORIAL

What is the purpose of launching *World Journal of Stem Cells*?

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Received: July 21, 2009 Revised: August 13, 2009
Accepted: August 20, 2009
Published online: December 31, 2009

Abstract

The first issue of *World Journal of Stem Cells* (*WJSC*), whose preparatory work was initiated on September 27, 2008, will be published on December 31, 2009. The *WJSC* Editorial Board has now been established and consists of 281 distinguished experts from 28 countries. Our purpose of launching *WJSC* is to publish peer-reviewed, high-quality articles *via* an open-access online publishing model, thereby acting as a platform for communication between peers and the wider public, and maximizing the benefits to editorial board members, authors and readers.

Key words: Maximization of personal benefits; Editorial board members; Authors; Readers; Employees; *World Journal of Stem Cells*

Ma LS. What is the purpose of launching *World Journal of Stem Cells*? *World J Stem Cells* 2009; 1(1): 1-2 Available from: URL: http://www.wjgnet.com/1948-0210/full/v1/i1/1.htm DOI: http://dx.doi.org/10.4252/wjsc.v1.i1.1

INTRODUCTION

I am very pleased to announce that the first issue of *World Journal of Stem Cells* (*World J Stem Cells*, *WJSC*, online ISSN 1948-0210, DOI: 10.4252) will be published on December 31, 2009. Originally, the journal was titled Stem Cells Review Letters when preparatory work was initiated on September 27, 2008. The *WJSC* Editorial Board has now been established and consists of 281 distinguished experts from 28 countries.

The role of academic journals is to exhibit the scientific levels of a country, a university, a center, a department, and even a scientist, and build an important bridge for communication between scientists and the public. As we all know, the significance of the publication of scientific articles lies not only in disseminating and communicating innovative scientific achievements and academic views, as well as promoting the application of scientific achievements, but also in formally recognizing the "priority" and "copyright" of innovative achievements published, as well as evaluating research performance and academic levels. To realize these desired attributes of a journal and create a well-recognized journal, the following four types of personal benefits should be maximized.

MAXIMIZATION OF PERSONAL BENEFITS

The maximization of personal benefits refers to the pursuit of the maximum personal benefits in a well-considered optimal manner without violation of the laws, ethical rules and the benefits of others.

Maximization of the benefits of editorial board members

The primary task of editorial board members is to give a peer review of an unpublished scientific article *via* online office system to evaluate its innovativeness, scientific and practical values and determine whether it should be published or not. During peer review, editorial board

members can also obtain cutting-edge information in that field at first hand. As leaders in their field, they have priority to be invited to write articles and publish commentary articles. We will put peer reviewers' names and affiliations along with the article they reviewed in the journal to acknowledge their contribution.

Maximization of the benefits of authors

Since *WJSC* is an open-access journal, readers around the world can immediately download and read, free of charge, high-quality, peer-reviewed articles from *WJSC* official website, thereby realizing the goals and significance of the communication between authors and peers as well as public reading.

Maximization of the benefits of readers

Readers can read or use, free of charge, high-quality peer-reviewed articles without any limits, and cite the arguments, viewpoints, concepts, theories, methods, results, conclusion or facts and data of pertinent literature so as to validate the innovativeness, scientific and practical values of their own research achievements, thus ensuring that their articles have novel arguments or viewpoints, solid evidence and correct conclusion[1].

Maximization of the benefits of employees

It is an iron law that a first-class journal is unable to exist without first-class editors, and only first-class editors can create a first-class academic journal[2,3]. We insist on strengthening our team cultivation and construction so that every employee, in an open, fair and transparent environment, could contribute their wisdom to edit and publish high-quality articles, thereby realizing the maximization of the personal benefits of editorial board members, authors and readers, and yielding the greatest social and economic benefits.

CONTENTS OF PEER REVIEW

In order to guarantee the quality of articles published in the journal, *WJSC* usually invites three experts to comment on the submitted papers. The contents of peer review include: (1) whether the contents of the manuscript are of great importance and novelty; (2) whether the experiment is complete and described clearly; (3) whether the discussion and conclusion are justified; (4) whether the citations of references are necessary and reasonable; and (5) whether the presentation and use of tables and figures are correct and complete.

SCOPE

The major task of *WJSC* is to report rapidly original articles and comprehensive reviews on basic laboratory investigations of stem cells and their application in clinical care and treatment of patients. *WJSC* is designed to cover all aspects of stem cells, including: embryonic, neural, hematopoietic, mesenchymal, tissue-specific, and cancer stem cells; the stem cell niche; stem cell genomics and proteomics; and stem cell techniques and their application in clinical trials. Papers published in *WJSC* will cover the biology, culture, differentiation and application of stem cells from all stages of their development, from germ cell to embryo and adult.

COLUMNS

The columns in *WJSC* will include: (1) Editorial: to introduce and comment on major advances in rapidly developing areas and their importance; (2) Frontier: to review recent developments, comment on current research status in important fields, and propose directions for future research; (3) Topic Highlight: this column consists of three formats, including: (a) 10 invited review articles on a hot topic; (b) a commentary on common issues associated with this hot topic; and (c) a commentary on the 10 individual articles; (4) Observation: to update the development of old and new questions, highlight unsolved problems, and provide strategies for their resolution; (5) Guidelines for Basic Research: to provide guidelines for basic research; (6) Guidelines for Clinical Practice: to provide guidelines for clinical diagnosis and treatment; (7) Review: to review systemically the most representative progress and unsolved problems, comment on current research status, and make suggestions for future work; (8) Original Articles: to report original and innovative findings; (9) Brief Articles: to report briefly on novel and innovative findings; (10) Case Report: to report a rare or typical case; (11) Letters to the Editor: to discuss and reply to contributions published in *WJSC*, or to introduce and comment on a controversial issue of general interest; (12) Book Reviews: to introduce and comment on quality monographs; and (13) Guidelines: to introduce consensuses and guidelines reached by international and national academic authorities on basic research and clinical practice.

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3 **Xiao H**. First-class publications can not do without first-class editorial talents. *Keji Yu Chuban* 2008; **3**: 192

S- Editor Li LF **E- Editor** Lin YP

World Journal of Stem Cells

Online Submissions: http://www.wjgnet.com/1948-0210office
wjsc@wjgnet.com
doi:10.4252/wjsc.v1.i1.3

World J Stem Cells 2009 December 31; 1(1): 3-7
ISSN 1948-0210 (online)

EDITORIAL

Microenvironment at tissue injury, a key focus for efficient stem cell therapy: A discussion of mesenchymal stem cells

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Received: May 26, 2009 Revised: July 31, 2009
Accepted: August 7, 2009
Published online: December 31, 2009

Abstract

Stem cell therapy is not a new field, as indicated by the success of hematopoietic stem cell reconstitution for various hematological malignancies and immune-mediated disorders. In the case of tissue repair, the major issue is whether stem cells should be implanted, regardless of the type and degree of injury. Mesenchymal stem cells have thus far shown evidence of safety, based on numerous clinical trials, particularly for immune-mediated disorders. The premise behind these trials is to regulate the stimulatory immune responses negatively. To apply stem cells for other disorders, such as acute injuries caused by insults from surgical trauma and myocardial infarction, would require other scientific considerations. This does not imply that such injuries are not accompanied by immune responses. Indeed, acute injuries could accompany infiltration of immune cells to the sites of injuries. The implantation of stem cells within a milieu of inflammation will establish an immediate crosstalk among the stem cells, microenvironmental molecules, and resident and infiltrating immune cells. The responses at the microenvironment of tissue injury could affect distant and nearby organs. This editorial argues that the microenvironment of any tissue injury is a key consideration for effective stem cell therapy.

Key words: Stem cell therapy; Microenvironment; Mesenchymal stem cells; Immune responses

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Rameshwar P. Microenvironment at tissue injury, a key focus for efficient stem cell therapy: A discussion of mesenchymal stem cells. *World J Stem Cells* 2009; 1(1): 3-7 Available from: URL: http://www.wjgnet.com/1948-0210/full/v1/i1/3.htm DOI: http://dx.doi.org/10.4252/wjsc.v1.i1.3

INTRODUCTION

The delivery of stem cells, regardless of their source, is expected to be within, or surrounding regions of tissue injuries. This editorial discusses the mechanisms by which stem cells could interact with different molecules at and within areas of tissue injury. For the purpose of this review, the area of injury and molecules found within the zones of injuries are referred as microenvironments. The sources of molecules with regions of tissue damage are varied. For example, cytokines can be produced by cells within and around the damaged tissue; neurotransmitters can be released from damaged and/or intact nerve fibers as well as from infiltrating immune cells. While the sources of molecules are varied, the types of molecules belong to different families. These include, but are not limited to, peptides, cytokines, and extracellular matrix proteins. Interactions between cells and soluble molecules are two-way processes. While the stem cells respond by producing other factors, these factors stimulate the stem cells, through autocrine and paracrine mechanisms, to induce further changes in the stem cells. The mechanisms

of these interactions could be positive and/or negative to the injuries. The questions that are pertinent for stem cells therapy include the method by which stem cells should be implanted at the region of injury. Intuitively, one should consider if the implanted stem cells should be delivered to allow for modulation of the damaged microenvironment or vice versa. The answer to this key question will depend on the goal of the therapy. Another question is whether the aim of stem cell therapy is to attain protection that prevents further damage to the tissue or to replace damaged cells. This would require the stem cells to generate differentiated or specialized cells. In any event, the stem cells or specialized cells will establish crosstalk with cells and molecules within the microenvironment.

Future therapies will need to consider the degree of changes within the microenvironment because tissue injuries differ. Furthermore, the milieu would differ at zones away from the area of injury (Figure 1). If future research studies show that altered tissue microenvironments could attain effective therapy by stem cells, such information will be crucial to stem cell therapies. Adjuvant treatments will be required for drugs to achieve the desired changes. Drugs are available to target a variety of molecules and, when administered with stem cells, will enhance stem cell treatments. This editorial discusses general issues facing adult human mesenchymal stem cells (MSCs). These discussions could be extrapolated for any type of stem cell and for methods to repair damaged tissues.

The immune properties of adult human MSCs have been well studied. A review of the list of clinical trials with these stem cells (clinicaltrials.gov) shows their global application for various diseases. Based on the current large numbers of trials with MSCs, it appears that these stem cells are safe for use in humans. However, a clear statement on their safety awaits longitudinal follow-up.

ALLOGENEIC VARIATIONS IN KEY CONSIDERATIONS FOR CHOICE OF STEM CELL TYPE

A major consideration in stem cell therapy is the availability of "off the shelf" sources of stem cells. In theory, all stem cells could be readily available for transplantation. The issue is whether particular "off the shelf" stem cells would be rejected by immunological reactions. While such rejections are expected for allogeneic stem cells, thus far, allogeneic MSCs seem to behave contrary to this dogma. It is important to discern the differences between the immune suppressive property of a stem cell and its ability to elicit an immune response. In the case of immune suppression, stem cells negatively regulate a reaction of immune stimulation. If a stem cell initiates an immune response from a host, that stem cell is perceived as foreign to the host. The latter response will be initiated by differences in the major histocompatibility class Ⅱ and/or class Ⅰ (MHC Ⅱ or MHC Ⅰ) molecules on the stem cells. On the other hand, although less studied, a stem cell can also act as

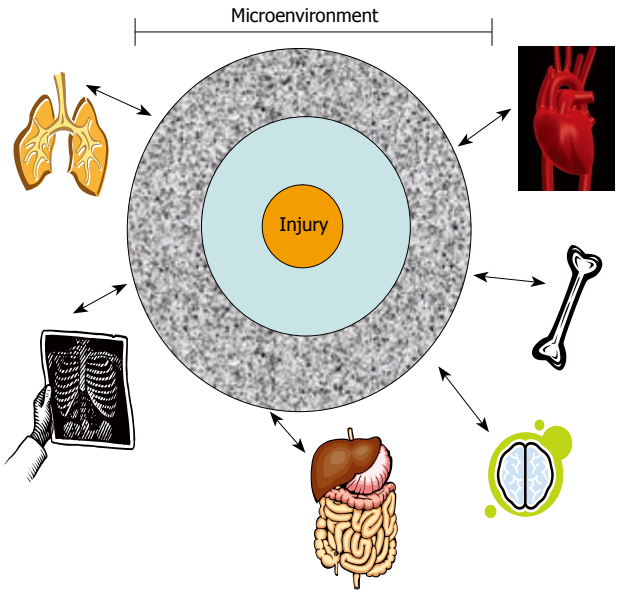

Figure 1 A representative focal point of tissue injury. This central point expands to zones of injury with each region showing varied degrees of tissue damage. The responses from the zones of injuries, through nerve fibers, soluble factors, or immune cells, could establish cross communications with other distant organs.

an immune cell and initiate inflammatory responses by autologous immune cells.

Autologous stem cells, or stem cells from fraternal twins, are ideal cases for avoiding rejection of the transplanted stem cells. However, probability of obtaining stem cells from fraternal twins would be low. Furthermore, in cases where the fraternal twin exists, if there is an injury that requires immune transplantation of stem cells, it would be problematic to wait for expansion of the stem cells. Immediate needs for stem cells include repair or protection of brain injuries and cardiac infarct. It could be argued that some forms of brain injuries could wait for the inflammation to subside, thereby providing health-care workers a few days for the use of autologous stem cells. There are two problems with this scenario; firstly, the stem cells might require weeks for expansion and characterization in a good manufacturing facility. Secondly, a particular organ is not mutually exclusive of the other; thus, injury to one organ could cause damage to another where the stem cells might be located (Figure 1). Thus, during injury, stem cells from a distant organ could be defective, thereby leaving autologous stem cells as the preferred source for therapy.

If organ-specific autologous stem cells are transiently damaged during injury, the time before these stem cells are ready for therapy would be significantly delayed. Additional studies are needed on the integrity of autologous stem cells at sites distant from injuries. Several reports show a brain-bone marrow connection, based on functional studies and anatomical evidence by nerve tracing analyses[1-3]. Furthermore, studies with surgical trauma patients reported dysfunctional bone marrow-derived hematopoietic stem cells (HSCs)[4]. The next section discusses the immune properties of MSCs and

argues for investigational studies on the bidirectional communication between these stem cells and inflammatory mediators within tissue microenvironments. It is imperative to dissect the responses of MSCs at areas of tissue injuries, especially because these stem cells are in various trials for immune-mediated disorders[5].

MSCs

MSCs are ubiquitously expressed, with the adult bone marrow as the major source[6-9]. MSCs differentiate into specialized cells, for example stroma, osteoblasts, adipocytes and chrondrocytes, *via* distinct lineages[10]. Stroma and osteoblasts are key supporting cells for hematopoietic stem cells functions, and form functional links between the two major bone marrow resident stem cells: hematopoietic and MSCs[6,11,12]. MSCs and HSCs are located at distinct regions of human bone marrow[13]. Hematopoietic stem cells prefer areas of low oxygen, close to the endosteum, whereas MSCs surround the blood vessels where oxygen levels are relatively high [6,14,15].

Blood vessels and nerve fibers generally follow each other into bone marrow. Therefore nerve endings would be in close contact with MSCs surrounding the abluminal blood vessels of bone marrow[6]. In fact, the anatomical literature shows nerve fibers forming synapse-like structures with reticular type cells of bone marrow[8]. MSCs have been referred to as reticular cells[6], indicating that MSCs are in contact with the nerve endings. In other reports, MSCs, are referred to as pericytes and form contacts with neurons[16]. The nerve contact with MSCs could be significant, based on the identification of neurotransmitter receptors on MSCs[17,18].

MSCs show potential for clinical application with evidence of tissue regeneration[19-23]. These stem cells could overcome the major obstacle associated with those of allogeneic sources. MSCs show unique immune properties, underscoring their clinical use for preventing graft *vs* host disease (GVHD)[24,25]. MSCs exhibit a veto property, indicating that they could thwart GVDH as third party cells[26,28]. Interestingly, the veto function of MSCs is specific for GVDH-type reactions because similar suppression has not been observed in responses to recall antigens[28].

MSCs express genes for different cytokines and their receptors and act as antigen presenting cells (APCs), underscoring the immune plasticity of MSCs as immune suppressor and immune enhancer cells[29-31]. The APC property occurs within a narrow window, followed by its reversion to an immune suppressor cell[29]. This bimodal property of MSCs is important to prevent exacerbated inflammation. It is suggested that this dual role of MSCs is responsible for homeostasis in bone marrow and prevents exacerbated hematopoietic suppression during inflammation[32-34].

The mechanisms by which MSCs exert immune suppression are complex. These functions involve reactions ranging from the production of cytokines with immune suppressor functions to the stimulation of regulatory T-cells and suppression of T cytotoxic activity[5,30,35]. The expression of MHC-Ⅱ, as well as the involvement of interferon-gamma (IFN-γ) in the immune function of MSCs, needs special attention[29,30]. A subset of MSCs expresses MHC-Ⅱ, which is decreased as the stem cells become specialized cells. This has been demonstrated in studies where the MSCs, transdifferentiated to neurons, show a gradual decrease in MHC-Ⅱ expression[36]. The addition of IFNγ to the MSC-derived neurons resulted in re-expression of MHC-Ⅱ. This indicates that in future stem cell therapy, the repair of organs with replaced cells could re-express MHC-Ⅱ. These patients will need to be followed for long-term tolerance. However, these findings underscore the need further investigation to determine methods of inducing anergy to the "foreign" MHC molecules.

PERSPECTIVE

Issues of allogeneic *vs* autologous stem cell delivery are key points when considering stem cell therapies. Another consideration for effective stem cell therapy is changes to stem cells by microenvironmental-induced responses by the implanted stem cells. Another major consideration is the possibility of tumor formation by the stem cells, even though the incidence of such an occurrence would be greater in embryonic stem cells. However, one cannot be certain that tumors would not be a problem for adult stem cells.

The maintenance of stem cells involves several genes, in particular those that are linked to cancer biology. During tissue injury, such as in traumatic brain injury, spinal cord injury, or myocardial infarction, the immune system will migrate towards the regions of insult. Once in the area, the immune cells will produce several soluble mediators, such as cytokines and chemokines. These two families of mediators can act locally through specific receptors on MSCs[37]. The resulting functions will depend on the concentrations of cytokines and the responses by the MSCs. The responses of the stem cells could be beneficial and assist in the repair process, or can be deleterious. The latter could occur if the reaction attracts additional immune cells to exacerbate inflammation, activates genes in the stem cells that can cause tumor formation, or activates genes in the stem cells to produce factors prematurely, which would be produced by specialized cells. On the other hand, exacerbated immune reactions could be protective. Thus, the biology of stem cells and the microenvironment of the area of tissue injury will determine the methods by which the therapies are developed.

The following are relevant questions when considering stem cell delivery within the context of varying microenvironments: (1) Should one type of stem cell serve as effective therapy for a particular type of tissue injury over another type? It is possible that one type of stem cell would be effective for a particular repair and another for a distinct injury; (2) Should the particular

type of stem cell depend on the extent of tissue injury? That is, one type of stem cell might be better for acute injury and another for chronic injury; (3) Would the microenvironment influence the developmental stage at which stem cells are delivered? It is possible that the factors present would determine if effective repair could be caused by undifferentiated *vs* partly *vs* completely differentiated stem cells; and (4) Would translational science be more effective by partnership between academia and pharmaceuticals? The latter would have drugs readily available to combine with stem cells for therapies.

In addition to variations among regions of tissue injuries, the method by which the stem cells are delivered is also an issue. While bioengineering has been an intense area of investigation, other methods are also available. These types of questions would entail inter- and multi-disciplinary teams to deliver stem cells to the clinic. There are numerous clinical trials of stem cells; thus, it is time for the scientific community to determine if stem cells can be referred to as drugs. This will be finally determined by the pharmaceutical companies as medicine moves to include stem cell therapies, perhaps for all types of disorders.

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S- Editor Li LF **L- Editor** Stewart GJ **E- Editor** Lin YP

World Journal of Stem Cells

Online Submissions: http://www.wjgnet.com/1948-0210office
wjsc@wjgnet.com
doi:10.4252/wjsc.v1.i1.8

World J Stem Cells 2009 December 31; 1(1): 8-10
ISSN 1948-0210 (online)

EDITORIAL

Tumor initiating cells in pancreatic cancer: A critical view

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Received: August 26, 2009 Revised: November 11, 2009
Accepted: November 18, 2009
Published online: December 31, 2009

Abstract

Emerging evidence points to the existence of pancreatic cancer stem cells (CSC) as the culprit in the initiation, maintenance, metastasis, and treatment resistance of pancreatic cancer. The existence of such a cell population would have an important impact on the design of novel therapies against this devastating disease. However, no *in vivo* validation or rebuttal of the pancreatic CSC hypothesis exists. Major backlashes in the discussion on CSC are firstly, the confusion between the terms CSC and cell of origin of pancreatic ductal adenocarcinoma (PDAC), secondly the ambiguity of the cell of origin itself and thirdly, the fact that the CSC hypothesis is based on cell sorting and xenografting experiments; the latter of which often precludes solid conclusions because of the lack of a natural microenvironment and differences in drug delivery. Nonetheless, recent studies in other cancers partially support the CSC hypothesis by demonstrating a link between epithelial-to-mesenchymal transdifferentiation/transition (EMT) and CSC properties. Such a link is again open to dispute as EMT is a reversible process which is highly dependent on major oncogenic pathways in PDAC [e.g. K-Ras, transforming growth factor-β (TGF-β)] rather than on presumed cancer stem cell pathways. Hence, the available evidence does not robustly support the CSC concept in PDAC and a thorough validation of this hypothesis in well-defined genetically engineered mouse models of pancreatic cancer is required.

Key words: Pancreatic cancer; Cancer stem cell; Tumor initiating cells; Mouse models

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Kong B, Michalski CW, Kleeff J. Tumor initiating cells in pancreatic cancer: A critical view. *World J Stem Cells* 2009; 1(1): 8-10 Available from: URL: http://www.wjgnet.com/1948-0210/full/v1/i1/8.htm DOI: http://dx.doi.org/10.4252/wjsc.v1.i1.8

INTRODUCTION

Pancreatic ductal adenocarcinoma (PDAC) is one of the most lethal cancers of the gastrointestinal tract with less than an overall 5-year survival rate of 5%. It is well known that human tumors including PDAC display significant heterogeneity in their respective cell populations. Based on normal hierarchical tissue architecture, the cancer stem cell hypothesis (CSCs) has been developed which suggests that only a specific subset of cancer cells in each tumor is responsible for initiation, maintenance and metastasis[1]. Specifically, the concept implies that a small number of stem or tissue-specific progenitor cancer cells give rise to the terminally differentiated or more committed progeny constituting the bulk mass of cancer, while at the same time these cells also maintain a process of self-renewal[2]. Therefore, following the first characterization of CSCs in acute myeloid leukemia (AML), the identification of pancreatic cancer stem cells was also reported[3,4]. However, a validation of the hypothesis, especially in the context of pancreatic cancer, does not exist. Confusion also derives from imprecise use of the terms "cancer stem cells" and "cell of origin" in PDAC which implies that cancer cells derive from normal tissue stem cells. This is of particular importance

in the development of PDAC because the cell or the cellular compartment of origin (i.e. ductal, acinar, centroacinar, endocrine, stem cells) is a subject of controversy. Genetically engineered mouse models of pancreatic ductal adenocarcinoma have made use either of mainly embryonically active, pancreas-specific promoters such as pdx1 or p48 (with specificity towards the exocrine pancreatic cellular compartment) or of inducible constructs driven by, for example, elastase expression to allow for Cre-recombination. Because neither a ductal- nor a centroacinar-specific promoter has been available so far, the cell of origin of PDAC remains obscure. However, initial data from genetically engineered mouse models have suggested that centroacinar cells which are considered to be pancreatic progenitor cells may be the cell of origin of PDAC. In a seminal paper, it has been shown that de-regulation of key pathways (e.g. PI3-K) in centroacinar cells might have contributed to the initiation of mouse PDAC[5,6]. Recently, some reports have argued that adult acinar cells can actually be transformed into pancreatic intraepithelial neoplasia and also into invasive adenocarcinoma , in the presence or in the absence of (chronic) inflammation[7-9]. These papers demonstrate that at least a subset of adult, differentiated cells is readily transformable into (pre-) malignant cells, suggesting that, if existing, a "pancreatic cancer stem cell" is rather derived from a differentiated compartment than from undifferentiated pancreas stem cells.

PANCREATIC CANCER STEM CELLS

So far, the CSC theory in pancreatic cancer largely relies on studies from FACS cell sorting according to the expression of specific "stem-cell" markers (CD133 or CD44/CD24/ESA) followed by xenografting of these cells into immune-compromised mice. Using such a system, a recent study reported that the combined blockade of so-called pancreatic CSC self-renewal pathways and standard chemotherapy eliminated the presumed pancreatic CSCs and resulted in prolonged survival of the transplanted mice[10]. Methodologically, this may not be an appropriate system for testing the CSC theory mainly because xenotransplantation itself has a number of disadvantages. For example, it does not provide the kind of real microenvironment which is usually required for the growth of pancreatic cancer cells[11-14]. Furthermore, drug delivery in xenograft PDAC models has been shown to be completely different than in genetically engineered mouse models[15], underscoring the difficulties in interpreting these results. Thus, the scarcity of so-called "tumor initiating cells" (i.e. CD133+ or CD44+CD24+ESA+) in human PDAC might in fact reflect the scarcity of human tumor cells that can readily adapt to growth in a foreign (mouse) milieu. The non-transplantable human PDAC cells may simply lack critical features for obtaining stromal support in the foreign microenvironment, such as responsiveness to mouse cytokines or chemokines that attract the cells to a nurturing niche rather than "stem-cell" properties[16,17]. Indeed, results from mouse leukemia and lymphoma challenge the general applicability of the cancer stem cell hypothesis to solid tumors because a substantial proportion of cancer cells can seed tumors in syngeneic animals and no functionally distinct subpopulation is evident[18,19]. It has recently also been shown that randomly selected single cells derived from mouse lung or breast cancer cell lines were able to produce tumors after allografting into histocompatible mice[20]. Furthermore, CD133 which is employed as a marker for (pancreatic) CSC is also expressed by endothelial cell precursors which were shown to be capable of enhancing growth of transplanted human cancer cells[21] Thus, using CD133 as a marker to isolate pancreatic cancer stem cells always carries the risk of enriching such a cell population.

EPITHELIAL-TO-MESENCHYMAL TRANSDIFFERENTIATION IN PANCREATIC CANCER

Recent evidence suggests that epithelial-to-mesenchymal transdifferentiation/transition (EMT) in PDAC marks an aggressive and, due to the expression of markers of CSC, a more "cancer stem cell-like" phenotype[22-24]. Accordingly, it has also been shown that highly metastatic pancreatic CSCs loose expression of the epithelial cell marker cytokeratin[3] illustrating a potential link between EMT and the cancer stem cell hypothesis. However, such a link may rather be a side-effect than a true effect since EMT is a reversible process that can be induced by various stimuli [e.g. transforming growth factor (TGF-β)] in the tumor microenvironment[25]. It is likely that in the stepwise malignant transformation process, pancreatic cancer cells have gained an ability to adjust their differentiation status to given environmental influences. This hypothesis seems to be supported by a recent report showing that pancreatic cancers can be divided into *K-ras*-dependent and -independent tumors. A comparison of these two classes of cancer cells revealed a gene expression signature in *K-ras*-dependent cells that was associated with a well-differentiated epithelial phenotype[26]. However, no changes in CSC marker expression has been reported after induction of EMT in such *K-ras*-dependent pancreatic cancer cells. Thus, the CSC "population" may also be considered as a transient state of the parental cancer cells which again would argue against the central concept of the cancer stem cell hypothesis.

CONCLUSION

Though the determination of the validity of the pancreatic CSC hypothesis would have an important impact on the design of novel therapies, the available evidence does not robustly support the CSC concept in PDAC. Therefore, we suggest analyzing the concept in well-defined genetically engineered mouse models of pancreatic cancer with the sole aim of eradicating the hypothesized minor population of CSC. Such experiments would determine

whether ablation of this presumed population of tumor-initiating cells has an effect on the development and potentially also the progression of the tumor.

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S- Editor Li LF **L- Editor** Hughes D **E- Editor** Lin YP

WJSC World Journal of Stem Cells

Online Submissions: http://www.wjgnet.com/1948-0210office
wjsc@wjgnet.com
doi:10.4252/wjsc.v1.i1.11

World J Stem Cells 2009 December 31; 1(1): 11-21
ISSN 1948-0210 (online)

TOPIC HIGHLIGHT

Andras Dinnyes, DSc, Professor, *Series Editor*

Embryoid body formation from embryonic and induced pluripotent stem cells: Benefits of bioreactors

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Supported by Grants from EU FP6 ("MEDRAT"- LSHG-CT-2005-518240; "CLONET", MRTN-CT-2006-035468), EU FP7 ("PartnErS", PIAP-GA-2008-218205; "InduHeart", EU FP7-PEOPLE-IRG-2008-234390; "InduStem", PIAP-GA-2008-230675; "Plurisys", HEALTH-F4-2009-223485); NKFP_07_1-ES2HEART-HU, No. OM-00202-2007 and CHE-TRF senior scholarship, No. RTA 5080010. Rungarunlert S was supported by grant under the program Strategic Scholarships for Frontier Research Network for the Joint Ph.D., and Program Thai Doctoral degree from the Office of the Higher Education Commission, Thailand, No. CHE-PhD-SW-2005-100
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Received: December 1, 2009 Revised: December 14, 2009
Accepted: December 21, 2009
Published online: December 31, 2009

Abstract

Embryonic stem (ES) cells have the ability to differentiate into all germ layers, holding great promise not only for a model of early embryonic development but also for a robust cell source for cell-replacement therapies and for drug screening. Embryoid body (EB) formation from ES cells is a common method for producing different cell lineages for further applications. However, conventional techniques such as hanging drop or static suspension culture are either inherently incapable of large scale production or exhibit limited control over cell aggregation during EB formation and subsequent EB aggregation. For standardized mass EB production, a well defined scale-up platform is necessary. Recently, novel scenario methods of EB formation in hydrodynamic conditions created by bioreactor culture systems using stirred suspension systems (spinner flasks), rotating cell culture system and rotary orbital culture have allowed large-scale EB formation. Their use allows for continuous monitoring and control of the physical and chemical environment which is difficult to achieve by traditional methods. This review summarizes the current state of production of EBs derived from pluripotent cells in various culture systems. Furthermore, an overview of high quality EB formation strategies coupled with systems for *in vitro* differentiation into various cell types to be applied in cell replacement therapy is provided in this review. Recently, new insights in induced pluripotent stem (iPS) cell technology showed that differentiation and lineage commitment are not irreversible processes and this has opened new avenues in stem cell research. These cells are equivalent to ES cells in terms of both self-renewal and differentiation capacity. Hence, culture systems for expansion and differentiation of iPS cells can also apply methodologies developed with ES cells, although direct evidence of their use for iPS cells is still limited.

Key words: Embryoid body; Embryonic stem cells; Induced pluripotent stem cells; Bioreactors; Differentiation

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Rungarunlert S, Techakumphu M, Pirity MK, Dinnyes A. Embryoid body formation from embryonic and induced

pluripotent stem cells: Benefits of bioreactors. *World J Stem Cells* 2009; 1(1): 11-21 Available from: URL: http://www.wjgnet.com/1948-0210/full/v1/i1/11.htm DOI: http://dx.doi.org/10.4252/wjsc.v1.i1.11

INTRODUCTION

Embryonic stem (ES) cells are capable of unlimited self-renewal *in vitro* and differentiate into cells constituting all three somatic germ layers. ES cells were first isolated from the inner cell mass of mouse blastocyst stage embryos[1,2], subsequently, followed by the derivation of non-human primate and human ES cell lines[3,4]. Currently, an alternative method has derived pluripotent cells by retroviral transduction of a combination of four transcription factors, Oct4, Sox2, C-myc and Klf4 into somatic cells; known as "induced pluripotent stem (iPS) cells"[5,6]. These cells are equivalent with ES cells in terms of both self-renewal and differentiation capacity[7,8]. The unique ability of pluripotent cells to generate a vast range of different cells makes both ES and iPS cells suitable for various cell transplantation, tissue engineering and drug testing applications. Efficient and controlled means of directing ES or iPS cell differentiation is crucial for the development of cell replacement therapies[9,10].

To realise the therapeutic potential of ES cells, it is essential to regulate their differentiation in a reproducible manner. Differentiation of ES cells is performed in two main ways; either by direct differentiation from pluripotent cells or through the formation of cell aggregates in non-adherent spheroids, called embryoid bodies (EBs)[11,12]. The molecular and cellular morphogenic signals and events within EBs recapitulate numerous aspects of the embryo development and result in differentiation to cells of three embryonic germ layers (endoderm, mesoderm, and ectoderm lineages), similar to gastrulation of an epiblast-stage embryo *in vivo*[13]. The precise number and spatial coordination of the various cell-cell interactions involved in EB formation are considered to influence the course of ES cell differentiation and, as a result, the control of cell number, size of EBs and quality of EB formation are important step directed differentiation strategies[14,15].

Methods of inducing EB formation are based on preventing ES cells from attaching to the surfaces of culture vessels, thus allowing the suspended ES cells to aggregate and form EBs. Standard methods of achieving EBs are *via* hanging drop and in static suspension culture to allow small scale formation of aggregates. These culture systems maintain a balance between ES cell aggregation essential for EB formation and prevention of EB agglomeration[16]. Even though hanging drop method is commonly used to prepare uniform-sized EBs (see details below), this method has disadvantages in the mass preparation of EBs due to its labor-intensive procedure, which hinders the use of differentiated ES cells for therapeutic application[17]. Mass EB production is easier from static suspension culture in which ES cells are suspended in a static Petri-dish. One drawback of this method, however, is that the EBs often fuse together to form large aggregates. This has negative effects on cell proliferation and differentiation, as well as causing extensive cell death. Hence, these methods are restricted as far as industrial applications are concerned because of their complication and difficult manageability[18].

Recently, novel bioreactors for large-scale production of ES-derived cells have been developed. A bioreactor is often defined as a device in which biological processes (cell expansion, differentiation or tissue formation on biomaterial scaffolds) occur in a tightly controlled environment *in vitro,* including the exchange of oxygen, nutrients and metabolites[19]. There are several types of bioreactors. For example, stirred suspension cultures (spinner flasks) have been successfully employed in some studies of mass scale production of ES-derived cells[20,21]. Conventional stirrer vessels may have the disadvantage of generating shear forces and, although manageable, these forces still can damage the cells[22]. Another bioreactor that allows agglomeration-free EB formation is the rotating cell culture system (RCCS) developed by the US National Aeronautics and Space Administration (NASA). This system is characterized by EB immobility in space, due to an extremely low fluid shear stress and oxygenation by diffusion[23]. EBs produced by bioreactors were more uniform in size and had less necrotic centers in comparison to static suspension culture. Furthermore, bioreactors can be also used for culturing iPS cells, which is expected to become a main further application of mass EB production in the near future. This review is focused on EB production in different systems, provides data on a number of existing bioreactors in comparison to conventional methods (hanging drop and static suspension culture) and describes differentiation of end-product EBs towards specific lineages.

METHODS FOR CULTURING EMBRYOID BODIES

At the present time, no universally accepted standard exists for measurement of EB formation although characteristics such as EB size, shape, homogeneity and the quality of EB formation, ratio of apoptotic and viable cell are typically used as benchmarks for evaluation[24]. There are several methods to generate EB formation, as schematically shown in Figure 1. A summary of all of the important methods (described below) is presented in Table 1. Traditionally the most common EB culture methods, such as hanging drop method and static suspension culture, were used for inducing differentiation.

Hanging drop method

The hanging drop method (Figure 1A) provides uniform sizes of EBs by dispensing equal numbers of ES cells in physically separated droplets of media suspended from the lid of a Petri-dish. This method offers a similar environment for forming individual EBs within each drop *via*

Table 1 Overview of current *in vitro* cell culture systems for production of EBs and other cell types

Cell culture methods	Benefit						Detriment						Propose	Yield	Note	Ref.
	a	b	c	d	e	f	g	h	i	j	k	l				
1 Hanging drop method	x							x	x	x			Differentiation into three germ layers	ND	Using mES cells	[26]
2 Static suspension culture					x		x						Differentiation into three germ layers and neural lineage	ND	Using mES cells	[30,31]
3 Entrapment of ES cells (methylcellulose)	x							x	x	x			Differentiation into hematopoietic lineage	ND	Using mES cells	[26]
4 Multiwell/ microfabrication																
4.1 Round bottomed, low attachment, 96 well plate	x				x			x					Differentiation into cardiac and neural lineage	94% of wells have a single EB with diameter of 415 microns	Using polyvinyl carbonate PCR plate without coating reagents	[37]
4.2 Low adherence, 96 well plate coated with MPC or CS	x				x			x					Differentiation into cardiac lineage	EB formed MPC and CS was increased cardiac differentiation	Using mES cells	[38]
4.3 Round bottomed, low attachment, 96 well plate	x				x			x					Differentiation into hematopoietic lineage	Single EBs were achieved from PC surface but not from PS surface	Comparison of EB formation derived various type of 96 well plate; PS and PS coated with MPC	[39]
4.4 Round bottomed, low attachment, 96 well plate polyvinyl carbonate PCR plate	x				x			x					Differentiation into cardiac lineage	Single EB achieved from PS coated with MPC was near 100%	Comparison of EB formation derived various type of 96 well plate; PS and PS coated with MPC	[40]
4.5 Round bottomed, low attachment, 96 well plate	x				x			x					Differentiation into hematopoietic lineage	Differentiation was achieved with blood cells formed in 90% of EBs	Force aggregation by using centrifugation; Using hES cells	[41]
4.6 V bottomed, 96 well plate	x				x			x					Differentiation into cardiac lineage	> 90% EB formation was achieved from this method	Force aggregation by using centrifugation; Using hES cells	[43]
5 Bioreactor																
5.1 A 2-L controlled spinner flask		x	x		x	x	x				x	x	Differentiation into cardiac lineage	4.6×10^9 of cardiomyocytes were produced in a single run	Using MHC-neo ES cells	[53]
5.2 Stirred		x	x	x	x	x	x				x	x	Expansion and differentiation into three germ layers	ES cells went through 13 passages over the same 28 d exhibiting higher pluripotency	Comparison of stirred and static suspension culture	[48]
5.3 Stirred		x	x		x	x	x				x	x	Differentiation into vascular lineage	ND	ND	[28]
5.4 Stirred		x	x	x	x	x	x				x	x	Expansion and differentiation into neural lineage	10 fold increase towards neural differentiation	Using hEC cells	[54]
5.5 Stirred		x	x	x	x	x	x				x	x	Expansion and differentiation into osteogenic lineage	10 fold of calcium per total grams of protein increase over the control culture	Comparison of stirred and static suspension culture; Transplantation	[75]
5.6 Stirred		x	x		x	x	x				x	x	Differentiation into hepatic lineage	No significant difference in the specific albumin productivity of EB derived from different groups	Comparison of stirred suspension culture and hanging drop	[81]
5.7 Stirred + encapsulation (HA and dextran)	x	x	x	x	x	x					x	x	Expansion and differentiation into three germ layers	Dextran can induce EB formation from ES cells	Using mES cells	[31]
5.8 Stirred + encapsulation (agarose) + perfusion	x	x	x		x	x					x	x	Differentiation into cardiac lineage	The cardiomyocytes production in encapsulated culture was higher than without encapsulation	Using MHC-neo ES cells; Comparison of O_2 tension	[72]
5.9 Two type of stirred, STLV and static suspension culture		x	x		x		x				x	x	Differentiation into cardiac lineage	EB formed GBI resulted in high EB yield with homogenous in size	Comparison of hydrodynamic condition (shear force)	[65]
5.10 RCCS (STLV and HARV)	x	x	x		x	x					x		Differentiation into three germ layers	3 fold enhancement in generation of EBs compared to static culture	Comparison of different type of bioreactors and suspension culture; Using hES cells	[23]
5.11 STLV	x	x	x		x	x					x		Differentiation into cardiac lineage	> 90% of the NTEBs generated beating area	Comparison of STLV and static suspension	[62]
5.12 HARV+ encapsulation (alginate)	x	x	x		x	x					x		Differentiation into osteogenic lineage	ND	Using mES cells	[70]

	a	b	c	d	e	f	g	h	i	j	k	l				
5.13 HARV+ encapsulation (alginate) + biograss	x	x	x		x	x					x		Differentiation into osteogenic lineage	ND	Using 70s bioglass	[71]
5.14 Rotary suspension culture using an orbital rotary shaker		x	x		x	x	x					x	Differentiation into three germ layers	20-fold enhancement in the number of cells incorporated into primitive EBs in rotary *vs* static conditions was detected in the first 12 h	Comparison of rotation, static suspension and hanging drop	[64]
5.15 Orbital shaker + microsphere fibrification	x	x	x	x	x	x						x	Differentiation into three germ layers	Degradable PLGA microspheres releasing RA were incorporated within EBs and induced cystic formation earlier than in non microspheres	Degradable PLGA microspheres releasing RA were incorporated within EBs and induced cystic formation	[68]
5.16 Perfused and dialyzed STLV	x	x	x	x	x	x							Differentiation into neural lineage	Perfused STLV can decrease in expression of markers of undifferentiated stage and increase in expression of markers of differentiation, specifically focusing on the neural lineage	Comparison of perfused and dialyzed STLV, perfused STLV, non-perfused STLV and suspension culture	[73]

a: Homogeneity of EB; b: Scalable production of EB; c: Controlled monitoring; d: Integrated single step of culture (expansion and differentiation); e: Easy to manage; f: Flexible culture cells; g: Heterogeneity of EB; h: Small scale production of EB; i: Labor-intensive procedure; j: Difficult to manage; k: Requires a lot of medium; l: Shear force; ND: No available data; EB: Embryoid body; MPC: Methacryloyloxyethyl phosphorylcholine; ES: Embryonic stem; hES: Human embryonic stem cells; mES: Mouse embryonic stem cells; PCR: Polymerase chain reaction; MPC plate: 96-well polystyrene plate coated with 2-methacryloyloxyethyl phosphorylcholine; PS plate: Polystyrene plate; CS plate: A polystyrene plate coated with a type of glycosaminoglycan; HD: Hanging drop; hEC: Human embryonic carcinoma stem cells; MHC-neo: Myosin heavy chain-neomycin resistance; O_2: Oxygen; RCCS: Rotating cell culture system; HARV: A high aspect rotating vessel; STLV: A slow turning lateral vessel; NTEB: EB derived from nuclear transfer ES; PLGA: Poly(lactic-co-glycolic acid)/poly (L-lactic acid).

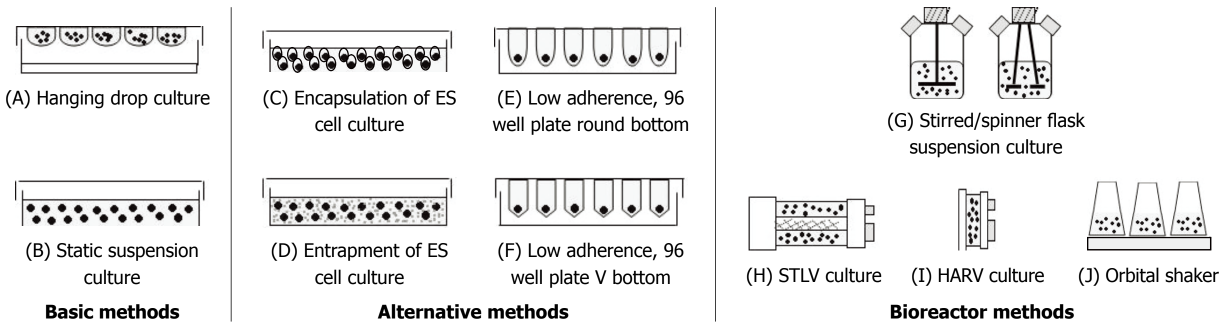

Figure 1 Schematic representation for vessels used in methods to form EBs from ES cells.

gravity-induced aggregation of the cells. For this reason, this technique has been used to generate plentiful cell types such as neuronal cells[25], hematopoietic cells[26], cardiomyocytes[27], vascular cells[28] and chondrocytes[29]. The hanging drop method is tremendously useful for appraisal of molecular mechanisms occurring in early embryogenesis in any cell type. However, this technique is mainly used for research purposes and is not suitable for large scale of EB production because of its laborious nature; a typical 100-mm Petri dish can contain no more than 100 drops and each drop usually creates only one EB[21]. Further limitations of this method include major difficulties in exchanging or manipulating the small volume of medium (less than 50 μL which can evaporate easily) without disturbing the EBs. Usually the hanging drop method is composed of two steps; the aggregation of ES cells in drops and maturation of aggregates to EBs in suspension culture using low adherence bacterial Petri-dishes. Several elements of the method may be troublesome such as losses of EBs during picking up the formed EBs by pipette and attachment of premature EBs on Petri-dishes[17].

Static suspension culture

Static suspension culture (Figure 1B) is used to produce a large number of EBs by simply inoculating a suspension of ES cells onto a bacteriological grade Petri-dish, ultra-low adherence plate or a Petri-dish coated with cell adhesion inhibitor such as *poly* 2-hydroxyethyl methacrylate (poly 2-HEMA), allowing the cells to spontaneously aggregate into spheroids[30]. Although simple, this method allows little control over the size and shape of EBs. The result is frequent agglomeration of EBs into large, irregular masses because of the probability that ES cells encounter each other accidentally[26]. An additional limitation of this technique is that EBs may prematurely attach to the plate because of the surface chemistry of the culture vessel, leading to a greater heterogeneity and loss of EBs from the suspension culture. On another hand, this method is popular for some applications such as differentiation of ES cells into the neuronal lineage[31,32].

Encapsulation/entrapment

Encapsulation/entrapment of a single cell suspension or

small clusters of ES cells in hydrogels (Figure 1C and D, respectively), such as methylcellulose[26], fibrin[33], hyaluronic acid, dextran[34], alginate[35], or agarose[36] represents a transition between hanging drop and static suspension approaches by generating individually separated EBs in a semi-solid suspension media. Entrapment of ES cells in methylcellulose, a temperature sensitive hydrogel, improves the overall synchrony and reproducibility of EB differentiation as it produces EBs of clonal origin. However, the efficiency of EB formation from individual ES cells can be rather low. In addition, soluble factor treatments and retrieval of differentiated cells may be complicated by the presence of the hydrogel material[26]. Interestingly, this method showed the possibility of designing a single cell culture system that would mimic the early developmental milieu and allow ES cells to switch between differentiation states within the same culture setting. When human ES (hES) cells are encapsulated in a 3D hyaluronic acid hydrogel, the hES cells can be maintained in an undifferentiated state. On the other hand, when hES cells are encapsulated in a dextran hydrogel, the hES cells are induced to differentiate and form EBs. Different types of hydrogels, therefore, act as a unique microenviroment for maintaining ES cells in either undifferentiated or differentiating state[31].

Multiwell and microfabrication

As an alternative approach for EB formation and culture, multiwell (Figure 1E and F) and microfabrication technologies have also been developed recently. Round-bottomed 96-well plates coated with or without reagents[37]; 2-methacryloyloxyethyl phosphorylcholine (MPC)[38-40], glycosaminoglycan (CS)[24] and *poly 2*-hydroxyethyl methacrylate (poly 2-HEMA), have been utilized to prevent cell adhesion to the plastic surfaces. This technique is among the tools for forming EBs with high uniformity similar to the hanging drop method as a defined number of ES cells is seeded in the separated wells. In contrast to the hanging drop method, this technique has no requirement to exchange or manipulate the medium (approximately 200 μL) and it is easier to observe directly the EB formation with a microscope during cultivation. Because of these advantages, this technique may be used instead of hanging drop method for laboratory research. The forced aggregation system, involving centrifugation of ES cells within round-bottomed (U-shaped)[41,42] and triangle-bottomed (V-shaped) 96-well plates[43], can induce aggregation more rapidly than hanging drops. This procedure improves the reproducibility of EB production. On the other hand, it still requires individual processing and manipulation of the resulting EBs due to the requirement of one more additional plating step. Microwells fabricated by lithographic methods yield EBs in an equivalent or at a much higher density than other methods and allow preparation of size-controlled EBs in a scalable manner for reproducible of EB formation[44]. Likewise, batches of EBs can be formed in microfluidic chambers and separated from the flowing culture medium by a semi-permeable membrane, allowing for temporal control of the molecular makeup of the medium. The cell patterning method is also useful for high-throughput screening assays, such as the exploration of biochemical agents to direct aggregate-induced differentiation into a specific lineage without plating EBs[45].

Bioreactors

Stem cell-based technologies and tissue engineering possibly permit a wide span of clinical and biotechnology applications in future. Nevertheless, realization of the potential of stem cells will require their large-scale generation in a robust system without any limitation[46]. This highlights the requirement for the *in vitro* expansion of stem cells used for therapy prior to their commitment into tissue-specific applications. The potential of bioreactors to address this is demonstrated by their capacity to support a robust and well defined scale-up platform for expansion of ES cells[47], EB formation[48,49] as well as differentiation[50]. The scaling up of the design, given mass transfer limitations, will depend on the type of bioreactor chosen[51]. The theory of selecting bioreactors for stem cell expansion and differentiation beyond bench scale is largely reliant on whether the cells are adherent, suspension grown as single cells or aggregates for EB formation[52]. Therefore, bioreactor culture systems must be designed according to the application. In addition, bioreactors have a significant advantage over static suspension culture which are as follows: (1) scale up of expansion and differentiation of ES cells; (2) no labor-intensive requirements; (3) no space requirement for available area of ES cell growth; and (4) the ability to monitor and control critical culture parameters (i.e. pH, dissolved oxygen, glucose consumption, and lactic acid production)[53]. At the present time, EB formation in hydrodynamic conditions has been achieved by using bioreactors. They comprise (1) spinner flasks; (2) RCCS; (3) rotary orbital culture; and (4) complex methods combining these techniques. All of these techniques generally improve ES cell aggregation and form EB faster and more homogeneously in size compared to typical static suspension cultures.

Spinner flasks: Spinner flasks (Figure 1G) have been pioneered, as promising *in vitro* systems for stem cell expansion, EB cultivation and differentiation of ES/iPS cells into specific cell types[54]. Spinner flasks provide attractive benefits due to their simple design, scalable configuration, the flexible culture of cells as aggregates on microcarriers[55] or scaffolds[56], and ease of continuous monitoring for tight regulation of the culture environment (e.g. O_2 tension, pH, shear forces, medium exchange rate)[57]. The simpler process in spinner flasks equipped with paddle-impellers results in the formation of large ES cells agglomerates within a few days[58]. The scaling-up is generally straightforward because of improved mass transport achieved by stirring. However, the flow environment created by the impeller renders them inappropriate, due to the shear stress[59]. Numerous culture parameters for this system have been optimized, including the agitation rate, cell initial concentration, me-

dium compositions, and different culturing approaches have been developed. In addition, a low rate of paddle-impeller stirring results in cell clumping in aggregation supporting EB cultures (leading to lower mass transport to the cells), while high rates of paddle-impeller stirring can be harmful for the cells. Consequently, an optimal fluid velocity promoting the suitable shear stress for the cell type being cultured is critical[60].

RCCS: Cells in conventional stirrer vessels are exposed to hydrodynamic shear stress resulting in damage to the cells. Another approach for controlling EB agglomeration employs RCCS which is comprised of a slow turning lateral vessel (STLV) (Figure 1H) and a high aspect rotating vessel (HARV) (Figure 1I), as a milder bioreactor. The advantages of these bioreactors are as follows: (1) horizontal rotation is characterized by extremely low fluid shear stress; (2) fluid-filled culture vessels are equipped with membrane diffusion gas exchange to optimize oxygen levels; and (3) membrane area to volume of medium ratio is high, thus enabling efficient gas exchange[61]. The type of rotating vessel had significant impacted on the process of hEB formation and agglomeration; hEBs formed small aggregates with no necrotic centers in STLV. Conversely, hEBs of extensive cell aggregation with large necrotic centers are formed in HARV[23]. STLV rotating bioreactors were used for cultivating mouse ES (mES) and hES cells to produce EBs and to compare both the quality and quantity of EBs with those from static suspension culture. ES cells grown in a STLV bioreactor were of higher quality and yielded a nearly 4-fold increase in the number of EB particles. EBs derived from a STLV bioreactor showed enhanced cardiac differentiation in comparison to static suspension culture[62].

Rotary orbital culture: Bioreactors may offer a more uniform differentiation environment capable of sustaining increased EB and differentiated cell yield. However, these methods may not be suitable solutions for assessing multiple experimental samples in parallel because of the requirement for larger-volume bioreactors. Orbital rotary shakers (Figure 1J) have been used to produce EBs as the constant circular motion provided by this simple system is good for improving the efficiency of EB formation[63]. The advantages of this technique include accommodation of cell culture dishes on the rotary platform, easily allowing production of numerous parallel samples and allowing comparison of different experimental parameters. EBs formed by using orbital rotary shakers appeared to differentiate more efficiently than those produced in static suspension culture on the basis of morphological appearance and gene expression profile patterns. A 20-fold enhancement in the number of cells incorporated into primitive EBs in rotary *versus* static conditions was detected after the first 12 h, and a fourfold increase in total cell yield was achieved by rotary culture after 7 d[64].

Complex methods combining these techniques: Recently, complex methods combining the above mentioned techniques have been adopted for solving the problems of these methods and keeping cells floating continuously in the culture medium. For example, the agglomeration of cells was avoided by keeping EBs in Petri-dishes for several days before transferring them into a different kind of environment; (1) spinner flasks; (2) a rotation culture system of Petri-dishes which were rotated on a horizontal rotation device; (3) rotary suspension culture in dishes on an orbital rotary shaker; (4) direct seeding ES cells into a spinner flask equipped with a glass ball bulb-shaped impeller or (5) two litres Stirred Tank bioreactor (STR) equipped with a newly developed pitched-blade turbine impeller[65].

In other cases, the encapsulation of ES cells was combined with transferring them into a bioreactor. For example, encapsulation of ES cells in defined conditions (i.e. number of cells per EB and capsule size); alginate[35], agarose[66], poly (lactic-co-glycolic acid)/poly (L-lactic acid) microsphere[67,68], hyaluronic acid[31] and Matrigel[69] was used to control agglomeration of cells. Then, after the initial period of EB formation, all encapsulated ES cells were transferred to a spinner flask. The encapsulation system allowed a 61-fold expansion in the number of cells, similar to the static control non-stirred culture but significantly higher than the stirred non-encapsulated system. Moreover, combination of the encapsulation of ES cells within alginate hydrogel, with or without 70s bioglass, followed by culturing cells in an HARV bioreactor directly enhanced both osteogenic differentiation in a functional test and generation of functional 3D mineralized constructs for further application of bone tissue engineering transplantation[70,71]. Finally, mES cells expanded as aggregates on microcarriers in stirred vessels retained expression of stem cell markers and could form EBs. Perfusion combined with frequent feeding has been shown to increase the expansion of ES cells and their differentiation into specific lineages, without compromising their stem cell performance[72]. Additionally, the effect of a rotary bioreactor promoted neural differentiation of hES cells in perfused and dialyzed STLV. The mean time delay for growing to so-called "neural rosette" formations was significantly shortened under STLV conditions compared to conventional static suspension culture. Likewise a perfused STLV bioreactor can decrease the expression of markers of undifferentiated stage and increase the expression of markers of differentiation, especially towards neural lineage commitments[73].

Recently, researchers have sought to develop culture systems with integrated bioprocesses, controlling stem cell expansion and differentiation tightly in a fully controlled bioreactor environment. For example, ten fold increase in expansion of ES cells as well as consequent neural differentiation was reported while drastically reducing, by 30%, the time required for the differentiation process[54]. Moreover, microcarrier spinner flasks have been used for the culture of mES and hES cell expansion and directed differentiation. Mouse ES cells were allowed to proliferate on microporous collagen-coated

dextran beads (Cytodex 3), glass microcarriers, and macroporous gelatin-based beads (Cultispher S) in spinner flasks[74]. Under different inoculated cell densities and microcarrier concentrations, mES cells on microcarriers showed increased yield of approximate 70-fold (8 d) to about 190-fold (15 d). These cultured cells also successfully expressed Oct4, Nanog, and SSEA-1, and when dissociated from the beads, they formed EBs yielding cells with differentiation markers such as Flk-1, CD34 and α-MHC (mesoderm), HNF-3b19 (endoderm), and b3-tubulin57 (ectoderm)[60].

Computer-controlled bioreactors

As mentioned before, the main advantage of computer-controlled bioreactors is process development by allowing online monitoring and control of specific culture parameters (temperature, pH, PO_2, lactic acid production and glucose consumption), and ensuring a fully controlled environment for stem cell cultivation[18]. Oxygen-controlled bioreactors have been used for culturing mES and hES cell-derived cardiomyocytes. These experiments also assessed the effect of oxygen tension on cardiac differentiation which is a main concern[72]. Moreover, this system was recently applied to culturing cells not only for stem cell expansion but also for differentiation. Expansion of a variety of stem cell types in bioreactors under defined and controlled conditions remains to be addressed. Future challenges also include the combination of expansion and directed differentiation steps in an integrated bioprocess that will ultimately result in scale-up of well differentiated cells to clinically relevant numbers.

It is worth mentioning that although differentiating cells in bioreactors have numerous benefits, these cells have been assessed for functionality by transplantation, and did not always perform well. Ten and twenty days post-implantation ES cells derived chondrogenic and osteogenic bioreactor aggregates showed no obvious influence on the healing process. In these experiments, all of the bioreactor derived cells showed higher Oct4 expression in the aggregates, even after 30 d of induced differentiation in a medium without LIF[75]. This emphasizes the importance of proper condition set-up and timing during cultivation of cells in bioreactors.

EMBRYONIC STEM CELL DIFFERENTIATION TO CARDIOMYOCYTES USING BIOREACTOR

Regenerative medicine based on cell transplantation therapies has attracted increasing attention as a potential alternative to organ transplantation[76]. Pluripotent stem cells (ES/iPS cells), because of their pluripotency and unlimited self-renewal capacity are promising cell sources to provide sufficient number of cells for therapeutic applications. However, the expansion and differentiation of these cells is still limited as a result of their complexity and difficult manageability in scale-up production for industrial purposes[77,78]. To solve these problems, bioreactor culture systems offer attractive advantages of ready scalability and relative simplicity[79,80].

Recently, a single-step bioprocess for ES cell-derived cardiomyocyte production have been developed by combining methods to prevent ES cell aggregation (hydrogel encapsulation) and to purify for cardiomyocytes from the heterogeneous cell populations by using genetic selection (myosin heavy chain-neomycin resistance; MHC-neo), with medium perfusion in a controlled bioreactor environment. It has been shown that the cardiomyocyte yield per input ES cells achieved in encapsulated culture was much higher than without encapsulation (3.17 ± 0.90 *vs* 0.16 ± 0.07). Furthermore, higher cardiomyocyte yield was achieved under hypoxic conditions (4% oxygen tension) *versus* normoxia conditions (20% oxygen tension), when cultured in the stirred culture system[72]. In addition, a 2-L bioreactor process enabling the controlled generation of EBs, derived from MHC-neo ES cell line, has been adopted for enhancing yield of ES-derived cardiomyocyte production. The fill-and-draw feeding protocol was replaced in a 2-L bioreactor, which allowed constant medium supply and avoided daily fluctuations of medium components. An optimized protocol resulted in more than five times greater cardiomyocyte yield, whereas medium consumption was 40% less than that in the control system[53].

For the controlled large-scale generation for clinical and industrial applications in humans, the efficacy of the dynamic process [Erlenmeyer, STLV bioreactor, Glass Ball Impeller (GBI) spinner flask and Paddle-Impeller (PI) spinner flask] was compared to static suspension culture in Petri-dishes by analyzing the quality of EB formation and subsequent differentiation into cardiomyocytes. The EB prearrangement in the static system and EB cultivation in the GBI spinner flask resulted in high EB yield, a round homogenous shape, the fastest growth rate and high contracting EB percentages over all other systems[65].

As noted above, cardiomyocytes derived from ES cells are anticipated to be valuable for cardiovascular drug testing and disease therapies. However, the overall efficiency and quantity of cardiomyocytes obtained by differentiation of ES cells is still low. Recently, to enable large-scale culture of ES-derived cells, we have tested a scalable bioprocess that allows direct EB formation in a well controlled STLV bioreactor system. Our laboratory has developed protocols of cardiomyocyte differentiation from mES cells by using STLV. We have optimized the initial ES cell seeding density into the bioreactor, the rotation speed and the day of transferring and plating of EBs on gelatin coated Petri-dishes. We have compared the quantity and quality of EB production, as well as the efficiency of cardiac differentiation of samples derived form STLV, static suspension culture and hanging drop method. We found that the optimized rotary suspension culture method can produce a highly uniform population of efficiently differentiating EBs in large quantities in a manner that can be easily implemented by basic research laboratories (Figure 2). Although EBs derived from STLV start rhythmically contracting later than static suspension

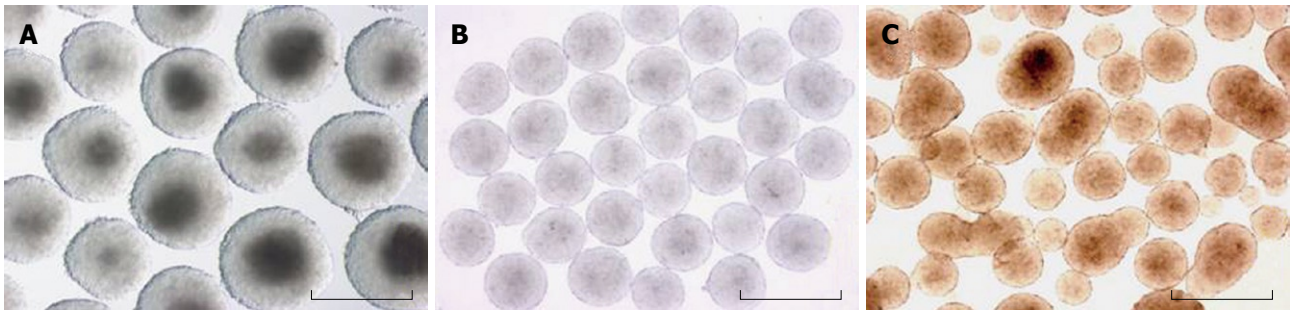

Figure 2 Formation of EBs in various methods. Gross morphology of EBs derived from slow turning lateral vessel (STLV) (A), hanging drop (B) and suspension culture (C). Scale bars correspond to 500 μm.

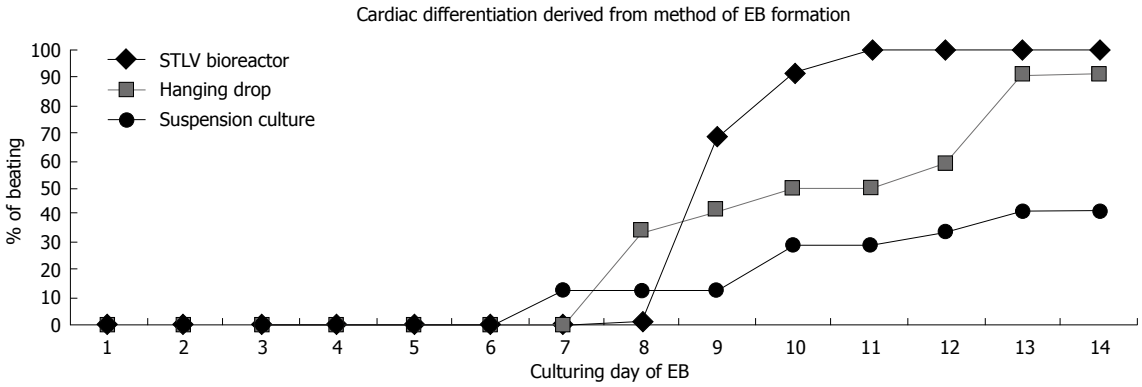

Figure 3 Illustration of the cumulative percentage of EBs containing contracting area derived from STLV, hanging drop and suspension culture.

culture and hanging drop method, they beat with nearly 100% efficacy (Figure 3). Furthermore, our results are similar to other reports of EBs formed in STLV which were more uniform in size, and contained mostly viable cells whilst lacking necrotic centers. Additionally, STLV-produced EBs differentiated into cardiomyocytes more efficiently than those from static suspension culture[62]. Hence, this method provides a technological platform for the controlled large-scale generation of ES-derived cells for clinical and industrial applications.

CONCLUSION

Bioprocessing and commercialization of ES or iPS cells and tissue engineering products in cell replacement therapy have the potential to facilitate and transform breakthroughs from the research bench to the patient bedside. This is expected to be a long process, however, as there are many key practical issues to be addressed before moving ahead from the laboratory-scale fundamental research level. Laboratory-scale suspension cultures in hanging drops or Petri-dishes are useful tools for process development and initial optimization, and encapsulation/entrapment of ES cells, multiwell and microfabrication methods can improve high-throughput EB production. However, these approaches are not suitable for further therapeutic application because of their labor intensive, time consuming nature, culture-to-culture variability and lack of monitoring. Bioreactor culture systems address many of these problems and offer several advantages over the conventional use of basic culture methods for expanding and differentiating ES cells into specific lineages, without compromising their stem cell performance. Future challenges in bioreactor development will include the design of advanced and sophisticated monitoring platforms that allow monitoring at the cellular level of parameters including temperature, pH and oxygen levels. With respect to ES or iPS cells, we envision a scenario, where a complete bioprocess would exist in the bioreactor for the expansion and subsequent differentiation of the ES or iPS cells to generate the specialized cell type of interest. For example, the current achievements with cardiomyocytes derived from ES cells would be developed into cardiovascular grafts tissue engineering, with an emphasis on its possible clinical use in cardiovascular surgery. The engineering of a human cardiac tissue patch would be used to illustrate the biological requirements and engineering approaches for human applications. For future therapeutic application, the specialized cells differentiated from ES or iPS cells could then be used for cell therapies or combined with scaffolds to produce tissue construct and transplants for patients.

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S- Editor Li LF **L- Editor** Hughes D **E- Editor** Lin YP

World Journal of Stem Cells

Online Submissions: http://www.wjgnet.com/1948-0210office
wjsc@wjgnet.com
doi:10.4252/wjsc.v1.i1.22

World J Stem Cells 2009 December 31; 1(1): 22-29
ISSN 1948-0210 (online)

TOPIC HIGHLIGHT

Andras Dinnyes, DSc, Professor, *Series Editor*

Germline competence of mouse ES and iPS cell lines: Chimera technologies and genetic background

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Author contributions: Carstea AC conceived the idea and prepared the original draft; all three authors participated equally in creating the final manuscript.
Supported by Grants from EU FP6 ("MEDRAT"-LSHG-CT-2005-518240; "Artemis", LSHM-CT-2006-037862; "AGLAEA", LSHM-CT-2006-037554, "CLONET", MRTN-CT-2006-035468), EU FP7 ("PartnErS", PIAP-GA-2008-218205; "InduHeart", EU FP7-PEOPLE-IRG-2008-234390; "InduStem", PIAP-GA-2008-230675; "Plurisys", HEALTH-F4-2009-223485); NKFP_07_1-ES2HEART-HU, No. OM-00202-2007 and NKTH/ANR TET Franco-Hungarian Bilateral Scientific and Technological Collaborative Project "Plurabit"
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Received: December 1, 2009 Revised: December 14, 2009
Accepted: December 21, 2009
Published online: December 31, 2009

Abstract

In mice, gene targeting by homologous recombination continues to play an essential role in the understanding of functional genomics. This strategy allows precise location of the site of transgene integration and is most commonly used to ablate gene expression ("knock-out"), or to introduce mutant or modified alleles at the locus of interest ("knock-in"). The efficacy of producing live, transgenic mice challenges our understanding of this complex process, and of the factors which influence germline competence of embryonic stem cell lines. Increasingly, evidence indicates that culture conditions and *in vitro* manipulation can affect the germline-competence of Embryonic Stem cell (ES cell) lines by accumulation of chromosome abnormalities and/or epigenetic alterations of the ES cell genome. The effectiveness of ES cell derivation is greatly strain-dependent and it may also influence the germline transmission capability. Recent technical improvements in the production of germline chimeras have been focused on means of generating ES cells lines with a higher germline potential. There are a number of options for generating chimeras from ES cells (ES chimera mice); however, each method has its advantages and disadvantages. Recent developments in induced pluripotent stem (iPS) cell technology have opened new avenues for generation of animals from genetically modified somatic cells by means of chimera technologies. The aim of this review is to give a brief account of how the factors mentioned above are influencing the germline transmission capacity and the developmental potential of mouse pluripotent stem cell lines. The most recent methods for generating specifically ES and iPS chimera mice, including the advantages and disadvantages of each method are also discussed.

Key words: Chimeras; Transgenic; Embryonic stem cells; Epigenetic changes; Germline competence; Induced pluripotent stem cells

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Carstea AC, Pirity MK, Dinnyes A. Germline competence of mouse ES and iPS cell lines: Chimera technologies and genetic background. *World J Stem Cells* 2009; 1(1): 22-29 Available from: URL: http://www.wjgnet.com/1948-0210/full/v1/i1/22.htm DOI: http://dx.doi.org/10.4252/wjsc.v1.i1.22

INTRODUCTION

Genetically altered mice offer researchers a powerful means to dissect and understand complex biological processes, as well as to manipulate gene expression, with the ultimate goal of developing therapeutic strategies for a variety of diseases including cancer, inflammatory and infectious diseases, and neurogenetic and cardiovascular disorders[1-5].

These mice are typically generated by means of the introduction of a specific population of cells called "embryonic stem cells", into a host preimplantational embryo. Embryonic Stem cells (ES cells) can self-replicate and are pluripotent. Originally, ES cells were isolated from blastocyst stage embryos[6], but recently a new method for generation of induced pluripotent stem (iPS) cells from somatic cells has been developed[7]. iPS cells are derived from autologous somatic cells after genetic reprogramming and were first described by Takahashi *et al*[7] and were independently confirmed later by others. Recently, iPS technology has opened new avenues for the generation of animals from genetically-modified somatic cells by means of chimera technologies. Results from many independent groups suggest that mouse and human iPS cells, once established, generally exhibit a normal karyotype, are transcriptionally and epigenetically similar to ES cells and maintain the potential to differentiate into derivatives of all germ layers. Injection of iPS cells into diploid (2n) blastocysts, similar to ES cells, frequently gives rise to high-contribution chimeras (mice that show major tissue contribution of the injected iPS cells in the host mouse), as has been shown by many different research groups[7,8]. A subset of these chimeras has successfully demonstrated germline contribution. However, only two reports so far have used the most stringent assay, that is, tetraploid embryo complementation[9,10].

Typically, genetic reprogramming of mouse and human somatic cells (iPS technology) can be achieved after ectopic expression of a defined combination of 4 transcription factors, namely c-Myc, Klf4, Oct4, and Sox2. It is known that c-Myc and Klf4 reprogramming factors are oncogenes and their expression or reactivation in iPS-derived mice causes tumors. The safety of iPS cell derivation can be improved by excluding c-Myc and Klf4 from the reprogramming cocktail or by selecting target cell types that already endogenously express these genes[11]. Recent studies provide a more efficient alternative that involves viral vector-free integration of reprogramming genes, followed by their removal. Recent adenoviral and plasmid-based strategies used in conjunction with latest generation transposon technology (e.g. PiggyBac and Sleeping Beauty transposons) may now potentially overcome some of these limitations[12-15].

The definition of "pluripotency" is that the cell can give rise to all three embryonic germ layers, i.e. mesoderm, endoderm, and ectoderm[16]. These three germ layers are the embryonic source of all cells of the body. The pluripotent nature of cells (either ES or iPS) is routinely tested by three methods. One test is to inject the cells into adult mice that are either genetically identical or are immune-deficient, so that the tissue will not be rejected. In the host animal, either when injected or when transplanted, these ES cells can become any cell in the body and form tumors called teratomas. A second test for pluripotency is to allow mouse ES cells to differentiate spontaneously *in vitro,* or to direct their differentiation along specific pathways. Within a few days after changing the culture conditions, ES cells aggregate and may form so-called embryoid bodies (EBs), further differentiating towards multiple cell lineages. Teratomas and EBs demonstrate that the ES cells are capable of developing into many cell types, derived from the three embryonic germ layers. Histological analysis has also demonstrated that iPS cells can give rise to teratomas comprising all three embryonic germ layers[17].

The third, *in vivo* method, is based on the capacity of cells to participate in the formation of germ cells when they are introduced into a preimplantational host embryo, resulting in the so-called "chimera mice".

Chimera mice - or in brief "chimeras" - were first created in the 1960's by Kristoph Tarkowski and Beatrice Mintz, by means of aggregating two eight-cell embryos, and were then produced by Richard Gardner and Ralph Brinster who injected cells into blastocysts. This revolutionary new technique opened up a new method for introducing any kind of cell (even genetically- modified) into the host embryo, thus creating a new chapter in mouse embryology, as well as in biotechnology.

The efficiency of producing live, transgenic and germline mice requires precise understanding of the mechanisms that could be vitally important for the maintenance of pluripotency, and therefore germline transmission of the ES cell lines. Despite successes in gene targeting in ES cells[18-20] during recent years, many factors that dramatically influence the efficiency of germline chimera mice generation have not been yet fully investigated.

One factor is the prolonged culture of cells. Once established and adapted to *in vitro* culture conditions, ES cells can be maintained for long periods of time. Stem cell derivation and maintenance imply extensive *in vitro* culture. This has raised the question of whether culture conditions could influence the developmental potential of stem cells and whether loss of germline capacity is due to the accumulated production of chromosome abnormalities and/or epigenetic alterations. In mouse and large animal models, extensive work has been performed on the epigenetic effects of *in vitro* culture. Indeed, recent work on ES cells has shown that stem-cell-derived tissues and embryos often fail to maintain stable epigenetic states, or the normal diploid karyotype. Several studies have reported that accumulation of epigenetic alterations, mostly in the imprinted genes, is the major cause of decreased or lost germline ability of ES cells. On the other hand, aneuploidy, rather than "loss of pluripotency", in ES cells, is the one major cause of failure in obtaining contributions to all tissues of the adult chimera, including the germline. Euploidy is

predictive for germline transmission and the karyotype analysis is crucial in any gene-targeting experiment.

Another factor is that, unfortunately, the founder mice are derived mostly from inbred strains, such as the C57BL/6 strain, which often shows poor viability or abnormalities due to developmental defects[2]. Still, the germline competency of the majority of ES cells (e.g DBA/1Ola, C3H/HeN, BALB/c, and FVB/N) is usually not comparable to the highly germline-competent 129 strains (129/Sv, 129/SvEv, and 129/Ola) derived ones[21,22]. Few ES cell lines are currently available from inbred strains (e.g. C57BL/6, BALB/c) and those have generally been produced with low success rates.

Another critical factor contributing to the success of germline-competent ES chimeras is the technique chosen to produce the chimeras. Attempts to improve the methods for generating ES mice chimeras were mainly carried out in order to establish ES cell lines with a higher potential for producing germline transmission. This strategy lead to the discovery that ES cell lines derived from hybrid mouse strains support the development of viable ES mice to a greater extent when compared with inbred ES cells[2] and significantly improved the technique. Although the effect of donor ES cells on the production of ES mice has been well studied, the technique still has a limitation in that ES mice can be generated only from specified ES cell lines. There are a number of options for generating chimeras from ES cells but each method has its advantages and disadvantages. In this review we will examine some of the conventional, and also the most recent methods for generating ES chimera mice, the advantages and the disadvantages of each method, and the factors that should be taken into account when deciding on one method in preference to another.

FACTORS INFLUENCING GERMLINE TRANSMISSION CAPACITY OF PLURIPOTENT CELLS

The efficiency of mouse ES cell germline transmission is strongly influenced by genetic background, and is maximized with ES cells that have spent a minimum amount of time in culture, that have a normal complement of chromosomes, and are not affected by epigenetic alterations. Here we give a brief account about how the factors mentioned above influence the germline transmission capacity and the developmental potential of mouse pluripotent stem cell lines.

Genetic background

It is still far from clear why certain strains are more amenable to ES cell derivation than others. In recent years, embryonic stem cells have been derived from various mouse strains. However, 129 ES cells (ES cell lines derived from different 129 backgrounds) are still widely used, partially due to poor germline transmission of ES cells derived from other strains. It is generally accepted that it is easier and more efficient to perform targeting for an ES cell line on a hybrid genetic background. A large number of inbred strains of mice exist, but only a small number are commonly used for establishing gene-targeted mice.

Genetic heterozygosity is presumed to be a crucial characteristic for postnatal survival of fully ES derived mice[23]. On the other hand, elimination of genetic background variability associated mostly with the use of 129 (129/Sv, 129/SvEv, and 129/Ola) embryonic stem (ES) cell lines, requires derivation of germline-competent ES cell lines from inbred mouse strains with specific genetic backgrounds, enabling generation of isogenic gene-targeted and control mice[24,25]. Mutagenesis by homologous recombination in ES cells[26] is an important means to the understanding of the molecular mechanisms of higher brain functions. This study requires gene targeting in embryonic stem (ES) cells derived from the strain suitable for brain function analysis and with a homogenous genetic background, such as the C57BL/6 strain. Auerbach *et al*[21] compared 129 and C57BL/6 ES cells and found that cells on C57BL/6 background are more sensitive to culture conditions and that it is more difficult to maintain them in culture than the 129 derived ones. Similar conclusions have also been reached by others[25].

Germline-competent ES cells have also been derived from other inbred strains, including C57BL/6, however, competency of germline transmission of these ES cell lines is not comparable to that of the 129 ES cells[20-24]. The developmental potential of C57BL/6 ES cells seems to be lost during cell culture *in vitro*[2], and seems to depend on several factors, such as the serum or even the feeder cells used for ES cell culturing. The quality of serum (even having the same catalogue number, but coming from different lots), pH of medium and the quality/origin of feeder layers used in different experiments can cause decreased developmental potential. Therefore care should be taken to introduce a broad variety of culture conditions in order to take ES cells germline. Mouse iPS cells are indistinguishable from embryonic stem (ES) cells in many respects and the production of germline-competent chimeras, and although this has not yet been studied, it is probable that it would also be influenced by the genetic background.

Some recent studies have described increased efficiency of derivation of germline- competent inbred ES cell lines, mostly by modifying current culture conditions[27,28] and have reported that using a culture medium conditioned by a rabbit fibroblast cell line and transduced with genomic rabbit leukemia inhibitory factor allows efficient derivation of ES cell lines from 10 inbred mouse strains (129/SvEv, 129/SvJ, C57BL/6N, C57BL/6JOla, CBA/CaOla, DBA/2N, DBA/1Ola, C3H/HeN, BALB/c, and FVB/N). Germline transmission was achieved by blastocyst injection of established ES cell lines after 10 or more passages from strains 129/SvJ, C57BL/6N, C57BL/6JOla, DBA/2N, DBA/1Ola, BALB/c and FVF/N. The efficiency of establishing ES cell lines and also generating germline chimeras from the C57BL/6 derived LK1 cell line was comparable with a widely used 129/SvJ derived GSI-1 ES cell line[28]. Sato *et al*[29] used leukemia inhibitory factor (LIF) and 6-bromoindirubin-30-oxime (BIO), a

glycogen synthase kinase-3 (GSK3) inhibitor, and showed that BIO treatment significantly increased the expression levels of 364 genes including pluripotency markers such as Nanog and Klf family members. Chimeras derived from cell lines from LIF, BIO or GSK3 inhibitor- enriched medium were germline-competent. The current hope is that ES cell lines from "non-permissive" mouse strains will become more widely derivable, possibly by means of modifying ES cell culture conditions.

Chromosomal abnormalities

A key property of ES cells is that they maintain their euploid karyotype. This is crucial because a balanced diploid chromosome complement is necessary for proper meiosis.

The chromosome make-up of mouse embryonic stem cells is predictive of somatic and germ cell chimerism. Over the years, several studies have reported that chromosome make-up correlates with the capacity of ES cell clones to contribute to the formation of all tissues, including the germline, of the adult chimaeras. The data support the notion that karyological instability, and not loss of pluripotency, is the major reason for the lack of contribution to chimaeras of individual ES cell clones, and that karyotype analysis is a predictor of the germline transmission capacity of ES cell lines[30-34]. Some studies suggest that the long-term culture of iPS cells, similar to the situation for ES cells, has to be monitored carefully for culture-induced chromosomal abnormalities[35].

Other studies have also reported that the number of aneuploid mitoses in ES cells expands with increasing culture time[30,36] and that the ES cell clones with less than 50% euploid metaphases generated only a few and weak chimeras and non- germline[37]. It was shown that in particular, trisomy 8 is associated with a selective growth advantage *in vitro* and represents a common cause for the failure of ES cells to contribute to the germline[38,39]. Multicolor karyotyping technologies, including both multi-color fluorescence *in situ* hybridization (M-FISH) and spectral karyotyping (SKY), are recently developed molecular cytogenetic techniques for rapid visualization of genomic aberrations at sub-cellular level. Guo and colleagues[40], using the M-FISH method, recognized various chromosomal abnormalities in two independent ES cell lines: trisomy 8 in some mitoses, trisomy 14q and the deletion 6q in 100% of the cells studied[40]. The deletion 6q affected only a part of the respective chromosome and therefore the total number of 40 chromosomes was still retained. Some of these chromosomal abnormalities might be overlooked by standard G-banding analysis alone[41]. Presently, it is not known whether such translocations are detrimental to the achievement of high levels of chimerism or germline transfer. On the other hand, some studies have reported that the presence of chromosomal aberrations may reduce, but not necessarily eliminate, the ability of ES cells to contribute to normal development[42].

In summary, these data demonstrate a strong correlation between losing the germline-competence of ES cell lines and accumulation of chromosome abnormalities. However, research should aim to link specific components of the aberrant phenotypes with specific epigenetic alterations in gene expression.

Epigenetic alterations

Long term culture and *in vitro* manipulation of the ES cells can induce epigenetic alterations, which in turn can have long- lasting effects on the transcription patterns of the ES cell genome. Indeed, recent work on ES cells has shown that stem cell-derived tissues and embryos often fail to maintain stable epigenetic states, especially in imprinted genes[43-47]. So, any epigenetic changes caused by the number of passages would most probably affect the developmental pluripotency of ES cells and thus the viability of ES mice. Two further mouse studies have also investigated the epigenome of iPS cells on a larger scale. Maherali *et al*[17] used ChIP-Chip to investigate the presence of H3K4me3 and H3K27me3 in the promoter regions of 16 500 genes in one iPS cell line. Their results suggested that iPS cells were highly similar in their epigenetic state to ES cells with 94.4% of 957 "signature" genes (defined as genes that have a different epigenetic state between MEFs and ES cells) being reset to an ES-cell state in the respective iPS cell line[48].

In ES cells, the effects of methylation on expression of specific genes, particularly imprinted ones[43] and some retrotransposons[49], have been demonstrated *in vivo*.

Dean *et al*[43] investigated whether the prolonged culture of ES cells affects their pluripotency and whether it is associated with epigenetic alterations in imprinted genes. Two maternally expressed genes (*Igf2r*, *H19*) and two paternally expressed genes (*Igf2*, *U2af1-rs1*) were analyzed in ES cells, and in completely ES cell-derived fetuses. Altered allelic methylation patterns were detected in all four genes, and these were consistently associated with allelic changes in gene expression. It was also demonstrated that all methylation changes that had arisen in the ES cells persisted on *in vivo* differentiation to fetal stages. Alterations included loss of methylation with biallelic expression of U2af1-rs1, maternal methylation and predominantly maternal expression of Igf2, and biallelic methylation and expression of Igf2r. In most of the ES derived fetuses, the levels of H19 expression were strongly reduced, and the biallelic repression was associated with biallelic methylation of the H19 upstream region. ES fetuses derived from two of the four ES lines appeared developmentally compromised, with polyhydramnios, poor mandible development and interstitial bleeding and, in chimeric fetuses, the degree of chimerism correlated with increased fetal mass. This study created a model for how early embryonic epigenetic alterations in imprinted genes persist to later developmental stages, and are associated with aberrant phenotypes. Generation of pluripotent cells with correct epigenetic profile after reprogramming of somatic cells by the iPS technology is crucial for their developmental competence. It is yet to be demonstrated whether insufficient reprogramming in iPS cells would increase the probability of epigenetic alterations and subsequent developmental abnormalities in

chimera embryos and fetuses.

Different studies have also reported that retrotransposon elements (REs) are transcribed during early mouse embryogenesis[50] and also in ES cell lines[51] and that transcriptional interference by active retrotransposons perturbs expression of neighboring genes in somatic cells, in a mosaic pattern corresponding to activity of each retrotransposon. Furthermore, the expression of REs also regulates host genes in preimplantation embryos[50]. Since ES cells are mostly isolated from the inner cell mass (ICM) of blastocysts, the expression of REs could be essential for *in vitro* and *in vivo* preservation of the genomic integrity and pluripotency of ES cells. Moreover, inadvertent alterations in the expression of two Res, i.e. intracisternal-A particle (IAP) and murine endogenous-retrovirus-L (MuERV-L), affected the pluripotency by losing the ability of germline transmission and started inducing the kinky tail phenotype in the chimera mice of high passage ES cell lines[50]. Therefore, the mechanism of epigenetic instability needs to be further explained and better understood, and consequently monitored when considering ES cells for transgenesis (chimera formation).

Es: mice chimera technologies

Tarkowski and Mintz made the first mouse embryonic chimeras by aggregating two eight-cell stage embryos. Since then, experimental manipulations have been modified in many different ways, for example, removing and/or reorienting cells, and adding them back at different stages. There are three commonly used methods for chimera production: (1) Diploid embryo (diploid embryo aggregation chimeras); (2) ES cells (diploid embryo aggregation and injection chimeras) (3) Diploid embryo (tetraploid embryo aggregation chimeras) and (4) ES cells (tetraploid embryo aggregation and injection chimeras). This section will focus on some of the conventional and also more recent methods for generating ES cell derived chimeras (ES chimera mice), the advantages and the disadvantages of each, and the factors that should be taken into account when one is chosen in preference to another.

ES cells - diploid blastocyst injection chimeras: This technique was initially developed by Gardner[52,53] and used the introduction of the whole ICM into the blastocysts cavity (blastocoel).

Later on, conventional blastocyst injection and assisted piezo blastocyst injection[54] was extensively used to generate progeny from ES cells. These techniques involve the microinjection of 7-15 ES cells into the blastocoel. Contribution of donor ES cells to the germline of chimera mice allows the generation of mouse strains carrying the haplotype of ES cells. The chimeras are a mixture of cells derived from both donor ES cells and the recipient embryos. The determination of all tissues in the chimeras, including cells derived from the donor ES cells, is extremely difficult. Moreover, because of the developmental potential of diploid embryos prior to ES cells, they may restrain the pluripotency of ES cells in

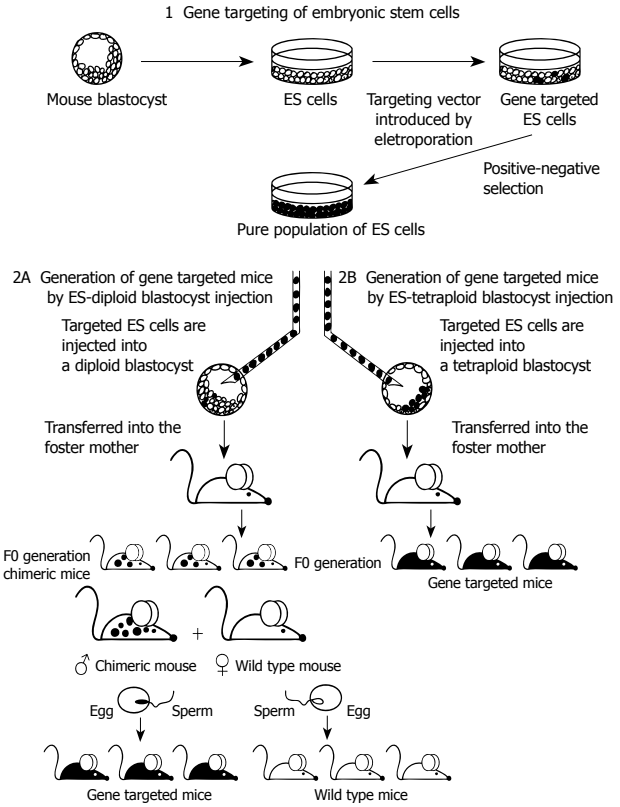

Figure 1 General strategy for producing gene targeted mice by different Embryonic Stem (ES), chimera methods. 1: Gene targeting of ES cells, followed by selection of the ES cell clones containing the desired mutations; 2A: The ES cells are injected into diploid blastocysts. F0 chimera mice are only partially derived from the modified ES cells and are bred to obtain F1 generation mice that are uniformly heterozygous for the mutation of interest; 2B: The ES cells are injected into tetraploid blastocysts. The F0 generation is fully derived from the gene targeted ES cells.

the chimeras[55]. In case of gene- targeted ES cell lines, the F0 chimera mice are only partially derived from the modified ES cells (Figure 1). If part of the germline is derived from the modified ES cells, these chimeras can be bred to obtain F1 generation mice that are uniformly heterozygous for the mutation of interest. Subsequent interbreeding of these heterozygous mice can result in F2 generation mice that are homozygous for the intended mutation. Because few mutant phenotypes can be detected in chimeric or heterozygous mutant mice, phenotyping requires derivation of homozygous mutant F2 mice. In addition, chimeras that are estimated to be 90% ES cell-derived based on coat color, can be inefficient germline transmitters, because coat color chimerism does not fully reflect ES cell contribution to internal organs (including germ cells).

In conclusion, the production of a mutant strain by using blastocyst injection method is a time-consuming task, often taking longer than 6-12 mo before the analysis of adult mutants can occur. It would be hoped that ES cell contribution is sufficient to enable germline transmission to result, with the transmission rates sufficient to enable heterozygote offspring to be obtained from 1st litters. Unfortunately, both the time and the number of mice generated to achieve that

milestone are low. It remains a challenge to achieve good and reliable results, particularly with C57BL6 ES cells, where greater variation in outcomes is likely.

ES cells - tetraploid blastocyst injection chimeras: In the chimeras produced by injection of ES cells into tetraploid (4n) embryo[56-59], the tetraploid host embryo contributes to trophoblast lineage of the placenta and the extraembryonic endoderm[5] whereas the ES cells give rise to the mesoderm layer of the yolk sac, the amnions, the embryo proper and the allantois/umbilical cord. Using this strategy, live new-born mice can be generated that are completely derived from ES cells[2,3]. Embryo electrofusion and tetraploid blastocyst microinjection is a modification of the traditional ES cell-based method to generate targeted mutant mice (Figure 1). The tetraploidy is mostly induced by passing an electrical current across 2-cell embryos, resulting in a single 4n cell produced by the fusion of the two 2n blastomeres[60,61].

The tetraploid method is limited by a number of factors, and its success appears to be highly variable, depending on host embryo blastocyst strain, ES cell strain, ES cell line passage number, and the quality of *in vitro* cell preparation. Most ES cells used to date for tetraploid blastocyst injection are of 129 mouse background strain or F1 hybrid ES cells (C57BL/6 × 129)[62]. The use of either pure 129 or C57BL/6 ES cells for tetraploid blastocyst microinjection is feasible[24,25] but to date F1 ES cells have proven to be more robust[57].

Viability of embryos from tetraploid injections is reportedly lower than with diploid embryos, with considerable strain variation[58] In addition, in one study, outbred Swiss Webster blastocysts exhibit greater developmental potential with the tetraploid technique than do blastocysts from 4n B6CBAF2 hybrid mice[57]. Post-implantation Swiss 4n embryos were observed more frequently and were more likely to develop advanced embryonic structures than 4n B6CBAF2 embryos in 4n:2n chimeras. The data show that the 4n component can persist at gastrulation and into midgestation in 4n:2n chimeras and that at later stages these 4n cells may colonize tissue sporadically throughout the embryo. The mechanism behind this difference in developmental potential is most likely explained by the presence of classes of alleles that promote or inhibit a cell's ability to regulate a duplicated genome.

A more recent retrospective study proved that outbred and hybrid tetraploid host embryos are more efficient for tetraploid complementation assay than inbred strains[23]. The reason could be that embryos used in the tetraploid procedure must not only survive *in vitro* for 3 to 4 d, but also withstand the additional electrofusion manipulation. Diminished ability of embryos to tolerate the additional manipulations would be expected with inbreeding depression. It was also shown that the use of 3 × 4n host embryos for aggregation with ES cells is more effective for generating ES mice than using 1 × 4n host embryo[63].

Another recent study reported the generation of several iPS cell lines that are capable of generating viable, live-born progeny by tetraploid complementation[9,10]. Therefore, even if the tetraploid method is limited by a number of factors, it has proven to be one of the most commonly used for mice generations fully derived from normal ES, gene targeted ES or even iPS cells.

ES cells - diploid eight-cell stage embryo injection chimeras: Interest in the ES cell injection into pre-blastocyst stage embryos was reawakened with a publication in 2007 from the US Company Regeneron[64]. Their "VelociMouse" methodology uses laser-assisted injection of ES cells into eight cell-stage host embryos, and generates fully ES cell -derived mice by an easier, more practical means from a variety of ES cell backgrounds. Further work in response to this publication has shown successful generation of fully ES cell- derived mice through the use of piezo injection[65] or through the use of standard beveled needles[66].

It was reported that F0 generation mice are able to efficiently transmit the mutation through the germline; they are fully derived from the modified ES cells and permit immediate phenotyping. The host contamination does not exceed 0.1% and demonstrates that the phenotypes of these and the eight-cell method is effective for either inbred ES cells, like C57BL/6 and 129, or hybrid ES cells[65].

The new methods were reported to be easier and more efficient than the tetraploid complementation method. On the other hand, these methods require expensive equipment and extensive experience, and demand more time than the conventional system, which may influence the quality of the micromanipulated embryos. In addition, the success of generation mice fully derived from ES cells could be, similar to the tetraploid complementation assay, highly variable. Factors like prolonged culturing, their genetic background, chromosomal abnormalities and/or the epigenetic profile of the ES cells could lead to a compromised developmental potential for high rate ES cells-derived fetuses.

CONCLUSION

In mice, ES cell lines can vary considerably in their germline transmission capacity and their developmental potential. The efficient production of live, transgenic and germline mice requires precise understanding of the essential mechanisms for the maintenance of pluripotency, and therefore germline transmission, of the ES cell lines. Retention of germline competence may be due, at least in part, to ES cell line genetic background, a reliable epigenetic profile, euploid karyotype and, last but not least, the most appropriate chimera method. Use of iPS cell lines for chimera production presents new challenges for investigators. A significant amount of further investigation must be undertaken to explore and understand the precise relationships between all the above factors in order to build appropriate mouse models and to develop therapeutic strategies for a variety of diseases. Continuous research on ES cells remains crucial in order to validate iPS cells and to determine which cells would be

most useful for specific purposes.

This review is focused on the germline chimera-forming potential of mouse ES and iPS cells. On the other hand, these findings might provide valuable insights for human cell therapy perspectives, but further clarification is needed on how ES and iPS cells differ in terms of biology, action mechanisms, and curative potential. The world will be watching these experiments to see if the field can live up to its promises. Also, for a better public understanding of science, the ethicists and non-specialists will need clear information regarding the basics of stem cell biology, and the predictive power of animal models.

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S- Editor Li LF **L- Editor** Herholdt A **E- Editor** Lin YP

World Journal of Stem Cells

Online Submissions: http://www.wjgnet.com/1948-0210office
wjsc@wjgnet.com
doi:10.4252/wjsc.v1.i1.30

World J Stem Cells 2009 December 31; 1(1): 30-35
ISSN 1948-0210 (online)

OBSERVATION

Twisting immune responses for allogeneic stem cell therapy

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Supported by CHOC Children's Foundation and CHOC Neuroscience Institute (to Li SC) and NIH grant 1R21CA134391-01A1 (to Zhong JF)
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Received: September 8, 2009 Revised: October 21, 2009
Accepted: October 28, 2009
Published online: December 31, 2009

Abstract

Stem cell-derived tissues and organs have the potential to change modern clinical science. However, rejection of allogeneic grafts by the host's immune system is an issue which needs to be addressed before embryonic stem cell-derived cells or tissues can be used as medicines. Mismatches in human leukocyte class I antigens and minor histocompatibility antigens are the central factors that are responsible for various graft-versus-host diseases. Traditional strategies usually involve suppressing the whole immune systems with drugs. There are many side effects associated with these methods. Here, we discuss an emerging strategy for manipulating the central immune tolerance by naturally "introducing" donor antigens to a host so a recipient can acquire tolerance specifically to the donor cells or tissues. This strategy has two distinct stages. The first stage restores the thymic function of adult patients with sex steroid inhibitory drugs (LHRH-A), keratinocyte growth factor (KGF), interleukin 7 (IL-7) and FMS-like tyrosine kinase 3 (FLT3). The second stage introduces hematopoietic stem cells and their downstream progenitors to the restored thymus by direct injection. Hematopoietic stem cells are used to introduce donor antigens because they have priority access to the thymus. We also review several clinical cases to explain this new strategy.

Key words: Stem cell therapy; Immune response; Allogeneic grafts

Peer reviewers: Domenico Capone, MD, Department of Neurosciences, Unit of Clinical Pharmacology, School of Medicine, Federico II University, Via S. Pansini 5, 80131 Naples, Italy; Philippe Bourin, MD, PhD, Laboratoire de thérapie cellulaire, EFS-PM, 75 rue de Lisieux, Toulouse, 31300, France

Li SC, Zhong JF. Twisting immune responses for allogeneic stem cell therapy. *World J Stem Cells* 2009; 1(1): 30-35 Available from: URL: http://www.wjgnet.com/1948-0210/full/v1/i1/30.htm DOI: http://dx.doi.org/10.4252/wjsc.v1.i1.30

INTRODUCTION

Ever since stem cells were discovered during the analysis of teratocarcinoma in 1964, stem cell-based therapy has conjured a hope for those who are suffering from previously incurable diseases, particularly degenerative diseases[1,2]. Since embryonic stem cells (ESC) are pluripotent, stem cell-derived cells and tissues are believed to be the best treatment for variety of degenerative diseases by replacing damaged tissues. However, there are still ethical and technical barriers which need to be overcome before these hopes become reality. For example, ESC-driven tumor formation is one technical barrier[3]. Immune rejection caused by foreign antigens expressed on the stem cell graft would be another major hurdle that needs an immediate solution for stem cell therapy[4-6].

Immune rejection concerns are raised when using stem cells that do not exactly match a recipient's immune system - such as existing human embryonic stem cell (hESC) lines that are not derived from the recipient[7]. For example, Wu's group showed that transplanted ESCs

died within about 7 to 10 d in mice with functioning immune systems while they survived and proliferated in immunocompromised mice[5]. They showed that secondary injections of ESCs into the immune-normal mice led to more rapid cell death, suggesting the immune system became more efficient at recognizing and rejecting the second dose of ESCs. It is believe that ESCs express certain surface proteins that trigger the recipient's immune system to attack donor ESCs as they differentiate into more-specialized tissues. Thus the first ESC injection primed the immune system to recognize the foreign molecules, and the immune system responded even more quickly to the second ESC injection. Combination of two antirejection compounds - tacrolimus and sirolimus - allowed the cells to survive for up to 28 d in mice with normal immune systems[4,5]. This is consistent with strategies to prevent T cell activation or effector function by immunosuppression in organ transplantation using pharmacological immunomodulatory agents[8-10].

Several new strategies have been developed to avoid immune rejection of stem cell-derived grafts. These include the use of novel immunosuppressants[11] and of autologous stem cells drawn from somatic cell nuclear transfer (SCNT) and inducible pluripotent stem cell (iPSC) technology[12,13]. However, little is known about the immune response toward these stem cells because of the lack of human clinical trials with these cells. Here, we discuss a new strategy to overcome the hurdle of immune rejection in stem cell therapy of human diseases.

MECHANISMS OF IMMUNE REJECTION

Immune rejection occurs when a transplanted stem cell is not accepted by the body of a transplant recipient. This is expected to happen, because the immune system is able to distinguish a foreign material within the body and try to destroy it, just as it tries to destroy infecting organisms such as bacteria and viruses[14,15]. Allogeneic graft rejection and organ maintenance are the two primary factors, which render donor organs competitive. To be eligible to receive a donor organ, an individual has to pass several compatibility tests[16]. However, rejections may occur even if a patient passes the needed compatibility tests.

Rejections are classified into three major types based on their severities: hyperacute rejection, acute rejection and chronic rejection. Hyperacute rejection happens within short duration after the transplantation process. Preexisting antibodies, which are reactive to the donor tissue, can cause a series of severe systemic inflammatory responses following by blood clotting. Therefore, the transplanted organ must be removed if hyperacute rejection occurs[17-19]. On the other hand, acute rejection and chronic rejection are less dangerous compared to hyperacute rejection. Acute rejection usually occurs within one week after the transplantation because of human leukocyte antigen system (HLA) antigen mismatch. Chronic rejection refers to mismatched minor histocompatibility complex, resulting in long-term rejection of the graft[20]. Since perfect matches between donor and recipient HLA antigens are rare, donor organ recipients often suffer from acute rejection. According to the Organ Procurement Transplant Network (OPTN) national registry in the United States, about 60%-75% of kidney recipients and 50%-60% of liver recipients will experience acute rejection[21-27]. The only available treatment for acute rejection now is either retransplantation or the use of chemotherapeutic immune suppressant like corticosteroids and calcineurin inhibitors. However, immune suppressants will affect the immune system as a whole and lead to immunocompromise complications.

Immune rejection is mediated through both T cell-mediated (direct) and humoral immune (antibodies, indirect) mechanisms. Direct rejections involve the contact between donor antigen presenting cells (APC) and recipient T cells. Antigens on the surface of donor APC can be recognized as foreign particles by recipient T cells through ligation of co-stimulatory molecules. Indirect rejections involve antigens released into the environment, which can be picked up by the recipient APCs and present to the recipient T cells[12,13]. The number of mismatched alleles determines the speed and magnitude of the rejection response. Different mechanisms act against different grafts.

ESCs and their derivatives express human leukocyte class I antigens (MHC I) and minor histocompatibility antigens, both have the potential to trigger host immune rejection[28]. Additionally, ESC may also differentiate into blood cells that express different ABO blood group antigens, which are also immunogenic. Blood group O is a universal donor, which can be selected to avoid rejections caused by ABO blood group antigen differences. In addition, ESCs also express embryo-specific antigens. These embryo-specific antigens, produced only in the embryo stage are treated as foreign particles in a fully developed human body[12,13].

Expressed xenogeneic proteins derived from culture medium may also be a source of immune rejection[29]. An immune response to these antigens may lead to a late graft loss or hastened rejection of subsequent stem cell grafts[30]. This immune rejection can be overcome either by physical immunoprotection of stem cells provided by polymer encapsulation[31-33] or by purification of stem cells before transplantation[34]. Alternative methods to eliminate this potential problem include growing cells in a serum-free medium[35-38].

TWISTING THE IMMUNE RESPONSES

The simplest way to avoid rejection is to use autologous adult stem cells (autologous bone marrow derived mesenchymal stem cells) instead of ESCs which are derived from embryos. Autologous adult stem cells express antigens identical with the hosts' cells and therefore are not subject to the rejections. However, it is difficult to isolate a sufficient amount of adult stem cells[39]. *Ex vivo* expansion and differentiation of adult

stem cells are another major challenge and roadblock for adult stem cell therapy[39]. Purifying adult stem cells from diseased cells (such as autologous bone marrow transplantation) is also technically difficult. Various methods such as SCNT and iPSC technology have been developed to yield sufficient amounts of patient-specific stem cells for cell therapy. However, these methods have not yet been achieved for the status of clinical application.

Manipulating the central self-tolerance pathway is a more immediately available approach for stem cell therapy[28]. There are two types of self-tolerance: central self-tolerance and peripheral self-tolerance[12,13]. Central self-tolerance refers to negative selection of T regulatory cells and T cells within the thymus. Under normal circumstances, T cells differentiated within the thymus do not leave the thymus immediately. Instead, they undergo a process called negative selection, which deletes the host-reactive T cells before releasing them into the circulation. On the other hand, peripheral self-tolerance refers to inappropriate co-stimulation between APCs and T cells which lead to failure to launch proper responses (inflammatory).

Technically, host-donor chimerism can be created to introduce self-tolerance to the donor cells with two steps. First, we can restore thymic function for T-cell selection in host. Second, we need to introduce donor cells into host thymus to delete T-cells that recognize donor antigens[40]. The thymus shrinks during puberty because of increasing production of sex hormones, and its functions are affected[41-44]. Therefore, thymic functions must be restored in order to manipulate self- tolerance in adults. The following strategy can be applied: first, sex steroid inhibitory drug (LHRH-A) can be used in combination with thymic growth factors including KGF, IL-7 and FLT3 to restore adult thymic functions[45]. Second, antigens derived from donor cells must be introduced into the restored thymus[46]. Hematopoietic stem cells (HSCs) can be used to deliver donor cell antigens since they have preferential access to the thymus[46]. However, injecting a large quantity of HSC may trigger serious graft-versus-host diseases, which result in side effects including hemolysis, lost of lymphocytes and possibly damaged tissues because of reactive donor T-cells. Therefore, direct injection is not ideal and an alternative method must be developed to avoid serious graft-versus-host diseases while supplying the HSC steadily to the restored thymus[47,48].

After introducing antigen, a phenomenon called mixed chimerism may occur within the thymus and the donor reactive T cells could be deleted by negative selection[49-52]. Also, T-regulatory cells formed from the thymus may migrate into the circulation and arrest those donor reactive T-cells in the circulation. This immune tolerance has been reported in experiments with mouse skin grafts[53-60].

In animal models, gradually introducing donor cells is a possible approach to create desirable chimeras for thymus restoration. Our previous study on tissue regeneration during mouse pregnancy showed that fetal stem cells repaired maternal skin injury and created fetal-maternal microchimerism[61]. This study suggests that slow release of stem cells that are minimally immunogenic could be used as an alternative method to create chimeras for donor specific immune tolerance. In another study of mouse hematopoietic stem cells, we used intraosseous infusion, a process of injection directly into the marrow of the bone, for introducing HSC into mouse[62]. Intraosseous infusion of HSC allows cells to home to bone marrow more efficiently and avoids circulating large amount of donor cells in recipient blood. This dramatically lessens the chance of immune rejection and may lead to more effective creation of chimerism[61,62].

CLINICAL SUCCESSES FOR MANIPULATING SELF-TOLERANCE

In humans, naturally occurring mixed chimerism self-tolerance has been reported[63]. In one case, a nine-year-old girl who acquired acute fulminant hepatitis from viral infection was given a liver transplant. However, the donor was a 12 year-old boy who died of brain injury having different HLA antigens (A34, 68; B50, 76; DR4, 13) from the girl's (A2, 24; B37, 62; DR7, 9). In addition, their blood types were also different. The girl was type O, RhD-negative but the boy was type O RhD-positive.

The allograft was thought to have a high potential for triggering acute and chronic rejections, which eventually might lead to destruction of the graft. Additionally both of them were cytomegalovirus positive, which may have negative effects on the immunocompromised patient. However, the liver was still transplanted since benefit was determined to be greater than the risk. Standard immunotherapy including tacrolimus and methylprednisolone was given to the girl after the transplantation. Ganciclovir was also given to the girl to try to get rid of the cytomegalovirus. Only 13 d after the transplantation, acute rejection was noted by acute biliary obstruction. The surgical formation of a communication between the common bile duct and the duodenum was performed. The immunosuppressant drugs were given continuously.

The girl suffered from moderate lymphopenia (lymphocyte count, 0.5×10^9 per liter) and anemia until about nine months after the transplantation. Her red blood cell and lymphocyte counts decreased while the white blood cell counts increased, which suggested that her B cells were making antibodies targeting the donor red blood cells resulting in hemolysis. Interestingly, her blood type changed from RhD-negative to RhD-positive, which was the donor's blood type nine months after the transplantation was given. The change showed she developed mix chimerism, which was possibly because of hematopoietic stem cells migrating from the allogeneic graft to the thymus. Therefore the physicians decided to do a series of follow-up studies including

analysis of XY chromosomes on the hematopoietic cells. The result was astonishing, 94% of her T cells, 98% of her B cells and 100% of her natural killer cells had XY chromosomes. The appearance of XY chromosomes on those cells was strong evidence suggesting HSC from the allogenetic graft had successfully migrated into her thymus and proliferated since the female only had XX chromosomes[64]. The surgeons then withdrew the immunosuppressive drug gradually to see if full engraftment could be achieved. The girl remained healthy for the next five years without any sign of rejection. Her liver was functioning normally, and her HLA antigen changed to the donor's type. In this case, the infection with cytomegaloviruses (CMV) in combination with her young age, may have resulted in a more immunocompromised state allowing the full engraftment to be achieved before the graft-versus-host diseases became lethal[63]. In such cases, intraosseous infusion of HSC may be a practical method to create the host-donor tolerance for following organ transplantation or stem cell therapy.

Another successful case was from a recipient of combined kidney and hematopoietic-cell transplants from an HLA-matched donor[65]. There was no rejection or clinical manifestations of graft-versus-host disease without immunosuppressive drugs for more than 24-mo post-transplantation. The blood analyses showed that the patient had persistent mixed chimerism and the function of the kidney allograft was normal.

CLINICAL IMPLICATION

The above case studies suggest that donor-host chimerism can be used to overcome tissue and organ rejection, and slowly introducing donor antigens into host is important to create such chimerism.

Twisting immune responses for allogeneic graft rejection will benefit millions of people worldwide. According to the OPTN (The Organ Procurement and Transplantation Network), there were about 400 000 allogeneic graft recipients in the United States of America alone during the last two decades. In addition, 100 000 patients were queuing on transplant waiting lists for various types of donor organs[26]. Unfortunately, donor organs are competitively allocated, and sometimes it takes years of waiting for an individual to finally be able to receive the donor organ. Sadly, some patients just cannot wait for that long with a dysfunctional organ.

Management of the central self-tolerance pathway provides a possible solution for not only stem cell therapy but all organ transplantation[66]. For example, the use of allogeneic "universal donor" mesenchymal stromal cells (MSCs) may be a great clinical convenience for treatment of autoimmune ailments such as multiple sclerosis[67]. The strategy of manipulating the central self-tolerance pathway may be able to change the appearance of modern clinical science and eventually benefit almost every individual worldwide.

FUTURE DIRECTIONS

The current objective is to reproduce mixed chimerism artificially within an adult with the use of HSCs and immunosuppressive drugs that can restore thymic functions. However, there are still some barriers, which need to be solved before a full engraftment can occur in an adult. Since the thymus atrophies during aging, a sex steroid inhibitory drug (LHRH-A) should be use in combination with thymic growth factors including KGF, IL-7 and FLT3 to restore adult thymic functions[43,68]. After restoration of thymic functions, HSCs need to be introduced into the thymus. Direct injection of HSC and its downstream progenitors (differentiated T cells/T regulatory cells) to the thymus in combination with immunosuppressive drugs (like tacrolimus and methylprednisolone) may allow time to eliminate donor reactive lymphocytes. This also reduces the risk and the severity of hemolysis caused by donor T-cells to the recipient red blood cells or vice versa.

In summary, host-donor chimerism can be used to introduce a specific tolerance for donor tissues. Such a self-tolerance not only makes stem cell therapy one step closer to reality, but also makes organ transplantation available for many patients who cannot find matching donors.

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S- Editor Li LF **L- Editor** Lalor PF **E- Editor** Lin YP

World Journal of Stem Cells

Online Submissions: http://www.wjgnet.com/1948-0210office
wjsc@wjgnet.com
doi:10.4252/wjsc.v1.i1.36

World J Stem Cells 2009 December 31; 1(1): 36-42
ISSN 1948-0210 (online)

REVIEW

Recent advances in stem cell research for the treatment of diabetes

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Author contributions: Noguchi H solely contributed to this paper.
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Received: September 18, 2009 Revised: October 15, 2009
Accepted: October 22, 2009
Published online: December 31, 2009

Abstract

The success achieved over the last decade with islet transplantation has intensified interest in treating diabetes, not only by cell transplantation, but also by stem cells. The formation of insulin-producing cells from pancreatic duct, acinar, and liver cells is an active area of investigation. Protocols for the *in vitro* differentiation of embryonic stem (ES) cells based on normal developmental processes, have generated insulin-producing cells, though at low efficiency and without full responsiveness to extracellular levels of glucose. Induced pluripotent stem cells, which have been generated from somatic cells by introducing Oct3/4, Sox2, Klf4, and c-Myc, and which are similar to ES cells in morphology, gene expression, epigenetic status and differentiation, can also differentiate into insulin-producing cells. Overexpression of embryonic transcription factors in stem cells could efficiently induce their differentiation into insulin-expressing cells. The purpose of this review is to demonstrate recent progress in the research for new sources of β-cells, and to discuss strategies for the treatment of diabetes.

Key words: Pancreatic stem cells; Embryonic stem cells; Islets; Pancreatic β-cells; Islet transplantation

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Noguchi H. Recent advances in stem cell research for the treatment of diabetes. *World J Stem Cells* 2009; 1(1): 36-42 Available from: URL: http://www.wjgnet.com/1948-0210/full/v1/i1/36.htm DOI: http://dx.doi.org/10.4252/wjsc.v1.i1.36

INTRODUCTION

The pancreas is a mixed exocrine and endocrine grand that controls many homeostatic functions. The exocrine pancreas consists of acinar cells and the ductal epithelium, while the endocrine pancreas consists of four cell types (α-, β-, δ-, and pancreatic-polypeptide cells). The pancreas controls body metabolism, including the digestion of foods by exocrine enzymes secreted from acinar cells and the regulation of blood glucose levels by insulin secreted from β-cells. Clinical studies have shown that transplantation of a pancreas or purified pancreatic islets can support glucose homeostasis in type 1 diabetic individuals, in whom the β-cells have been destroyed by an autoimmune reaction[1-5]. Islet transplantation carries the special advantages of being less invasive and resulting in fewer complications compared with the traditional pancreas or pancreas-kidney transplantation. However, islet transplantation efforts have limitations including the short supply of donor pancreata, the paucity of experienced islet isolation teams, side effects of immunosuppressants and poor long-term results[6]. These limitations have led to the search for other stem/progenitor cell sources of β-cells and intense interest in how the differentiation of such progenitors can be directed, or "programmed", efficiently.

The programming efforts are based on understanding how β-cells are normally generated in the embryo and how they arise during regeneration in adults, in response to tissue damage and disease. Here, we review recent studies on β-cell development and regeneration, and highlight unresolved issues in the field.

PANCREATIC β-CELL DEVELOPMENT

The pancreas is specified from the endoderm germ layer and develops from a dorsal and ventral protrusion of the primitive gut epithelium[7-9]. These two pancreatic buds grow, branch, and fuse to form the definitive pancreas. Initially, broad suppression of mesodermal Wnt and fibroblast growth factor (FGF) signaling in the foregut enables pancreas induction, whereas active mesodermal Wnt signaling in the posterior gut suppresses these tissue fates[10,11]. Retinoic acid signaling, apparently from paraxial mesoderm cells, helps to further refine the anterior-posterior position, in which the liver and pancreas can develop from the gut endoderm[12-15]. Subsequently, in the ventral foregut, FGF from the cardiac mesoderm and bone morphogenetic protein/transforming growth factor-β (BMP/TGF-β) from septum transversum mesenchyme cells coordinately induce the liver program and suppress the pancreas program[16-19]. In the dorsal foregut, signals from the notochord, including activin and FGF, suppress sonic hedgehog (shh) signaling within the endoderm and allow the pancreatic program[20,21].

The newly specified pancreatic endoderm is initially marked by the expression of the pancreatic and duodenal homeobox gene 1 (Pdx1; also known as Ipf1) and then by the pancreas specific transcription factor 1a (Ptf1a)[22,23]. Both proteins are crucial for pancreatic development. Pdx1 marks all pancreatic and midgut progenitors[22] and is crucial for development after the bud stage[24,25]. Pdx1 levels also help to control the balance between the endocrine and exocrine (acinar and duct) progenitors that differentiate within the pancreas[26]. Notch signaling also helps to regulate the balance of exocrine and endocrine cells, probably by allowing the expansion of an undifferentiated pancreatic-progenitor population[27-29]. Loss of Notch signaling allows the endocrine lineage to develop, which is marked by and requires the basic helix-loop-helix (bHLH) transcription factor, neurogenin 3 (Ngn3)[22,27,30]. Ngn3 directly influences the expression of another islet specific bHLH gene, neurogenic differentiation (NeuroD; also known as BETA2)[31]. A loss of function assay of NeuroD/BETA2 implicates a phenotype similar to, but less severe than, Ngn3, leading to a diminished number of all endocrine cell types[32]. Then, definitive β cells are generated under the influence of the v-maf musculoaponeurotic fibrosarcoma oncogene homolog A (MafA) transcription factor[33,34].

EMBRYONIC STEM (ES) CELLS

ES cells, which are pluripotent diploid cells and can be induced to differentiate into cells of all three germ layers both *in vivo* and *in vitro*[35,36], are a potentially abundant source of β-cells. It has been reported that ES cells from mouse[37-40], monkey[41], and human[38,42] were able to differentiate into insulin-positive cells, a potential source of new β-cells. Numerous groups have been developing ES cell differentiation protocols that attempt to mimic normal embryonic development. The first step of pancreatic development is the induction of a definitive endoderm using high concentrations of activin A treatment[43,44]. Further treatment in sequential stages with keratinocyte growth factor (KGF), retinoic acid, Noggin, and cyclopamine (the hedgehog-signaling inhibitor) can then direct definitive endoderm toward Pdx1-expressing posterior foregut endoderm cells[45,46]. Treatment with DAPT and exendin-4 recruited the Pdx1-expressing posterior foregut endoderm cells to the pancreatic and endocrine lineages, which expressed Pdx1, Nkx6-1, Nkx2-2, Ngn3, and/or Pax4. After treatment with exendin-4, IGF-1, and HGF, endocrine cells expressing the pancreatic hormones insulin, glucagon, somatostatin, pancreatic polypeptide and ghrelin are produced (Figure 1).

Melton's group recently reported small molecules that efficiently direct endodermal differentiation of mouse and human embryonic stem cells[47]. In a screen of 4000 compounds, they identified two cell-permeable small molecules that direct differentiation of ES cells into the endodermal lineage. The efficiency of differentiation into definitive endoderm using these compounds was higher than that achieved by Activin A or Nodal, which commonly used protein inducers of endoderm. The definitive endoderm induced by these compounds was able to participate in normal development when injected into developing embryos, and was able to form pancreatic progenitors. These small molecules could induce reproducible and efficient differentiation of ES cells into endoderm.

On the other hand, a significant number of problems remain unsolved in terms of clinical application of ES cells, such as the risk of tumorigenicity and immunosuppression after transplantation. The ethical issue is another major obstacle to the clinical use of ES cells.

INDUCED PLURIPOTENT STEM (iPS) CELLS

iPS cells are also pluripotent diploid cells that can be induced to differentiate into cells of all three germ layers both *in vivo* and *in vitro*. Moreover, iPS cells have fewer ethical issues compared with ES cells, because iPS cells can be established from somatic cells. Initial iPS cells have been generated from mouse and human somatic cells by introducing Oct3/4 and Sox2 with either Klf4 and c-Myc or Nanog and Lin28, using retroviruses[48-51]. Recently, it has been reported that valproic acid (VPA), a histone deacetylase inhibitor, enables reprogramming of primary human fibroblasts with only two factors, Oct4 and Sox2, without the need for the c-Myc or Klf4[52]. The results support the possibility of reprogramming by chemical means, which would make therapeutic use of reprogrammed cells safer and more practical. Another

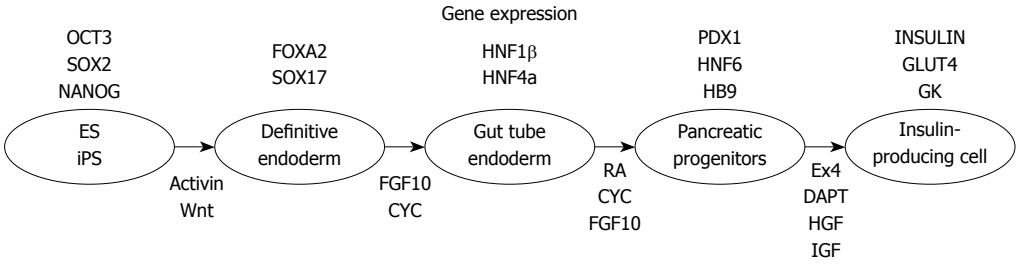

Figure 1 Schematic representation of the stepwise differentiation of embryonic stem (ES) and induced pluripotent stem (iPS) cells to insulin-producing cells. CYC: KAAD-cyclopamine; RA: All-trans retinoic acid; DAPT: γ-secretase inhibitor; Ex4: Exendin-4; FGF: Fibroblast growth factor.

group showed that adult mouse neural stem cells (NSCs) expressed higher endogenous levels of Sox2 and c-Myc than embryonic stem cells[53] and that exogenous expression of the germline-specific transcription factor Oct4 was sufficient to generate pluripotent stem cells from adult mouse NSCs[54]. These data suggest that, in inducing pluripotency, the number of reprogramming factors can be reduced when using somatic cells that endogenously express appropriate levels of complementing factors.

On the other hand, retroviral integration of the transcription factors might activate or inactivate host genes, resulting in tumorigenecity, as was the case in some patients who underwent gene therapy. Yamanaka's group reported the generation of mouse iPS cells with transient expressions of Oct3/4, Sox2, Klf4, and c-Myc from plasmids. Repeated transfection into mouse embryonic fibroblasts of two expression plasmids, one containing complementary DNAs (cDNAs) for Oct3/4, Sox2, and Klf4 and the other containing the c-Myc cDNA, resulted in iPS cells without evidence of plasmid integration[55]. At the same time, another group demonstrated the generation of mouse iPS cells from fibroblasts and liver cells using non-integrating adenoviruses transiently expressing Oct4, Sox2, Klf4, and c-Myc[56]. Moreover, Ding's group reported generation of protein-induced pluripotent stem cells (piPSCs) from murine embryonic fibroblasts using recombinant cell-penetrating reprogramming proteins without transfection of any genes[57]. For efficient transduction of four reprogramming factors, Oct4, Sox2, Klf4, and c-Myc, into cells, they used protein transduction technology[58-61]. A poly-arginine (11R) protein transduction domain (PTD) fused to the C terminus of these reprogramming factors efficiently delivered the proteins into cells and induced iPS cells, which demonstrated long-term self-renewal and were pluripotent *in vitro* and *in vivo*. These reports provide strong evidence that insertional mutagenesis is not required for *in vitro* reprogramming. The production of iPS cells without integration into the host genome addresses a critical safety concern for potential use of iPS cells in regenerative medicine.

Although some papers have shown the generation of insulin-secreting islet-like clusters from human iPS cells[62,63], the efficiency of the method seems low. The method, as detailed in this review in the ES cells section, might represent a critical step in the development of insulin-producing cells from iPS cells (Figure 1). Indeed, Melton's group recently reported generation of iPS cells from patients with type 1 diabetes and differentiation from the iPS cells into insulin-producing cells using this method[64].

PANCREATIC STEM/PROGENITOR CELLS

Although it is clear that the majority of new β-cells derive from pre-existing insulin-expressing cells after surgical injury[65,66], several *in vitro* studies have shown that insulin-producing cells can be generated from adult pancreatic ductal tissues[67-71]. A recent study has shown that duct ligation can activate Ngn3-positive β-cell precursors in the ductal epithelium[72]. The Edmonton group has shown that, in clinical islet transplantation, a significant positive correlation exists between the number of ductal-epithelial cells transplanted and long-term metabolic success, as assessed by an intravenous glucose tolerance test at approximately two years post-transplantation. No significant correlation was observed between the total islet equivalents and long-term metabolic success[73]. Cells in the pancreatic anlage migrate from the ducts while differentiating to form clusters that will eventually become islets during embryonic development [74]; therefore, the post-natal pancreatic duct might harbor islet precursor/stem cells. Inada *et al*[75] generated transgenic mice expressing human carbonic anhydrase Ⅱ (CAⅡ) promoter-Cre recombinase or inducible CreER to cross with ROSA26 loxP-Stop-loxP LacZ reporter mice. CAⅡ-expressing cells within the pancreas act as progenitors that give rise to both new islets and acini normally after birth and after injury (ductal ligation). This identification of a differentiated pancreatic cell type as an *in vivo* progenitor of all differentiated pancreatic cell types has implications for a potential expandable source of new islets for replenishment therapy for diabetes[75]. Such interesting results suggest the possibility of multipotent progenitors in adult pancreatic ducts.

Mouse pancreatic stem cells have been isolated from duct-rich population, which are capable of self-renewal and multipotency[76,77]. On the other hand, human cells from the duct-rich population were unable to divide after 30 d under several culture conditions, although the cells were able to differentiate into insulin-producing cells[78]. There are some differences between the methodologies

used by the two groups, such as culture conditions, isolation stresses, and/or species themselves. The ability of β-cells to expand is limited, especially in the adult, and the partial growth ability is insufficient to permit recovery from cell loss in type 1 diabetes[79]. Therefore, it is important to isolate human pancreatic "stem" cells comprising a sufficient number of β-cells for the treatment of diabetes.

The transdifferentiation of acinar cells to islets has also been proposed[80-82]. Melton's group showed *in vivo* reprogramming of adult pancreatic exocrine cells to β-cells by viral delivery of the developmental transcription factors Pdx1, Ngn3, and MafA[83]. Pancreatic exocrine cells greatly outnumber β-cells; therefore, the transdifferentiation of acinar cells to β-cells is also an interesting possibility.

MESENCHYMAL STEM CELLS

Another interesting stem cell in this field is the mesenchymal stem cell (MSC). It has been reported that marginal mass islet transplantation with autologous MSCs promotes long-term islet allograft survival and sustained normoglycemia[84]. MSCs also prevent the rejection of fully allogenic islet grafts by the immunosuppressive activity of matrix metalloproteinase-2 and -9[85], and protect NOD mice from diabetes by inducing regulatory T cells[86].

PERSPECTIVES

Several reports have suggested that epitopic transdifferentiation is also possible. *In vivo* transduction of mice with an adenovirus expressing Pdx-1[87,88], and both betacellulin and NeuroD[89], or a modified form of Pdx-1 carrying the VP16 transcriptional activation domain[90], or MafA together with Pdx-1 and NeuroD[91], markedly increased insulin biosynthesis and induced various pancreas-related factors in the liver. The existence of potential β-cell precursors in the adult liver is of obvious medical interest. Moreover, overexpression of embryonic transcription factors in stem cells could efficiently induce their differentiation into insulin-expressing cells. We reported that transduction of Pdx-1 and NeuroD proteins induces insulin gene expression[67,92,93]. Other groups also showed that transduction of NeuroD *in vivo* or TAT-Ngn3 fused TAT-PTD induced insulin-producing cells[94,95]. The production of insulin-producing cells using protein transduction technology without gene transduction addresses a critical safety concern for potential use of the cells in regenerative medicine. Further investigations to induce differentiation of stem/progenitor cells into insulin-producing cells will help to establish cell-based therapies in diabetes.

ACKNOWLEDGMENTS

The author thank Dr. Steven Phillips for editing the manuscript.

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S- Editor Li LF **L- Editor** Stewart GJ **E- Editor** Lin YP

World Journal of Stem Cells

Online Submissions: http://www.wjgnet.com/1948-0210office
wjsc@wjgnet.com
doi:10.4252/wjsc.v1.i1.43

World J Stem Cells 2009 December 31; 1(1): 43-48
ISSN 1948-0210 (online)

REVIEW

The famous *versus* the inconvenient - or the dawn and the rise of 3D-culture systems

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Supported by The European Union Grant STREP NMP3-CT-29005-013811 (to Welle A); the Bundesministerium für Bildung und Forschung Grant 03ZIK-465 (to Altmann B), Germany
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Received: November 28, 2009 Revised: December 9, 2009
Accepted: December 16, 2009
Published online: December 31, 2009

Abstract

One of the greatest impacts on *in vitro* cell biology was the introduction of three-dimensional (3D) culture systems more than six decades ago and this era may be called the dawn of 3D-tissue culture. Although the advantages were obvious, this field of research was a "sleeping beauty" until the 1970s when multicellular spheroids were discovered as ideal tumor models. With this rebirth, organotypical culture systems became valuable tools and this trend continues to increase. While in the beginning, simple approaches, such as aggregation culture techniques, were favored due to their simplicity and convenience, now more sophisticated systems are used and are still being developed. One of the boosts in the development of new culture techniques arises from elaborate manufacturing and surface modification techniques, especially micro and nano system technologies that have either improved dramatically or have evolved very recently. With the help of these tools, it will soon be possible to generate even more sophisticated and more organotypic-like culture systems. Since 3D perfused or superfused systems are much more complex to set up and maintain compared to use of petri dishes and culture flasks, the added value of 3D approaches still needs to be demonstrated.

Key words: Three-dimensional cell culture; Microbioreactors; Chips; Scaffold; Perfusion

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Altmann B, Welle A, Giselbrecht S, Truckenmüller R, Gottwald E. The famous *versus* the inconvenient - or the dawn and the rise of 3D-culture systems. *World J Stem Cells* 2009; 1(1): 43-48 Available from: URL: http://www.wjgnet.com/1948-0210/full/v1/i1/43.htm DOI: http://dx.doi.org/10.4252/wjsc.v1.i1.43

INTRODUCTION

Since Petri published his methodology of growing bacteria in flat glass dishes in 1887[1], scientists have used this culture format for growing, not only prokaryotes, but all kinds of eukaryotic cells and tissues. Even if this culture technique is simple and convenient in daily cell culture routines, it is undisputable that growing cells on flat substrates is insufficient to reflect complex systems like tissues or whole organs. With this consideration, the introduction and systematic characterization of new

culture techniques, generating spherical aggregates of isolated embryonic cells by Holtfreter in 1944[2] and Moscona in 1952[3-5], revealed new insights in tissue morphogenesis and opened a new chapter in cell culture technique. During the following years, studies on cell aggregates were extended to the research field of tumor biology and were advanced in the 1970s by Sutherland *et al*[6], who used "multicellular spheroids" as a tumor model for radiation experiments. Three-dimensional (3D)-culture models more closely resemble the *in vivo* situation concerning cell shape and the microenvironment. Compared to traditional monolayer techniques, it was shown that three-dimensionality is able to restore and maintain the differentiated status of adult cells, such as hepatocytes[7-9], cardiac myocytes[10,11], chondrocytes[12,13], and endocrine pancreatic islet cells[14] *in vitro*. Moreover, this culture configuration was applied to the study of the growth and differentiation of progenitor cells such as osteoblasts[15,16], hematopoietic progenitor cells[17], and embryonic and mesenchymal stem cells[18-23]. More importantly, a considerable amount of stem or progenitor cell cultivation techniques require, at least temporarily, aggregation into embryoid bodies for proper differentiation[24].

STATE OF THE ART OF 3D-CULTURE SYSTEMS

Systematic analysis of various cell types in conventional monolayer- and 3D-culture revealed that parameters like spatial and temporal gradients of soluble factors (growth factors, cytokines and hormones), homologous and heterologous cell-cell contacts, cell-matrix interactions, which are undoubtedly coupled with the molecular and physical properties of the matrix, mechanical forces like fluid flow, as well as surface topography and chemistry of the cell culture substrate are of particular importance for cellular behavior *in vitro*[25-30]. Based on this knowledge, numerous 3D-culture systems have been developed to restore and maintain or induce cellular differentiation *in vitro*: (1) explant cultures of tissue slices or perfused whole organs, which retain tissue architecture; (2) cultivation of reaggregated cells (e.g. spheroids, embryoid bodies) or simple micro-mass cultures in which isolated cells are pelleted; (3) three-dimensional cultivation of isolated cells embedded in gels or immobilized on porous matrices in stationary culture; and (4) systems using micro-bioreactors for high density 3D-cultures with active nutrient and gas supply. In the remainder of this manuscript, only the latter two systems, using isolated cells together with synthetic scaffolds in 3D culture configurations, will be discussed in detail.

3D-MATRICES

One frequently used culture technique is to entrap cells in natural or synthetic hydrogels consisting of extracellular matrix (ECM) components (e.g. collagen, laminin, Matrigel, hyaluronic acid), natural polymers like alginate and chitosan or synthetic polymers comprising polyethylene glycol, synthetic self-assembling peptides or artificial DNA molecules[31,32]. Due to their mechanical and biochemical properties, hydrogels mimic the nature of soft tissues and provide a 3D network for cell-matrix interactions. Furthermore, the vast number of biocompatible natural and synthetic materials, which can be utilized in combination, turns hydrogels into many useful 3D-substrates. However, as hydrogels lack a distinct porous structure corresponding to blood and lymphatic vessels, mass transport in gels depends on slow diffusion through the gel and consequently leads to the formation of gradients of oxygen, nutrients, metabolites, and soluble factors (e.g. growth factors, hormones) within the gel-matrix. Therefore, gel-based systems without any forced medium flow are limited to rather small setups, at least in one dimension, as exemplified by several thin gel sandwich constructs or very low densities of cells with low metabolism-like cartilage[33]. The lack of mechanical stability of gel based tissue models often hampers the use of preformed tissues for implantations, especially if a certain load bearing is needed with the beginning of the *in vivo* application. Therefore, approaches to culture cells in a 3D configuration in combination with porous 3D-matrices based on sponge-like structures, usually prepared from biodegradable polymers, became attractive. Sponge-like structures exhibit larger pores than pure hydrogels and thus facilitate cell seeding and colonization of the substrates. Important parameters for their application in cell culture are the number of pores, pore size, as well as interconnectivity and distribution of the pores[31]. If the fenestrations are smaller than the cell size or the interconnectivity of the pores within the scaffold is poor, cell migration into the 3D-matrix is limited and thus cell distribution is restricted to near-surface layers of the substrate. Instead, perfused open porous foams from polylactic-co-glycolic acid that are collagenized and inoculated with immortalized bovine capillary endothelial cells and a hepatoma cell line (C3A), show a spatial separation upon *in vitro* culture; endothelial cells invade the foam completely whereas the hepatoma cells form a dense layer on the inflow side of the spongeous matrix (Figure 1).

Depending on the cell type and pore size, a monolayer formation within the scaffold can be observed. Thus, optimal size and interconnectivity of the pores may vary and must be determined for each cell type used. Although a variety of materials can be used to produce porous sponge-like scaffolds, the most common are natural polymers often used for hydrogels (e.g. alginate, chitosan, collagen), synthetic polymers like polylactic acid, polyglycolic acid and their copolymers or composite material[31]. Experiments in our laboratory with the hepatoma cell line HepG2 in alginate sponges revealed that, despite a larger pore size compared to hydrogels, mass transport between sponge and culture medium was limited in stationary culture conditions (unpublished data).

Another approach to culture cells in a 3D configuration came along with new technologies for scaffold

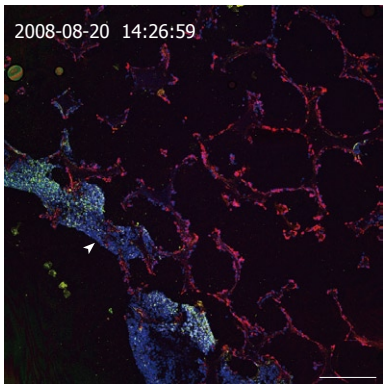

Figure 1 Cross section of a resin embedded co-culture of a hepatoma cell line (C3A) and immortalized BCE in polylactic-co-glycolic acid foam. Medium inflow from the lower left side into the polymer foam (arrowhead). Staining of cytokeratin 18 (green, C3A cells), vimentin (red, BCE cells), Draq5 nuclear stain (blue, both cell types). Scale bar: 250 μm.

fabrication that comprises the immobilization of cells in fibrous 3D-matrices. Fibrous scaffolds can be produced in an electrospinning process that allows the creation of micro- and nano-fibers from a polymer solution and the subsequent deposition of a non-woven fibrous mesh on a collector[34,35]. This technique allows the fabrication of two dimensional or 3D-matrices depending on the thickness of the deposited fiber network on the collector. Fiber diameter can range from 3 nm to greater than 5 μm[34] and therefore electrospun nanofibers reflect, in part, the fibrous structure of natural extracellular matrix components. Commonly used materials for nanofiber scaffolds are synthetic polymers like polylactic-co-glycolic acid, poly-L-lactic acid-co-ε-caprolactone, poly-ε-caprolactone, polyamide and natural polymers like collagen, elastin, fibrinogen, alginate or hyaluronic acid or even combinations thereof[34]. One example of 3D tissue culture scaffolds is based on electrospun biodegradable aliphatic polycarbonates comprising photochemical post-treatments[36]. These scaffolds exhibit important advantages when compared with foams since the interconnectivity of voids available for tissue ingrowth is perfect. This is realized by a photopatterning of the non-woven fabric selectively lowering the molecular weight of the used polymer and, in turn, speeding up biodegradation. Within a couple of days, voids are formed in the scaffold, opening ways for increased perfusion and tissue/vessel ingrowth. In addition, ultrathin fibers produced by electrospinning offer an unsurpassed surface to volume ratio among applied tissue scaffolds. This has important consequences on the availability and presentation of polymer bound signaling molecules and on degradation rates of biodegradable scaffolds. Finally, electrospinning offers new 3D scaffolds with double length scale features with combinations of microfibers and electrospun nanofibers[37].

In contrast to the above mentioned techniques, we use a culture system developed at the Karlsruhe Institute of Technology that is based on a microstructured polymer chip that serves as a scaffold for the 3D cultivation of cells[38-41]. Currently, the chip is manufactured in two

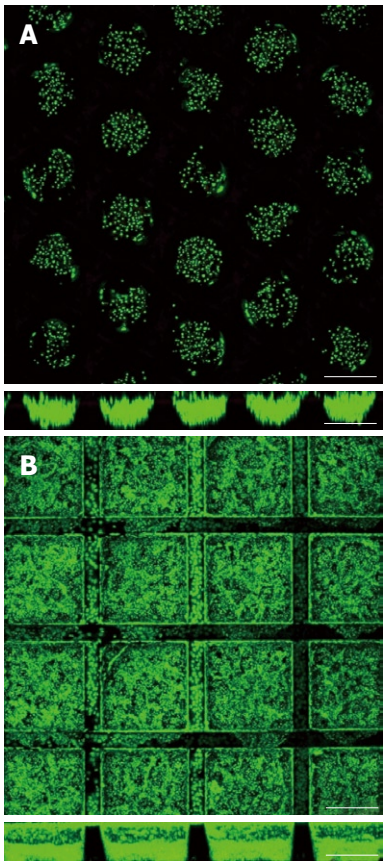

Figure 2 Primary human hepatocytes and Hep G2 hepatoma cells 5 d and 24 h after cell seeding into r- (A) and cf-KITChips (B) respectively (upper panels: top view, lower panels: cross section). The r-KITChip (20 mm × 20 mm in total) is comprised of up to 625 round microcontainers (diameter up to 300 μm, depth up to 300 μm) or 1156 cubic microcontainers (300 μm × 300 μm × 300 μm in w × l × h) for the cf-KITChip of which 5 × 5 can be seen in 2A and 4 × 4 can be seen in 2B. Live cell staining with Syto 16. Scale bar: 250 μm.

different designs varying mainly in geometry and the manufacturing process. The so-called cf-KITChip has outer measures of 20 mm × 20 mm and a central grid-like microstructured area of 10 mm × 10 mm to 14 mm × 14 mm with cubic microcavities in which cells can organize into multicellular aggregates (Figure 2B). The cavities of the chips are open to the top and are 300 μm in each direction (w × l × d). However, the size and the shape of the microcavities can be adjusted to experimental needs. The bottom of the chip consists of a track etched polycarbonate (PC) membrane (10 μm thickness) with a high pore density (2×10^6 pores per cm^2) and a pore size of 3 μm. Thus, mass exchange by diffusion through the membrane is facilitated and cell migration onto the back of the chip is prevented at the same time. The manufacturing process comprises a microreplication technique, such as microinjection molding or vacuum hot embossing of polymethylmethacrylate (PMMA) or polycarbonate (PC), to produce the container array of the scaffold and a solvent-vapor-welding technique to bond the perforated membrane to the back of the chip. The so-called r-KITChip represents another variant of the polymer chip. It differs in its current design from the cf-KITChip by the round geometry of the microcavities

and the fabrication process called SMART (Substrate Modification and Replication by Thermoforming), which allows the production of chips from thin polymer films[42,43]. SMART consists of a combination of microtechnical thermoforming or microthermoforming and various material modification techniques, and thus allows a site-specific functionalization of 3D cavities. By the combination of microthermoforming and ion track technology, for instance, highly porous thin-walled microcavity arrays can be produced. Compared to hydrogels and nanofibrous or sponge-like scaffolds, the uniform geometry of the microstructured polymer chip allows the formation of cell aggregates with defined size in the microcavities (Figure 2). This is of particular importance in terms of a homogenous mass transport and diffusion gradients within the cell aggregates and the whole scaffold. Moreover, the influence of aggregate size on cell differentiation could be recently demonstrated for human embryonic stem cells[44]. Therefore, chips with defined geometries for cellular aggregation may be helpful tools in stem cell research. Another important advantage of the chip is the defined surface area, which permits the application of known cell densities in the chip cavities and defined surface modifications like coating with extracellular matrix components, leading to distinct culture conditions and reproducible experiments. In this context, simple coating of the chip surface with extracted extracellular matrix proteins, such as collagen, represents a rather simple surface modification, whereas more sophisticated modification techniques have been developed in our laboratory, for example, the integration of defined nanotopographies on the inner surface of the curved microcavity walls[45].

MICRO-BIOREACTORS FOR 3D-CULTURE

As a result of cellular metabolic activity, 3D high density cell culture can lead to limited nutrient and oxygen supply as well as accumulation of toxic metabolites in the tissue construct. Furthermore, it has been shown that fluid flow or shear stress can influence cell behavior like osteogenic differentiation of human mesenchymal stem cells[46-49]. Micro-bioreactors specifically designed for 3D cell culture provide an opportunity to overcome these mass transfer limitations in high density cell cultures and offers the possibility of studying the influence of mechanical forces like fluid flow or hydrodynamic pressure on cell responses. For this purpose numerous bioreactor designs have been developed, which can be divided in stirred flasks like spinner-flask or rotating-wall vessel (RWV) bioreactors, fluidized or fixed bed bioreactors, hollow-fiber bioreactors and systems using perfused scaffolds. All these systems to some extent use a combination of common 3D cell culture techniques like gel-based techniques, spheroids, encapsulated or immobilized cells on various types of 3D-matrices. However, many bioreactor systems must cope with difficulties like large death volumes, heterogeneous cell distribution in the scaffold or bioreactor, large diffusion distances and non-uniform perfusion of the scaffolds due to different flow resistances inside the matrices[50]. For instance, in hollow-fiber bioreactors cells may be embedded in gels to improve cell distribution and are cultured inside or outside of semi-permeable hollow fibers, while culture medium flows on the reverse side, respectively. In these systems mass transport takes place by diffusion and it has to be considered that fiber diameter and length play an important role since radial and longitudinal gradients may be formed.

Systems using encapsulated cells like fluidized or fixed bed bioreactors show similar mass transfer limitations due to slow diffusion across the capsules[50]. Bioreactors based on perfused scaffolds show a better nutrient supply compared to the above mentioned systems since cells immobilized on 3D-matrices are in direct contact with the culture medium. However, not all of the systems, termed "perfusion systems", use a setup where scaffolds are directly perfused with culture medium. More precisely, one should differentiate between perfused culture chambers where culture medium flows around the scaffold[51,52], and perfusion through the scaffold and the tissue inside[46,53,54]. For better differentiation between the two different setups, we have coined the term superfusion for the flow around the scaffold. The KITChip-culture system is comprised of a chip and a bioreactor that allows the use of both superfusion and perfusion and even a combination of the two. Moreover, sensors for oxygen and other determinations can easily be integrated.

CONCLUSION

Since the early days of 3D-culture a vast number of investigations have been performed to identify the factors relevant for cell survival, proliferation and/or differentiation *in vitro*. Based on progress in the research fields of biology, material science and engineering, a multitude of different culture techniques, sophisticated cell culture scaffolds and micro-bioreactors have been developed that are nearly as diverse as the tissues of the body.

Based on the advances in surface modification and micro- and nano-structuring techniques, new applications and, therefore most likely, new concepts for 3D-tissue cultures will arise. Today, scientists already provide a tool box for the design of appropriate 3D-culture configurations depending on the cell type and experimental setup, thus moving closer to *in vivo* conditions. This is of particular importance with regard to control stem cell maintenance, expansion and differentiation, as well as the generation of artificial tissue for applications in medicine or high-throughput screening systems in the pharmaceutical and chemical industry.

However, if 3D cell culture techniques better reflect the natural microenvironment of tissues and current advanced technologies allow the design and fabrication of numerous 3D-culture systems, why then is the Petri dish, or rather the monolayer culture, still the standard

technique in most cell culture labs? The reasons are simple and convincing: monolayer culture devices are easy to manufacture and thus they are inexpensive to produce, which in turn allows mass production. Many companies have a large portfolio of related products and, last but not least, they are easy to handle. Especially the latter and the fact that many 3D-culture systems are of academic nature and not commercially available are the major obstacles that prevent faster distribution of organotypic culture systems and their becoming new standards. For instance, commercially available 3D-culture systems comprise mainly sponges (e.g. collagen or calcium-phosphate sponges), hydrogels made of natural polymers like alginate or extracellular matrix components or more rare synthetic peptide hydrogels and cell culture flasks coated with nanofibers representing a synthetic substrate for cells in monolayer culture. All these systems are designed for stationary culture in multiwell cell culture plates, while available fluidic 3D-culture systems using bioreactors are based on encapsulated cells or cells immobilized on microcarriers in rotating bed/wall vessel bioreactors displaying in part the already discussed limitations. Furthermore, many standardized techniques for cell analysis used so far in conventional monolayer culture, like cell lysis for mRNA or protein extraction, immunostaining or quantification of secreted proteins into the culture medium, are often difficult to transfer to 3D-culture systems, especially in gel-based systems as gels often hinder the accessibility of the cells. However, as more and more academic systems become commercially available, the increasing number of standard protocols adapted to 3D-cultures will help to improve their acceptance and diffusion.

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S- Editor Li LF **L- Editor** Lutze M **E- Editor** Lin YP

World Journal of Stem Cells

Online Submissions: http://www.wjgnet.com/1948-0210office
wjsc@wjgnet.com
doi:10.4252/wjsc.v1.i1.49

World J Stem Cells 2009 December 31; 1(1): 49-54
ISSN 1948-0210 (online)

REVIEW

Targeting leukemia stem cells: The new goal of therapy in adult acute myeloid leukemia

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Received: July 2, 2009 Revised: August 20, 2009
Accepted: August 27, 2009
Published online: December 31, 2009

Abstract

The most popular view of hematopoietic cell lineage organization is that of complex reactive or adaptative systems. Leukemia contains a subpopulation of cells that display characteristics of stem cells. These cells maintain tumor growth. The properties of leukemia stem cells indicate that current conventional chemotherapy, directed against the bulk of the tumor, will not be effective. Leukemia stem cells are quiescent and do not respond to cell cycle-specific cytotoxic agents used to treat leukemia and thus contribute to treatment failure. New strategies are required that specifically target this malignant stem cell population.

Key words: Acute myeloid leukemia; Leukemia stem cells; Targeted therapy; Prognosis

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Thomas X. Targeting leukemia stem cells: The new goal of therapy in adult acute myeloid leukemia. *World J Stem Cells* 2009; 1(1): 49-54 Available from: URL: http://www.wjgnet.com/1948-0210/full/v1/i1/49.htm DOI: http://dx.doi.org/10.4252/wjsc.v1.i1.49

INTRODUCTION

Acute myeloid leukemia (AML) is a paradigm of cancer stem cells with a hierarchy analogous to that seen in normal hematopoiesis. Leukemia stem cells have long-term repopulating potential and the ability to propagate and maintain the AML phenotype. By their ability to hijack homeostatic mechanisms and take refuge within the sanctuary of the bone marrow microenvironment, they consequently contribute to disease resistance. Thus, targeting this stem cell population and its microenvironment is a new goal for therapy of adult AML.

THE LEUKEMIA STEM CELL HYPOTHESIS

Leukemia, as with all malignant diseases, undergoes a series of genetic events that result in the activation or overexpression of genes promoting proliferation, the silencing of genes involved in the inhibition of proliferation, and the development of the ability to elude apoptosis. However, this does not explain self-renewal, clonal expansion, and additional mutations. Over the past few years, it has been recognized that malignant diseases contain a particular subpopulation of cells with biological features that are reminiscent of stem cells[1]. The modern concept of "cancer stem cell" was promoted by John Dick and colleagues, who showed that cells with the ability to transfer human AML to NOD/SCID mice are frequently found exclusively in the $CD34^+$ $CD38^-$ compartment[2,3]. Stem cells modulate tissue formation, and maintenance and repair, based on a complex interaction of cell-autonomous and cell-non autonomous regulatory mechanisms[4]. In the hematopoietic system, there are three different populations of multipotent progenitors: (1) stem cells with a capacity for long-term renewal; (2) stem cells with a capacity for short-term renewal; and (3) and multipotent progenitors that cannot renew but differentiate into the varied lineage and undergo rapid division, allowing them to populate the bone marrow[5]. Classically, a slow cycling fraction of cells is generating a fast cycling fraction. However, an

alternative hypothesis predicts that all tumor cells have the potential to self-renew and recapitulate the tumor, but with a low probability that any tumor cell enters the cell cycle and finds a permissive environment[5].

THE LEUKEMIA THERAPEUTIC CHALLENGE

AML is one of the most common leukemias in adults. The outcome of therapy for AML has improved over recent years, mainly in younger patients. However, the challenges in this area remain considerable. The incidence of AML increases with age. AML is therefore primarily a disease of the elderly. This patient population has a very poor prognosis, which is attributed to having a disease that is inherently more resistant to current standard cytotoxic agents in relationship with acquired genetic characteristics of the leukemia, and/or relatively poor tolerance of these agents because of comorbidity and reduced tolerance of adverse effects. In contrast to the molecular mechanisms of leukemogenesis in children and younger adults, recent studies indicate that the majority of cases of AML in the elderly have quite distinct biological and molecular genetic features[6]. These features include trilineage dysplasia, complex unfavorable cytogenetic abnormalities (involving chromosomes 5 and/or 7, del(5q), del(7q), abnormalities of 11q, inv(3), and complex or multiple abnormalities), the potential for clonal remissions and reversion to an MDS marrow picture at remission. There is also a high incidence of drug-resistant phenotypes (mediated by MDR-1/P-glycoprotein or other members of the ABC transporter family). Treatment options for the older AML patient population (≥ 65 years) are limited. The traditional chemotherapeutic approach to a patient with AML has been based on treatment with a combination of an anthracycline (or anthracenedione) with cytarabine. The unmet therapeutic need is therefore greatest among older patients with AML, in whom response rates are comparatively low (50% for those over 60 years old), relapse rates are exceedingly high (more than 85%), and long-term survival rates are less than 10%[7,8]. Current chemotherapeutic options provide essentially no chance for durable remission, and toxicity of the treatment is significant. Nearly all patients relapse, and the median survival is approximately nine months. Consequently, many older patients with AML are not offered, or choose to decline, traditional intensive chemotherapy and receive supportive care only. More effective therapies to provide durable remissions in a significant proportion of patients and less toxic therapies, which could be offered to more patients, are desperately needed for the treatment of AML in the elderly. Biological insights into the mechanisms of defective molecular pathways in malignant cells have recently resulted in the identification of novel targets for drug development. New drugs are currently in early clinical development with the aim of circumventing chemotherapy resistance.

The possible existence of a rare stem cell-like population of cells within a much larger pool of malignant cells has presented new questions as to the biology of leukemia relapse and resistance. Most stem cells are assumed to be quiescent at steady state, and to express a number of membrane transporters with broad specificity linked to drug resistance. Assuming that leukemia stem cells recapitulate these two aspects of stem cells, quiescence and inherent drug resistance are likely to make the leukemia stem cell population the most difficult to eradicate fraction.

MODELS OF LEUKEMOGENESIS

Two models of leukemogenesis have long been proposed. The "stem cell model" or the "hierarchy model", suggesting that leukemias originate from stem or progenitor cells through deregulation of self-renewal pathways. Theoretically, the leukemia stem cell model is based on the idea that pluripotency and maturation are mutually exclusive. Leukemia stem cells maintain themselves and the clone by self-renewal, and they mature to generate progeny that lack stem cell properties. In contrast, in the "stochastic model", any cell could be the target of leukemogenesis. This model predicts that a tumor is biologically homogeneous and the behavior of malignant cells is influenced by intrinsic or extrinsic factors. Transformation results from random mutation and subsequent clonal selection[9,10]. All leukemia cells are equally sensitive to such stochastic influences and can revert from one state to another. Recently, a third model was proposed. In this model, leukemia cells can dedifferentiate and regain leukemia stem cell capacity, thereby sustaining the disease[11]. In all cases, both bone marrow and stromal cells may have abilities to differentiate into different cell types, suggesting that pluripotency and maturation might be influenced by the micro environmental stimuli[12]. This is encapsulated in the concept of the "niche" in the bone marrow that is required to maintain the status of the bone marrow stem cells[13].

Although hematopoiesis has been considered hierarchical in nature, recent data suggest that the system is functionally quite plastic[14]. Rather than a hierarchical transition from stem to progenitor cell, it appears that a fluctuating continuum exists, in which the phenotype of primitive marrow cells shifts from one state to another and back again. A primitive progenitor cell can actually make very different lineage choices during one cell cycle transit[15]. It has also been suggested that hematopoietic stem cells are functioning concurrently, continuously cycling and contributing to blood cell production. This suggests that hematopoietic stem cells are not completely dormant, cell quiescence being relative[16]. Cell cycle passage could determine the fate of cells derived from stem cell division and renew stem cell multipotency[17]. Cell cycle transit is associated with a continually changing stem cell phenotype[14]. The identity of the stem cell could be masked at certain points in the cycle. In these models, stem cells would show asymmetric division. The other alternatives would lead to either hyperproliferation or stem cell exhaustion. In some models, less primitive cells

could give rise to more primitive cells[18]. Some daughter cells could have greater pluripotency than the parent cells. Here the stem cell population is viewed as a continuum, rather than being composed of discrete states.

These last models are more compatible with the view of the cell lineages representing complex reactive or adaptive systems[19], in which self-organization arises on the macro-scale from micro-scale interactions of the individuals that constitute the system. Complex adaptive systems often have multiple ground states or points of equilibrium, and transitions between these states may lead to small or large instabilities, including system collapse. Mathematical modeling of stem cell lineage systems, taking into account a limited number of parameters, such as affinity for a growth environment (the stem cell niche) and cycling status of the cell, produces clonal fluctuation patterns that are a precise match for those seen experimentally in human leukemias[20]. This model includes stochastic elements. In the setting of cell lineages as complex reactive or adaptive systems, fluctuations are necessary for self-organizing systems to explore new states.

IMPLICATIONS FOR THERAPY

The concept of leukemia stem cells has implications in leukemia therapy, most particularly for the development of targeted therapies. Based on the understanding of the molecular basis of cancer, current therapeutic strategies focus on inhibiting the molecular drivers of cancer. Research should therefore focus more on leukemia stem cells than on the bulk cells that makes up the majority of the tumor. Characterization of the biological features that initiate and maintain leukemia is an essential step in the development of novel effective agents. The challenge is to identify proapoptotic stimuli that spare the normal hematopoietic stem cells while exerting the desired effect on leukemia stem cells[21]. Malignant stem cells have a number of biological features that are different from their normal-tissue counterparts and these might be exploited for therapeutic benefits. The identification of genes that regulate self-renewal might provide rational targets for therapeutic intervention, particularly if their requirement is more critical for self-renewal in leukemia stem cells than hematopoietic stem cells. New agents, such as kinase inhibitors, histone deacetylase inhibitors, cyclin D kinase inhibitors, nuclear factor κB (NF-κB) inhibitors, methylation inhibitors, heat shock protein inhibitors, farnesyltransferase inhibitors, and proteasome inhibitors, showing specific mechanisms that target leukemia cells, are now available. However, there is little or no evidence that inhibiting these different pathways is relevant to inhibiting proliferating leukemia stem cells.

Targeting drug efflux pumps

A therapeutic approach could be to target drug efflux pumps. ATP-dependent drug efflux has been linked to the increased expression of ABC transporter proteins[22]. At least 22 ABC transporters have been identified in leukemia stem cells, and all were expressed at lower levels in $CD34^+$ $CD38^+$ cells in comparison with the $CD34^+$ $CD38^-$ cells[23]. Several agents effective in overcoming the inherent drug efflux pumps have been studied, but found to have limited efficacy because of the high expression of the targeted receptors in normal hematopoietic stem cells, making them equally susceptible to the inhibitors[24,25]. Third-generation multidrug resistance modulators that are more powerful are currently under clinical investigations[26].

Targeting cell cycle

Another approach could be to target the self-renewal machinery of leukemia stem cells, by inducing the quiescent leukemia stem cells into the cell cycle. Leukemia stem cells respond to depletion of the leukemia cell mass that occurs when antiproliferative drugs are administered to leukemia patients. Thus, one way to eliminate dormant leukemia stem cells would be to find the window when they cycle and kill them at that point. Unfortunately, little is known of the biology of these cells. It has been proposed that recruitment of leukemia stem cells from G0 to the S phase of the cell cycle might contribute to their eradication by cell cycle-specific agents.

A priming strategy might improve the efficacy of cell cycle-dependent cytotoxic agents. Recent clinical trials have shown that sensitization of leukemia cells and their progenitors by granulocytic growth factors can improve the outcome of patients with newly diagnosed AML[27,28]. Inhibition of CXCR4 (the receptor for bone marrow stroma derived SDF-1) has been shown to overcome resistance to numerous drugs in leukemia/stromal co-cultures *in vitro*[29]. CXCR4 inhibition also affects CXCR4-mediated signaling events that are induced by leukemia/stroma co-culture conditions. In a recent clinical trial using an anti-CXCR4 in patients with AML in complete remission, massive mobilization (up to 80%) of leukemic cells was observed when hematopoietic growth factor application was followed by anti-CXCR4[30]. It is expected that mobilization of leukemic stem cells with CSF and anti-CXCR4, accompanied by chemotherapy, will result in increased anti-leukemic effects. Thus the mobilization of leukemic stem cells is a concept that is presently being revisited.

Targeting molecular pathways including PTEN, p21, and PML might also be an attractive proposition[31]. The *BMI1* oncogene-driven pathway is one of the key regulatory mechanisms of pluripotency. The polycomb group gene *BMI1* influences the proliferative and self-renewal potential of normal and leukemia stem cells[32].

Conversely, prolonging the quiescent phase could also be beneficial. The existence of patients with indolent forms of AML and the wide variation in the duration of relapse-free interval among patients can sustain this hypothesis.

Targeting cell surface antigens

Although both leukemia stem cells and normal stem cells express CD34 but not CD38, there are differences

between their surface phenotype that could be useful for targeting leukemia stem cells. Recent data suggest that the majority of leukemia stem cells express CD33, the target of gemtuzumab ozogamicin[33]. However, the expression is not specific to leukemia stem cells. Conversely, CD123 (IL3α receptor) is expressed on most leukemia stem cells, but not on normal stem cells[33,34]. A specific fusion of IL3 and a diphtheria toxin has therefore been generated showing interesting results in NOD-SCID mice[35] and encouraging data in a phase I / II clinical trial in patients with relapsed or refractory AML[36]. An anti-IL3 receptor alpha chain (CD123)-neutralizing antibody (7G3) has been shown to target AML leukemia stem cells, impairing homing to bone marrow and activating innate immunity in NOD/SCID mice[37]. Studies also reported that leukemia stem cells could be targeted with monoclonal antibodies to CD44, CLL-1, or CXCR4[38-40].

Targeting NF-κB activity

Recent studies have described means of differential activation of apoptosis mechanisms in leukemia stem cells[41-43]. The transcription NF-κB has been found to be constitutively activated in leukemia stem cells but not in normal hematopoietic stem cells. Molecules able to inhibit NF-κB activity might selectively target the leukemia stem cell. The combination of idarubicin with proteasome inhibitors has been shown to mediate selective apoptosis in leukemia stem cells while sparing normal cells[43]. Recently, parthenolide, a sesquiterpene lactone with potent NF-κB inhibitory activity, was found to kill AML progenitors selectively while sparing normal progenitors[42]. Unfortunately, it is not water-soluble and is not, therefore, a candidate for pharmacologic development. However, the development of soluble analogs is ongoing[44]. Another molecule, TDZD-8 (4-benzyl-2-methyl-1,2,4-thiadiazolidine-3,5-dione),which also has NF-κB inhibitory activity, showed promising activity on AML progenitor cells expressing $CD34^+$ $CD38^-$[44].

Targeting other pathways involved in self-renewal

Other pathways are involved in self-renewal. Pathways such as the HOX gene, WNT/β-catenin, PTEN, Hedgehog, and BMI-1 are frequently mutated and could be selectively targeted in leukemia stem cells. Another transcriptional pathway that appears to alter self-renewal is that associated with the AP-1 transcriptional factor, JunB[45]. The Notch pathway might also be deregulated in leukemia stem cells. Inhibition of γ-secretase (which is necessary for Jagged and Notch signaling) by N-[N-(3,5-difluorophenacetyl)-L-alanyl]-S-phenylglycine t-butyl ester (DAPT) has been shown to inhibit leukemia stem cell growth[46]. The phosphatidylinositide-3 kinase (PI3K) pathway is the major signaling pathway downstream of oncogenic tyrosine kinases, and is activated in AML[47]. Inhibition by LY294002 of the activation of the PI3K also leads to a dose-dependent decrease in survival of leukemia stem cells[48]. The use of mTOR inhibitors such as rapamycin and its derivative has demonstrated a loss of clonogenic potential of AML blasts, while sparing normal progenitors[49]. Phases I and II studies of mTOR inhibitors, in combination with standard chemotherapy, are ongoing[50,51].

Targeting cell differentiation

A differentiation block is a main feature of AML. Malignancy can be suppressed in certain types of leukemia stem cells by inducing differentiation with cytokines that regulate normal hematopoiesis, or with other compounds that use alternative differentiation pathways. The suppression of malignancy by inducing differentiation can bypass genetic abnormalities that give rise to malignancy and shows that leukemia stem cells can be genetically reprogrammed. CD44, a mediator of the stem cell/niche interaction, also represents a potential target for differentiation of leukemia stem cells. Targeting CD44 with an activating monoclonal antibody has led to eradication of human leukemia stem cells in NOD/SCID mice[38]. This could lead to a new therapeutic approach targeting the leukemia stem cell/niche interaction.

Targeting leukemia stem cells *via* active specific immunization

Targeting leukemia stem cells *via* active specific immunization has also been proposed, based on the development of immunoconjugates with toxic moieties. Immunotherapy, aiming at the generation of anti-leukemia T-cell responses, could provide a new therapeutic approach in eliminating minimal residual disease cells in leukemia. Leukemia stem cells could be targeted with a $CD8^+$ cytotoxic T lymphocyte clone specific for minor histocompatibility antigens; an approach that might be useful in relapsing AML patients after allogeneic transplant[52]. The presence of cytotoxic T lymphocytes directed against leukemia blasts emphasizes their suitability as immunological targets. Increased immunogenicity can also be achieved by the differentiation of leukemia blasts into leukemia dendritic cells, which have been demonstrated to induce antileukemic T-cell responses *in vitro*. However, it remains to be established whether AML-dendritic cells are able to elicit profound immune responses *in vivo*[53].

CONCLUSION

In conclusion, most cytotoxic therapeutic strategies currently used for leukemia therapy damage DNA or disrupt mitosis to induce cell death in highly proliferative cells that represent the bulk of malignant cell populations. Stem cells tend to be more resistant to chemotherapy. This involves the presence of multidrug resistance, antiapoptotic proteins, and DNA repair mechanisms. Furthermore, most current therapies do not target the signaling pathways that regulate self-renewal. Selectivity of targeting leukemia stem cells over normal stem cells is needed to avoid systemic toxicity. An important endpoint will therefore involve assessing changes in the size of the leukemia stem cell population. With the development

of clinical trials involving more targeted therapies, time progression or patient survival will thus become the ultimate clinical endpoints. However, a survey of markers for leukemia stem cells, *via* micro array or proteomic profiling, will also be important. The development of mathematical modeling could also be useful to understand responses to treatments that target malignant stem cell complexes in reactive or adaptative systems[54,55].

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S- Editor Li LF **L- Editor** Stewart GJ **E- Editor** Lin YP

World Journal of Stem Cells

Online Submissions: http://www.wjgnet.com/1948-0210office
wjsc@wjgnet.com
doi:10.4252/wjsc.v1.i1.55

World J Stem Cells 2009 December 31; 1(1): 55-66
ISSN 1948-0210 (online)

REVIEW

Effects of nanotopography on stem cell phenotypes

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Received: September 7, 2009 Revised: October 30, 2009
Accepted: November 6, 2009
Published online: December 31, 2009

Abstract

Stem cells are unspecialized cells that can self renew indefinitely and differentiate into several somatic cells given the correct environmental cues. In the stem cell niche, stem cell-extracellular matrix (ECM) interactions are crucial for different cellular functions, such as adhesion, proliferation, and differentiation. Recently, in addition to chemical surface modifications, the importance of nanometric scale surface topography and roughness of biomaterials has increasingly becoming recognized as a crucial factor for cell survival and host tissue acceptance in synthetic ECMs. This review describes the influence of nanotopography on stem cell phenotypes.

Key words: Stem cells; Nanofibers; Nanotopography; Biomaterials; Extracellular matrix; Differentiation

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Ravichandran R, Liao S, Ng CCH, Chan CK, Raghunath M, Ramakrishna S. Effects of nanotopography on stem cell phenotypes. *World J Stem Cells* 2009; 1(1): 55-66 Available from: URL: http://www.wjgnet.com/1948-0210/full/v1/i1/55.htm DOI: http://dx.doi.org/10.4252/wjsc.v1.i1.55

INTRODUCTION

Stem cells are a natural choice for cell therapy due to their pluripotent nature and self-renewal capacity. In humans, stem cells have been identified in the inner cell mass of the early embryo, in some tissues of the fetus, the umbilical cord and placenta, and in several adult organs. The microenvironment in which the stem cells exist is called the stem cell niche. There are several factors which regulate the stem cell niche *in vivo*, such as extracellular matrix (ECM) molecules, growth factors, cytokines, and cell secreted metabolites. Molecular signals are exchanged between the stem cells and other neighbouring cells within the stem cell niche. The niche saves stem cells from depletion, while still protecting the host from excessive stem-cell proliferation. In short, the stem niche encompasses all of the elements immediately surrounding the stem cells when they are in their naive state, including the non-stem cells that might be in direct contact with them, as well as the ECM and proximal

soluble molecules[1]. Typically, a niche contains a few stem cells with high potential of differentiation into different kinds of mature cells. These stem cells are supported by, or incorporated into, the niche walls formed by the neighbouring cells. After asymmetrical division, a stem cell remains in the same position, while a daughter cell with a narrower potential for differentiation migrates, divides symmetrically or asymmetrically, and eventually leaves the niche[2].

Stem cells can be broadly classified, based on their origin, into two types - embryonic stem cells (ESCs) and adult stem cells (ASCs). Their potency may be classified into three types - totipotent, pluripotent and multipotent stem cells (Table 1)[3].

ASCs can be employed for various tissue regeneration applications for the following reasons: (1) They are naturally poised to generate a particular tissue, which might consist of several cell types; (2) They are able to migrate to injured tissue or other discrete sites in the body; and (3) Some cells secrete growth factors that mobilize or protect other cells residing in the tissue. However they are rare, difficult to identify and purify, and, when grown in culture, are difficult to maintain in the undifferentiated state.

Hematopoietic stem cells (HSCs) are capable of self-renewing continuously. HSCs reside in two different niches-the endosteal niche and the perivascular niche. In the endosteal niche, HSCs are associated with a subset of osteoblasts that line the inner surface of the cavities of trabecular bone. It supports quiescence and self-renewal of the HSCs[4]. HSCs that are found in the vicinity of sinusoidal endothelial cells are referred to as the vascular niche. The vascular niche forms a milieu that supports the proliferation, differentiation, and trans-endothelial migration of HSCs[5]. Ma *et al*[6] demonstrated that topographical and biological cues are responsible for early adhesion of bone marrow derived HSCs. They showed that the adhesion of the HSCs was faster onto the collagen blended poly-lactic-co-glycolic acid (PLGA) nanofibrous scaffold compared to the tissue culture polystyrene (TCP) (Figure 1)[6].

Human bone marrow-derived mesenchymal stem cells (hBM-MSCs) have attracted substantial attention in the field of tissue engineering and regenerative medicine due to the following advantages: firstly, the techniques for collecting and purifying MSCs from bone marrow are relatively convenient[7]; secondly, they are naturally poised to generate a particular tissue, which might consist of several cell types such as adipocytes, chondrocytes, osteoblasts, tenocytes, myoblasts, or neurocyte[8-11]; thirdly, MSCs can escape the immune system[12]; and fourthly some cells secrete growth factors that mobilize or protect other cells residing in the tissue[13]. There is little ethical controversy in the application of MSCs. Besides the perivascular areas in the bone marrow, where the MSCs could be in close association with HSCs, the MSCs can also be isolated from other tissues, such as the periosteum, synovial membrane, and synovial fluid[12]. Muguruma *et al*[13] demonstrated that upon insertion of human MSCs into the bone marrow of immunodeficient mice, the human MSCs differentiated into stromal cells, bone-lining osteoblasts, and endothelial cells, all functional constituents of the marrow hematopoietic microenvironment. Thus, understanding how niche cells and the ECM control stem cell fate will provide new tools to stimulate the differentiation of stem cells into desired cell types.

Factors influencing stem cell behaviors

The influence of the substratum on cell migration was first reported by Harrison in 1911 when he grew cells on a spider web and found that the embryonic cells followed the fibers of the web. This phenomenon was called stereotropism or physical guidance[14]. The role of topography on cells such as endothelia, fibroblasts, epithelia and epitena, was first explained by Curtis *et al*[15,16]. A very wide range of cell types, such as fibroblasts, osteoblast, nerve cells, and mesenchymal stem cells respond profoundly to nanotopography[17,18]. Cells seeded onto artificially produced micro- and nano-grooves aligned their shape and elongated in the direction of the groove. However, it was reported by Wilkinson *et al*[19] that cells do not respond to groove width other than depth of size greater than 2 micrometer. Cells adhere well onto surfaces having structures on the nanoscale range of 58 nm, but do not adhere that well on structures with diameter of more than 73 nm[20]. It was also reported that cells can recognize symmetries in the nanorange[21].

There are five key design parameters that influence cell behaviour in a biomaterial, depending on the surface molecules present in the biomaterial, including: ligand identity, presentation, and density; material architecture; and material mechanical properties. Together, these material properties coordinate the interplay between intrinsic and extrinsic determinants of stem cell fate to produce a desired phenotype[22]. In addition, the properties of the scaffold surface that must be taken into careful consideration include the rate of degradation of the scaffold, optimal fluid transport, and delivery of bioactive molecules, cell-recognizable surface chemistries, mechanical integrity, and the ability to induce signal transduction. The ultimate success of a scaffold is dependent on these properties because they influence cell adherence, nutrient/waste transport, matrix organization and cell differentiation[23].

The nanostructured surfaces of nanometallic and nanoceramic materials have several advantages compared to conventional surfaces. These include: (1) they possess greater surface roughness resulting from both decreased grain size and possibly decreased diameter of surface pores; (2) enhanced surface moisture retention due to greater surface roughness; and (3) greater numbers of grain boundaries. For example, nanoceramics are commercially available as new bone grafts or as implant coating materials (i.e. nano-HA paste-Ostim® from Obernburg, Germany; nano-beta-tricalcium phosphate-Vitoss from Orthovita, USA)[24].

The types of biomaterials used commonly in stem cell cultures ranges from polymers [polystyrene, polysulfone, polytetrafluoroethylene, cellulose acetate, PLGA, Collagen, and PCL (polycaprolactone)] to metals (titanium,

alumina, and stainless-steel) and glasses. Many polymers do not have the desired surface properties to be used as biomaterials in tissue engineering; therefore, surface modification is used to improve surface characteristics, such as hydrophilicity, cell attachment, expansion, proliferation, and differentiation[25]. Cell response is affected by the physicochemical parameters of the biomaterial surface, such as surface energy, surface charges or chemical composition. Topography is one of the most crucial physical cues for stem cells and recently it has been proven that nanotopography is the main influencing factor, rather than microtopography[26].

Nanofibrous scaffolds present a 3D nanostructured topology that resembles the fibrillar ECM proteins *in vivo*. Polyglycolic acid (PGA), polylactic acid (PLA) and the copolymer PLGA have been extensively used as nanofibrous scaffolds. These materials are hydrolytically degradable and their by-products are physiologically removed *via* metabolic pathways[27,28]. The mechanics of the nanofibrous scaffold are determined primarily by its composition, water content, and structure, which affect intermolecular and intramolecular forces and stress distributions[29-31]. Common methods of altering the mechanical properties of biomaterials include modulating the molecular composition and connectivity, thermal processing, and creating reinforced and porous composites. The mechanical properties of a material affect cell behaviors such as proliferation and migration[31-35].

Fabrication of scaffolds with various nanotopographies

There are several techniques for the fabrication of nano- and microsurfaces suitable for the growth of cells, as depicted in Table 2. These include laser deposition and etching, soft lithography, electrospinning, and colloidal lithography[36-39].

Electrospinning is the most widely used technique to create fibrous structures with favourable mechanical and biological properties. Electrospun nanofibers have been incorporated in stem cell cultures, to provide the desired microenvironment for their growth and differentiation, and to ultimately mimic the stem cell niche. Electrospun nanofibrous matrices provide integrated networks of nanoscale fibers with a specified pattern, high porosity, high spatial interconnectivity, and a high surface area to volume ratio[40].

There are a number of electrospinning parameters that affect both the fibers and the scaffold. These include solvent type, material concentration and viscosity, distance of the collecting target from the spinning nozzle, the gauge of the needle, and the voltage. The above parameters should be optimized depending on the desired application, as cell proliferation and differentiation are influenced by the fiber diameter[41,42]. HFP (1,1,1,3,3,3-hexafluoro-2-propanol) is a commonly used solvent for electrospinning. It is an organic solvent allowing full extension of the polymer, without leaving any residue on the electrospun fibers. However, some proteins, such as collagen, tend to lose their 3D molecular structure when using HFP as the solvent. Hence cross-linking agents like glutaraldehyde or stabilizers are proposed to be applicable[43]. Recently, it has been found that adding PCL not only reduced the potential cytotoxicity that a chemical cross-linking reagent such as glutaraldehyde can cause, but also produced a new composite with improved mechanical and biological properties[44-47]. Heydarkhan-Hagvall *et al*[48] demonstrated that electrospinning of natural proteins like collagen/gelatin with synthetic polymers like PCL/PLGA can be used to produce tissue-engineered scaffolds that better recapitulate key features of the native ECM, including its mechanical and biochemical properties.

The biocompatible scaffold materials can be synthetic or natural. Collagen, fibrinogen, hyaluronic acid, glycosaminoglycans (GAGs), hydroxyapatite (HA), cellulose, chitosan, and silk fibroin are the most commonly used biomaterials. Although the natural biomaterials have the advantage of being biocompatible and bioactive, they have certain disadvantages compared to synthetic biomaterials such as the difficulty in modifying degradation rates, difficulty in sterilization and purification. Grafting of polymers with collagen is said to increase the surface hydrophilicity and thereby facilitates cell attachment and proliferation on the modified surface[49-52]. In addition, plasma surface treatment of scaffolds with N_2, O_2, and NH_3 makes the polymer surface more hydrophilic, more polar, and more bio-adhesive[53,54].

Surface modification of implants with nanotopographies

Using bone/dental implants as an example, once an implant is placed into the body, the adjoining bone will interact with the surface of the load bearing implant. This process is called osseointegration. The success of an implant depends on how early osseointegration is achieved[55]. Hence, the surface of the implants ought to be modified, to create a nanostructured surface matching native bone ECM and enhancing osteoblast incorporation, to improve early osseointegration. Various techniques have been attempted to improve the surface roughness of the implant, such as plasma treatment, acid-etching, and heat treatment. For example, the TPS (titanium plasma sprayed) surfaces used by the Straumann Company, recommended a healing period of 12 wk[56] and this was reduced to six to eight weeks with the introduction of the SLA (sand blasted, acid etched) surface[57]. Alternatively, nano-hydroxyapatite (n-HA) has been widely used as a bioceramic in orthopaedics and dentistry due its osteoconductive properties[58], which makes the combination of a load bearing biomaterial like titanium with the osteoconductive properties of n-HA very attractive.

The current time required for osseointegration ranges from three to six months. This delay might be because the osteoprogenitor cells and/or stem cells need a long time to recognize the implant surface, attach onto it, followed by proliferation and differentiation. The surface creation of nanotopography such as a nanofiber offers the possibility to optimize cell capture as well as other cell functions, because both the substrate topography and the biological cues enhance the initial attachment of MSCs, which might be very helpful for osseointegration.

Table 1 Different types of stem cells, their properties, and functions

Stem cell type	Properties	Functions
MSCs	Multipotent and pluripotent. Bone marrow is the major source of MSC	MSCs are capable of differentiating into bone, cartilage, fat, muscle, marrow stroma, and other tissue types
ESCs	Derived from an early stage embryo and can differentiate into derivatives of all three primary germ layers. ESCs are multipotent and pluripotent	Can differentiate into brain and nervous system cells, insulin producing cells of the pancreas, bone cells, hematopoietic cells, endothelial cells, cardiomyocytes
ASCs	Multipotent, oligopotent, or unipotent progenitor cells. Derived from a more mature tissue, such as the umbilical cord, bone marrow, or skin	To treat leukemia and related bone/blood cancers through bone marrow transplants
HSCs	Found in the bone marrow. Multipotent	All types of blood cells
iPS	Derived from epithelial cells. Pluripotent	The iPS cell lines could be differentiated into heart muscle and neuronal cells, in addition to basic cell types (ectoderm, mesoderm, and endoderm)
Mammary stem cells	Isolated from human and mouse tissue	Growth of mammary glands
Endothelial stem cells	Multipotent cells found in the bone marrow	Can differentiate into endothelial cells, the cells that make up the lining of blood vessels

Table 2 Various fabrication techniques to achieve nanotopography

Fabrication technique	Advantages	Drawbacks
Laser deposition	Uniform distribution of pore size, simple and fast	Reduced resolution and poor surface finish
Self assembly	Can generate fibrous networks capable of supporting cells in three dimensions. Cell-seeding problems associated with using prefabricated nanofibrous scaffolds eliminated owing to spontaneous assembly	Lack mechanical strength, Limited amphiphilic materials, random and very short nanofibers
Lithography	Relatively good resolution	Time consuming and expensive.
Electrospinning	The properties of electrospun nanofibers, such as fiber diameter, can be controlled readily *via* manipulation of spinning parameters. Capable of mimicking the stem cell niche	Electrospinning yields a flat mat that has limited three dimensionality and suffers from cell infiltration problems because of the small pore size of the mats
Phase separation	A nanofibrous (fibers with diameters of 50-500 nm) three-dimensional scaffold can be constructed. Has controllable high porosity, surface-to-volume ratios, and well as defined mechanical properties	Nanofiber distribution and uniformity is subject to the controllability of the processing

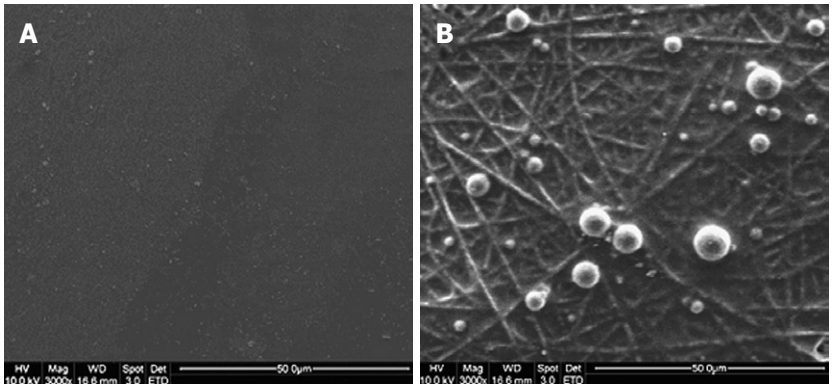

Figure 1 Capture of BM-HSCs by different substrates after 30 min of incubation. A: No BM-HSCs captured on tissue culture polystyrene (TCP); B: Rounded morphology of BM-HSCs captured on E-selectin-coated collagen-blended polylactide-co-glycolide (PLGA) nanofiber[6].

EFFECTS OF NANOTOPOGRAPHY ON STEM CELLS

Nanotopography is of critical importance in various biomedical applications. The nanoscale surface morphology, along with mechanical and biochemical cues, determines stem cell attachment, proliferation, and differentiation. Nanotextured scaffolds, besides providing structural support to the cultured stem cells, can also provide the topographical signals to influence cell differentiation, particularly through the nanostructural architecture provided by the fibers. Li *et al*[59] showed that a 3D electrospun nanofibrous scaffold was capable of supporting multilineage differentiation of MSCs into adipogenic, chondrogenic, and osteogenic lineages, as shown in Figure 2. Stem cells use transmembrane actin-integrin adhesion complexes as mechanosensors to probe the rigidity of the extracellular environment, mediate adhesion, trigger signaling, and remodel the ECM[60]. Culturing hESCs in the presence of actin disrupting agents proved that cytoskeleton remodelling through actin polymerization is critical for the morphological and proliferative behaviour of hESCs cultured on nanotopographic surfaces[61].

Effect of nanotopography on embryonic stem cells

Gerechta *et al*[61] recently reported the influence of surface topography on the morphology and proliferation of

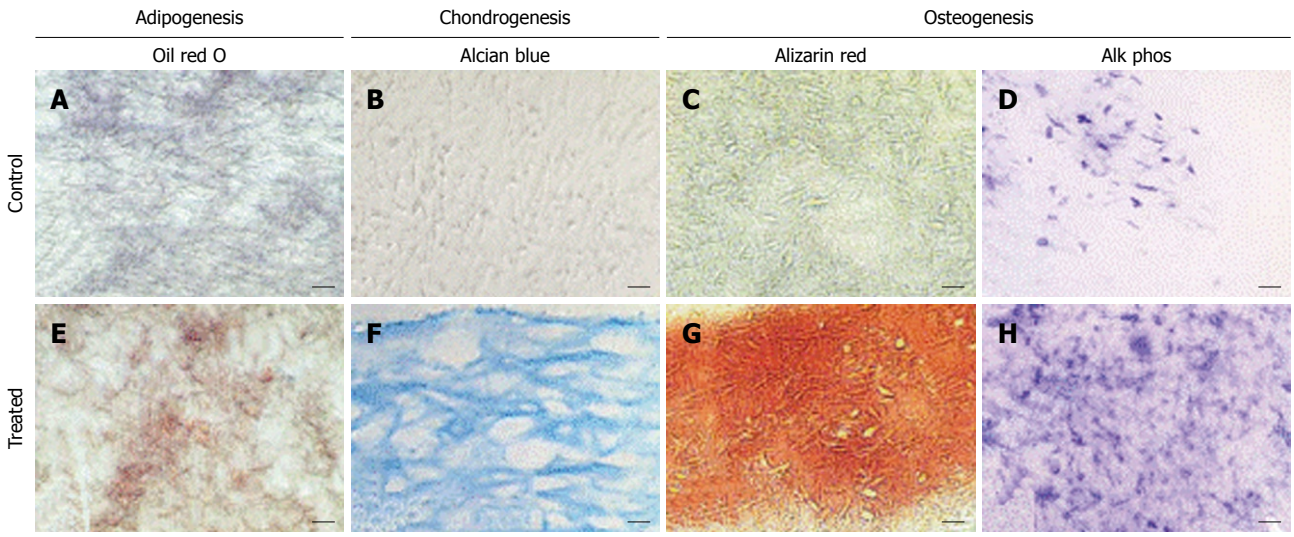

Figure 2 Histological analysis of cell-polymer constructs maintained in adipogenic, chondrogenic, or osteogenic medium. Sections of the constructs were stained with (A,E) oil red O, (B,F) alcian blue, or (C,G) alizarin red, or histochemically stained for alkaline phosphatase (D,H). In adipogenic cultures (E), oil red O-positive lipid droplets were seen, compared to the lack of staining in the control culture (A). In chondrogenic cultures (F), intense alcian blue was seen, showing cells surrounded by a sulfated proteoglycan-rich ECM (F), whereas control cultures (B) showed little staining. In osteogenic cultures (G,H), mineralization (G) and alkaline phosphatase activity (H) were both significantly higher than in control cultures (C,D). Bar: 20 μm (B,F), 40 μm (A,C,E,G), or 80 μm (D,H)[59].

hESCs. They demonstrated that poly (di-methyl siloxane) substrates with nanoscale line-grating (in the range of 600 nm ridges with 600 nm spacing and 600 ± 150 nm feature height) induced more hESC alignment and elongation, compared to the flat surface[61]. These were characterized by the cytoskeletal proteins actin, vimentin, and tubulin.

The maintenance and differentiation of hESCs is mainly dependent on the use of feeder cells, which are obtained from animal sources. Hence, there is always a risk of immune recognition. The mechanism of how feeder cells maintain the proliferation of undifferentiated ESCs is unknown. Such *in vitro* culturing presents certain theoretical hazards to the use of stem cells for regenerative medicine, such as the spread of viruses and other infectious agents not normally found in humans. However, it is believed that the nanotopographical substrates can maintain the proliferation of undifferentiated rhesus ESCs without the use of feeder cells[38].

Effect of nanotopography on mesenchymal stem cells

Mesenchymal stem cell adhesion and migration: The initial adhesion of the cells to the surface determines its long term cell viability. Different aspects, such as surface moisture retention and free energy[62], surface roughness, material composition[63,64], and method of preparation[65] of various materials have been studied and were determined to be the major factors influencing the attachment of cells, including MSCs, *in vitro*. Oha *et al*[66] found that hMSCs cultured on TiO_2 nanotubes of diameter less than 50 nm adhered more strongly compared to the cells cultured on TiO_2 nanotubes of 100 nm diameter. This was because nanotubes of diameter less than 50 nm had more surface area and hence a higher amount of ECM proteins can be deposited. In addition, he also showed that hMSCs adhered more effectively onto the smallest nanotube diameter, which was 30 nm, within two hours[66]. Park *et al*[67] also demonstrated that the adhesion of MSCs was reduced when the diameter of the TiO_2 nanotubes increased beyond 50 nm[67]. These directly indicate the influence of nanotopography on the biological processes. Larger adhesions are usually associated with increased indirect mechanotransductive signalling (adhesion and cytoskeleton-related) such as integrin-related signalling, extracellular receptor kinase (ERK), and focal adhesion kinase (FAK).

Cells will migrate along topographical features when plated onto a chemically uniform surface, a phenomenon known as contact guidance, which is crucial in embryonic morphogenesis and wound healing[68]. Emerging evidence indicates that the surface topography, stiffness, and electrical properties play important roles in neuron adhesion and neurite outgrowth[69]. Fan *et al*[70] studied the adhesion of neural cells of prenatal rats on silicon wafers with different nanotopographies in the range of 20 nm to 70 nm. Cell adhesion and viability were significantly improved on the nanofeatured surface. Moreover, 15% of the cells remained dopaminergic after five days of culture. Massia *et al*[71] analyzed the cell adhesion kinetics and demonstrated that the surface threshold spacings for focal contact and stress fiber formation occurs at 140 nm, indicating that cell adhesion can be enhanced on a nanostructured surface. Kommireddy *et al*[72] proved that MSC attachment and migration increased after the deposition of TiO_2 nanoparticle layers.

The surface modification of implants for cell attachment and migration has been the focus of much research, as the initial cell adhesion is critical for the functionality and the lifetime of the implant.

Mesenchymal stem cell proliferation and differentiation: Nanotopography not only enhances adhesion, but

Table 3 Various cell types and the nanotopographies on which they are cultured

Cell type	Nanotopography	Advantages	Ref.
Chondrocytes	(a) PCL nanofibrous scaffold (200-800 nm) in the presence of TGF-β1; (b) Collagen nanofibers of diameter 110 nm-1.8 μm	The differentiation of the stem cells into chondrocytes in the nanofibrous scaffold was comparable to an established cell pellet culture. Nanotopography supports chondrocyte growth and infiltration	[82,90]
Osteoblasts	(a) Ceramics like HA, alumina and titania having nanostructures of grain sizes less than 100 nm and nanophase zinc oxide (23 nm); (b) PLGA, PLLA and PCL nanofibers (diameter 200-800 nm); (c) Nanotubes of diameter less than 100 nm	Enhanced proliferation and differentiation of MSC to osteoblasts	[67,77-79,105-113]
Smooth muscle cells (SMC)	(a) PLGA and PCL, PLLA-CL nanofibers (diameter 200-800 nm); (b) Nanogratings of 350 nm in width, spacing, and depth imprinted on PMMA or PDMS	SMC adhesion was enhanced on the nanostructured substrates compared to the conventional submicron substrates	[114-118]
Fibroblasts	(a) PLGA (85:15 ratio) nanofibers of diameter 500-800 nm; (b) Nanocolumns	Increased endocytic activity. Nanotopography can be used to improve hemocompatibility of blood-contacting biomaterials	[82]
Nerve cells	(a) Silicon wafer in the range of 20-70 nm; (b) PLLA or PCL scaffolds *via* electrospinning and phase separation	The cell adhesion and viability significantly improved on the nanofeatured surface	[70,91]

PCL: Polycaprolactone; TGF-β: Transforming growth factor-β; HA: Hydroxyapatite; PLGA: Poly-lactide-co-glycolide; PLLA: Poly-L-lactide acid; MSC: Mesenchymal stem cell; PMMA: Poly-methylmethacrylate; PDMS: Polydimethylsiloxane.

also improves proliferation efficiency of MSCs. Nanotopography induces certain biochemical and structural cues, which cause the differentiation of the MSCs into certain desired phenotypes. Table 3 gives an outline of the influence of various substrates and their topographies on several cell lineages, which might give some hints on the differentiation of stem cells into those cell types.

The influence of nanotopography on the osteogenic, chondrogenic, and neural differentiation of MSCs will be discussed in detail in the following sections. MSCs develop into osteoblasts *via* a series of developmental stages - osteoprogenitor cell, preosteoblast, and finally osteoblast cells. Osteoblast adhesion on nanostructured surfaces was first reported in 1999 by Webster *et al*[73]. He reported that osteoblast adhesion was improved when they were cultured on nanostructured surfaces, compared to the conventional micro surfaces. Specifically, alumina with grain sizes between 49 nm and 67 nm and titanium with grain sizes between 32 nm and 56 nm enhanced osteoblast proliferation and differentiation compared to their respective micro-grained materials. This can be measured by monitoring ECM protein synthesis, such as collagen and alkaline phosphatase (ALP). Enhanced bone formation was reported on the nanophase HA coated tantalum compared to the microscale HA coated tantalum, and the non-coated tantalum[73]. Webster *et al*[74,75] demonstrated that osteoblast adhesion increased by 146% on nanophase zinc oxide (23 nm) compared to microphase zinc oxide (4.9 nm). Nanophase metals have been extensively investigated for orthopedic applications due to their higher surface roughness, energy, and the presence of more particle boundaries at the surface compared with conventional micron metals. Moreover, osteoblasts were even further increased on nanofiber structures compared to nanospherical structures of alumina; this was believed to occur because, compared to nanospherical geometries, nanofibers more closely approximate the shape of HA crystals and collagen fibers in the natural bone[76]. Woo *et al*[77] observed enhanced osteoblast attachment on nanofibrous scaffolds when compared to solid pore walls.

Yoshimoto *et al*[78] cultured rat MSCs on PCL nanofibrous scaffolds of diameter 400 nm. ECM production (Collagen) and the multiple cell layer formation occurred within a short span of one week. Hosseinkhani *et al*[79] investigated mesenchymal stem cell (MSC) behavior on self-assembled peptide-amphiphile (PA) nanofiber scaffolds. Significantly enhanced osteogenic differentiation of MSCs occurred in the 3D PA scaffold compared to 2D static tissue culture. It was characterized by enhanced collagen synthesis, alkaline phosphatase activity, and calcium mineral deposition. It was demonstrated that when hMSC loaded constructs made of PCL nanofibers were cultured in an osteogenic differentiation media comprising of β-glycerolphosphate, ascorbic acid, and dexamethasone, a dense, opaque bone-like tissue was observed, indicating the osteogenic differentiation of hMSCs. Polygonal-shaped osteoblast/osteocyte-like cells with upregulated expression of alkaline phosphatase, bone sialoprotein, and osteocalcin were observed[59].

Dalby *et al*[80] demonstrated the use of nanoporous topography to stimulate hMSCs to produce bone mineral *in vitro*, in the absence of osteogenic supplements. Their results demonstrated that highly ordered nanoporous topographies produce low to negligible cellular adhesion and osteoblastic differentiation. Cells on random nanoporous topographies however exhibited a more osteoblastic morphology. This enhanced differentiation was due to the nanodisorder. This work demonstrated that topographical strategies provide further orthopedic approaches to be exploited and harnessed. However, the intracellular events controlling the differentiation of hMSCs into osteoblasts have still not been clearly analyzed. Salasznyk *et al*[81] suggested that focal adhesion kinase signalling plays an important role in regulating ECM-induced differentiation of hMSCs into osteoblasts.

Li *et al*[82] investigated the chondrogenesis of MSCs on a PCL nanofibrous scaffold in the presence of TGF-β1 *in vitro*. The differentiation of the stem cells into chondrocytes in the nanofibrous scaffold was comparable to an established cell pellet culture. It was advantageous to use nanofibers rather than a cell pellet system, owing to their better mechanical properties, oxygen/nutrients exchange, and easy fabrication[83-87]. The findings reported suggested that the PLLA nanofibrous scaffold is a practical carrier for MSC transplantation, and represents a candidate scaffold for cell-based tissue engineering approaches to cartilage repair[88].

Cheng *et al*[89] reported that human cartilage cells attached and proliferated well on HA nanocrystals homogeneously dispersed in PLA, and collagen fibers of diameter 110 nm to 1.8 μm were proved to support chondrocyte growth and infiltration[90]. Such data shows the promise of nanomaterials for promoting cartilage regeneration.

Koh *et al*[91] fabricated various nanofibrous PLLA or PCL scaffolds *via* electrospinning, which demonstrated excellent cytocompatibility properties for neural tissue engineering applications. When laminin was incorporated into the nanofibrous scaffold, the neurite outgrowth improved on the laminin-PLLA scaffold produced by facile blended electrospinning. Yang *et al*[92] showed that the direction of Neural stem cell (NSC) elongation and its neurite outgrowth was parallel to the direction of aligned PLLA fiberous scaffolds. They also demonstrated that the differentiation rate of NSCs was higher for nanofibers than for micro fibers.

Most recently, Prabhakaran *et al*[93] demonstrated the potential of hMSCs for neuronal differentiation *in vitro* when cultured on poly (l-lactic acid)-co-poly-(3-caprolactone)/Collagen (PLA-CL/Col) nanofibrous scaffolds (Figure 3). The differentiation of MSCs into neuronal lineages was carried out using neuronal inducing factors, including β-mercaptoethanol, epidermal growth factor, nerve growth factor, and brain derived growth factor, in DMEM/F12 media. These supplements, in addition to the nanoscaffold, induced the differentiation of the MSCs into neuronal cells.

Stem cells have the potential to differentiate and self-renew into the desired cell types. Therefore, many efforts have focused on impregnating multi-potential stem cells into the nanofibrous scaffolds, which can be directly transplanted into injury sites and assist neural tissue recovery. In addition, the development in nerve repair grafts for peripheral nerve injuries to bridge nerve gap has advanced to the next level where the nanofibers were been used as guidance channel[94]. However, a challenging problem has been to determine how to effectively deliver and selectively differentiate stem cells into nerve cells at injury sites to regenerate desirable tissue. Although the underlying mechanisms triggering differentiation of stem cells are not entirely clear, previous research has indicated that novel biomimetic nanomaterials might contribute to selective stem cell differentiation[95].

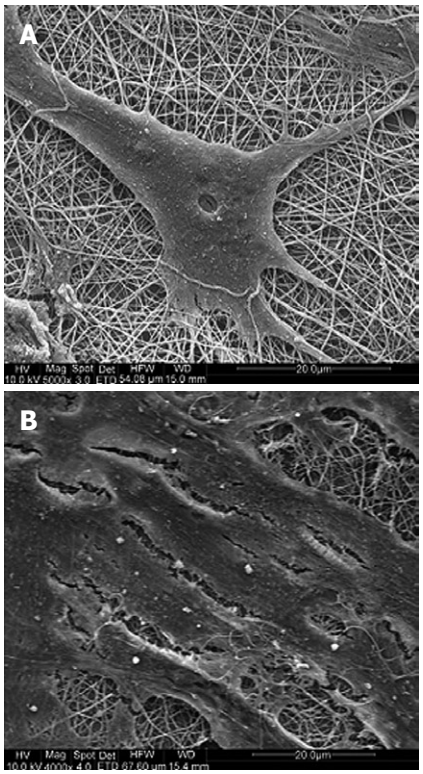

Figure 3 SEM images of (A) MSCs induced to neuronal cells grown using neuronal induction medium and (B) undifferentiated MSCs on electrospun PLA-CL/Collagen nanofibers grown using MSC growth medium[93].

Combined effect of nanotopography and other factors

The fate of multipotent stem cells can be desirably controlled when they are cultured on nanopatterned substrates. It was recently demonstrated that FGF-2 could allow long-term self-renewal of hESCs and maintain their pluripotent status[96]. The addition of growth factors, such as retinoic acid and activin A, have demonstrated success in promoting *in vitro* differentiation of murine ESCs into cells of pancreatic lineage like α, β, γ, and δ cells. A commonly used cell adhesive ECM peptide is the RGD protein, which is an arginine-glycine-aspartic acid sequence. Holtorf *et al*[97], proved that when titanium fiber mesh scaffolds were coated with RGD peptides, MSCs attached more strongly to these RGD-coated scaffolds. However. no significant change was observed in ECM secretion. Murine MSCs seeded onto fibronectin(FN)-functionalized scaffolds created by an LbL (Layer by Layer) microfabrication system, adhered more strongly to the scaffold and readily differentiated into osteoblasts[98]. The addition of a phosphoester group to photo-polymerizable PEG-based hydrogels not only provides biodegradability but has also been shown to promote mineralization of encapsulated MSCs. The use of such phosphoester (Phosph-) groups is said to significantly increase the ALP and osteocalcin levels in differentiated cells[99]. Peter *et al*[100] demonstrated that MSC adhesion, proliferation, and differentiation into osteoblasts increased when TGF-β1 was encapsulated within polymer blends of PEG-PLGA particles (sized at an average of 158 μm). Thus we find that the cell-

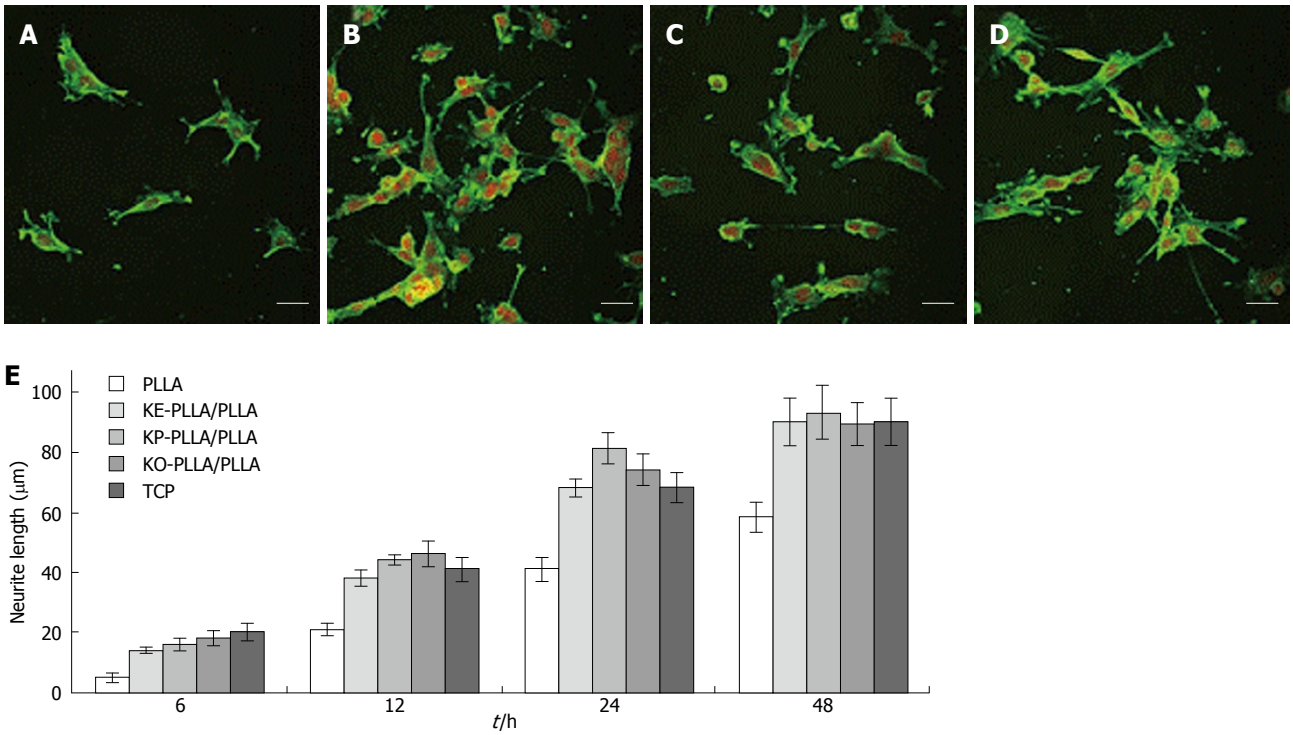

Figure 4 Laser scanning confocal microscopy (LSCM) micrographs of immunostained neurofilament 200 kDa in C17.2 after 24 h of culture on different films. A: Poly-L-lactide acid (PLLA); B: KE-PLLA/PLLA; C: KP-PLLA/PLLA; D: KO-PLLA/PLLA; E: The average length of the longest neurite per cell from 50 randomly selected cells on different films from PLLA and K-$(CH_2)_n$-PLLA/PLLA (10/90, w/w) over cultivation[101]. The neurite was stained by FITC and nuclei was stained by PI. Scale bar: 40 μm.

scaffold interactions increased in the presence of factors like RGD, FN, Phospho-group, and TGF-β1.

Chemical guiding cues were exploited to stimulate neuron adhesion and neurite outgrowth, using amino-functioned PLLA after phase separation with nanotopography. It was found that improved viability and neurite outgrowth were obtained on the peptide-grafted PLLA films compared to the ordinary PLLA films. Here, the neonatal mouse cerebellum C17.2 stem cells were cultured onto the K-$(CH_2)_5$-PLLA/PLLA peptide-grafted films and the PLLA films were used as controls. The enhanced neurite outgrowth of the C17.2 stem cells was shown to be due to the addition of laminin-derived peptide sequences (Figure 4)[101]. It is thus possible to further mimic the stem cell niche by covalently linking certain ligands or growth factors to the nanostructured scaffold.

APPLICATIONS

The list of medical achievements of stem cells seems to be expanding at an incredible pace. ESCs have the advantage of multipotency and can be readily cultured in the laboratory. The degree of plasticity of adult stem cells is still unknown and there are difficulties in purifying and culturing them. The only proven stem cell-based medical therapies that are currently available rely on adult-derived stem cells from bone marrow and skin. The idea of employing adult stem cells for many applications is for the following reasons: (1) They are naturally poised to generate a particular tissue, which might consist of several cell types; (2) They are able to migrate to injured tissue or other discrete sites in the body; and (3) Some cells secrete growth factors that mobilize or protect other cells residing in the tissue[102]. Pluripotent stem cells could be used to create an unlimited supply of cells, tissues, or even organs that could be used to restore function without the requirement for immunosuppressive drugs. Such cells, when used in transplantation therapies, would in effect be suitable for "universal" donation.

Neural application

Nerve stem cells can be used to treat the neurodegenerative diseases such as Parkinson's disease. Parkinson's disease involves the loss of cells which produce the neurotransmitter dopamine. Recent clinical studies using fetal cell transplants reported survival and release of dopamine from the transplanted cells and a functional improvement of clinical symptoms[103]. Thus it opens yet another frontier for stem cell therapy.

Orthopaedic application

Bone marrow transplantation is a well known clinical application of stem cells in orthopedics and blood diseases. Nanostructured biocomposites provide alternatives that have not yet been fully explored for orthopedic applications such as implants. They may be fabricated to possess similar micro- and nanoarchitecture as that of healthy, physiological bone. The behavior of cells depends on their interactions with their environment. Consequently, the interactions between cells and implantable materials will determine the

success or failure of a medical device. Thus, to achieve proper osseointegration, it is necessary that the implant has a nanostructured surface, ensuring early adhesion of stem cells. Biomaterials in the form of implants (sutures, bone plates, joint replacements, ligaments, vascular grafts, heart valves, and dental implants) and medical devices (for example pacemakers and biosensors) are widely used to replace and restore the functions of degenerated tissues or organs, to assist in healing, improve functionality, and thus improve quality of life[56]. Their improved mechanical and biocompatibility properties promise future greater orthopedic implant efficacy.

Cell based therapies

Perhaps the most important potential application of human stem cells is the generation of cells and tissues that could be used for cell-based therapies. Today, donated organs and tissues are often used to replace repaired or destroyed tissue, but the need for transplantable tissues and organs far outweighs the available supply. Hence stem cells, directed to differentiate into specific cell types using nanotopography, offer the possibility of a renewable source of replacement cells and tissues to treat a number of diseases. For example, it might become possible to generate healthy heart muscle cells in the laboratory and then transplant those cells into patients with chronic heart disease. Recent studies have demonstrated that stem cells that are injected into the circulation or directly into the injured heart tissue appear to improve cardiac function and/or induce the formation of new capillaries[104].

CONCLUSION

By carefully controlling the nanotopography and surface chemistry, in principle, one could design a device that enhances a selective cell population to grow in specific regions of the device. The literature presented in this review clearly indicates that cells respond to the topography of substrates in the nanometer range in terms of adhesion, proliferation, and migration. The substratum, besides providing mechanical support, acts as an intelligent surface, providing the necessary topographical cues and signals to guide cell adhesion, proliferation and differentiation. Although many challenges lie ahead, the nanofibrous scaffold having excellent cytocompatibility and controllable mechanical properties, can mimic properties of the natural ECM and thus, shows great potential for numerous tissue regeneration applications. Scaffolds with advanced technologies, by incorporating nanotopography, can be used to create complex guidance channels, which can be used to mimic the natural repair process of the human body. Recent advances made in the field of nanotopography mediated stem cell regeneration provide optimism for nerve tissue engineering and bone tissue engineering to create a permissive environment for nerve and bone regeneration. Of particular interest in tissue engineering is the creation of reproducible and nanotopographic scaffolds for stem cell migration and differentiation, resulting in bio-matrix composites for various tissue repair and replacement procedures. Though stem cell based-therapy seems to be very remarkable, there are many legal and social questions that must be addressed before stem cell-based therapies become clinically available.

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World J Stem Cells 2009 December 31; 1(1): I
ISSN 1948-0210 (online)

Acknowledgments to reviewers of *World Journal of Stem Cells*

Many reviewers have contributed their expertise and time to the peer review, a critical process to ensure the quality of *World Journal of Stem Cells*. The editors and authors of the articles submitted to the journal are grateful to the following reviewers for evaluating the articles (including those published in this issue and those rejected for this issue) during the last editing time period.

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World J Stem Cells 2009 December 31; 1(1): I
ISSN 1948-0210 (online)

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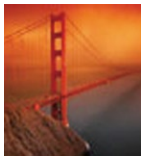
May 12-15, 2010
Stem Cells, Tissue Homeostasis and Cancer
EMBL Heidelberg, Germany
http://www.embl.de/training/courses_conferences/conference/2010/STM10-01/



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World J Stem Cells 2009 December 31; 1(1): I-IV
ISSN 1948-0210 (online)

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Published by
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Supportive foundations: The complete name and number of supportive foundations should be provided, e.g., Supported by National Natural Science Foundation of China, No. 30224801

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15 Morse SS. Factors in the emergence of infectious diseases. Emerg Infect Dis serial online, 1995-01-03, cited 1996-06-05; 1(1): 24 screens. Available from: URL: http//www.cdc.gov/ncidod/EID/eid.htm

Patent (list all authors)

16 **Pagedas AC**, inventor; Ancel Surgical R&D Inc., assignee. Flexible endoscopic grasping and cutting device and positioning tool assembly. United States patent US 20020103498. 2002 Aug 1

Statistical data

Write as mean ± SD or mean ± SE.

Statistical expression

Express *t* test as *t* (in italics), *F* test as *F* (in italics), chi square test as χ^2 (in Greek), related coefficient as *r* (in italics), degree of freedom as υ (in Greek), sample number as *n* (in italics), and probability as *P* (in italics).

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Italics

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