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REVIEW

Culture Systems for Pluripotent Stem Cells

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Pluripotent stem cells have the capacity to self renew and to differentiate to cells of the three somatic germ layers that comprise an organism. Embryonic stem cells are the most studied pluripotent stem cells. Pluripotent stem cells have also been derived from adult tissues. Both embryonic and adult stem cells represent valuable sources of cells for applications in cell therapy, drug screening and tissue engineering. While expanding stem cells in culture, it is critical to maintain their self-renewal and differentiation capacity. In generating particular cell types for specific applications, it is important to direct their differentiation to the desired lineage. Challenges in expansion of undifferentiated stem cells for clinical applications include the removal of feeder layers and non-defined components in the culture medium. Our limited basic knowledge on the requirements for maintaining pluripotency of adult pluripotent stem cells and the lack of appropriate markers associated with pluripotency hinders the progress toward their wide spread application. *In vitro* differentiation of stem cells usually produces a mixed population of different cell lineages with the desired cell type present only at a small proportion. Use of growth factors that promote differentiation, expansion or survival of specific cell types is key in controlling the differentiation towards specific cell lineages. A variety of bioreactors for cell cultivation exist and can be readily adapted for stem cell cultivation and differentiation. They provide a well-controlled environment for studying the process of stem cell propagation and differentiation. Their wide use will facilitate the development of processes for stem cell application.

[**Key words:** embryonic stem cell, adult stem cell, bioreactor, pluripotent]

Stem cells, with their extensive regeneration potential and functional multilineage differentiation capacity, are highly attractive for many applications in tissue engineering, cellular therapies and drug screening. Implementation of technologies based on these cells requires a readily available source of stem cells and/or their differentiated derivatives outside a living body. Such a requirement poses a challenge in their cultivation. Unlike many traditional processes which use cell's capabilities to produce a protein product or virus this new technology aims to generate the cells itself as the product. Some of those target applications will benefit from expansion of stem cells (for example, hematopoietic stem cell transplantation), whereas others will require the production of a specific differentiated cell type with defined characteristics either by controlling the differentiation in a very specific path or by elimination of undesirable cell types that could arise during the production (differentiation) process.

Stem cells are classified according to their differentiation capability. Pluripotent stem cells are capable to differentiate into the three somatic germ layers that comprise an organism: mesoderm (muscle, bone, etc.), ectoderm (neurons, skin, etc.) and endoderm (hepatocytes, pancreatic beta cells, etc.), whereas multipotent cells can differentiate only into cells of one tissue or germ layer. In this review, we will address the issues related to expanding undifferentiated pluripotent stem cells and the strategy of controlling or modulating their differentiation from a cell bioprocessing perspective.

STEM CELL TYPES

It is now well accepted that a stem cell must fulfill three criteria (1): First, it must be capable of self-renewal, *i.e.*, undergoing symmetric or asymmetric divisions through which the stem cell population is maintained. Symmetric division implies that both the daughter cells retain full stem cell characteristics, whereas asymmetric division refers to only one of the two daughter cells remains a stem cell while the other continues on a differentiation pathway. Second, a single cell must be capable of multilineage differentiation. In other words, the ability to differentiate to multiple lineages must not be due to the presence of a mixture of cells having different differentiation potential. The third criteria is *in vivo* functional reconstitution of a given tissue. Cells

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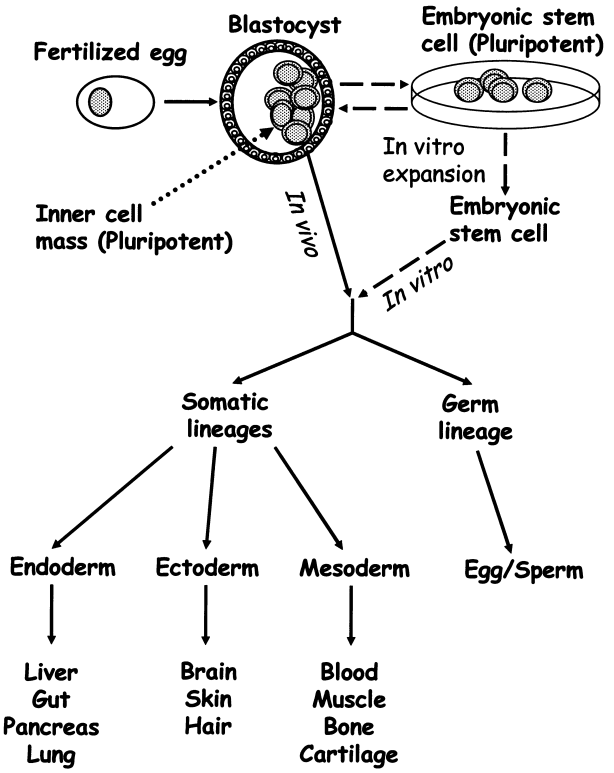

FIG. 1. Embryonic stem cells are isolated from the inner cell mass of the blastocyst. Upon injection into a blastocyst, ES cells contribute to cells of the different cell lineages in the mouse. Specification of cultured ES cells towards cells of the different cell lineages with phenotypical and in some cases functional characteristics of specific cell types have been shown *in vitro*.

derived from many different sources have been shown to fulfill those criteria and are considered stem cells including embryonic stem cells and adult stem cells from different tissues.

Embryonic stem cells Embryonic stem (ES) cells have unlimited self-renewal and differentiation potential. They are capable of giving rise to cells of the three somatic germ layers that constitute an organism: mesoderm, ectoderm and endoderm, so they are pluripotent. These cells are derived from the inner cell mass of the blastocyst (Fig. 1). They were first derived from mice and more recently from non-human primates and humans (2–5). One remarkable aspect of mouse embryonic stem cells is that, even after extended propagation and manipulation *in vitro*, they are capable of re-entering embryogenesis when injected into a pre-implantation embryo, producing functional differentiated progeny in all tissues and organs (6). In principle, human ES (hES) cells should be able to produce the same results; however, for ethical reasons, this cannot be demonstrated. It has been shown that when transplanted into post-natal animals, mouse and hES cells generate tumors consisting of different types of tissue, as of skin, hair, and muscle that are called teratomas (7, 8). The presence of cells of the three germ layers in these tumors demonstrates their pluripotency. This property however posses a challenge on ES cell application for clinical therapies because of the need to eliminate undifferentiated cells to avoid teratoma formation after transplantation.

ES cells have been differentiated *in vitro* to cells of the three somatic cell lineages (ectoderm, endoderm and mesoderm) as well as germ cells (Fig. 1). The *in vitro* differentiation usually renders a mixture of cells with some percentage of these cells showing specification towards different lineages (*e.g.*, hepatocytes, hematopoietic cells, neurons); however, whole organs or tissues have not been obtained *in vitro*. Transplantation of these cells has been utilized to prove functional differentiation or maturation of the cells.

Adult stem cells Adult stem cells also fulfill the criteria for stem cells outlined before. However, their degree of self-renewal and differentiation potential is more restricted when compared to ES cells. Although they differentiate into multiple lineages, adult stem cells are not pluripotent. The most extensively studied adult stem cell is the hematopoietic stem cell (HSC) (9, 10). The HSC undergoes self-renewal, differentiates into many different blood-forming units at the single cell level, and when transplanted functionally repopulates the hematopoietic system of an ablated recipient. A hierarchical description of the hematopoietic system is known with well-characterized cell surface markers for the progenitors and the eight types of differentiated blood cells. A number of other adult stem cells have been studied although they are not as well characterized as HSC. Neural stem cells (NSC) give rise to neurons, astrocytes, and oligodendrocytes (11). Mesenchymal stem cells (MSC) differentiate into fibroblasts, osteoblasts, chondroblasts, adipocytes, and skeletal muscle (12–14). Other stem cells have been identified, including gastrointestinal stem cells (15), epidermal stem cells (16), and hepatic stem cells (also called oval cells) (17).

Until recently, adult stem cells derived from a particular lineage are believed to be capable of differentiating into cells of that same lineage. Recent observations indicate that greater differential potential might persist in post-natal adult stem cells than previously thought. This phenomenon is generally referred to as stem cell plasticity. Some important observations of stem cell plasticity include bone marrow derived stem cells giving rise to hepatocytes *in vivo* (18). HSC contribution to tissues outside of the hematopoietic lineage when transplanted into animals has been challenged by different studies and needs to be further revisited (19, 20). Neuronal stem cells have been reported to differentiate to hematopoietic cells after being injected into lethally irradiated mice (21). There are reports of *in vitro* differentiation of cells isolated as MSC into neurons and hepatocytes (22, 23). These findings are still highly controversial and the mechanism by which the cross lineage differentiations occur may not be stem cell plasticity. For example, since the general protocol for isolating MSCs relies on cell attachment to a culture surface, it is possible that different non-mesenchymal progenitors or pluripotent stem cells existing in bone marrow were co-isolated and expanded. Another mechanism that may lead to the observation of stem cell plasticity is cell fusion, for example, bone marrow cells fusing with hepatocytes (24). The issue of stem cell plasticity will not be discussed here. For a recent review, see Lakshmipathy and Verfaillie (25).

Recently, a population of pluripotent stem cells termed multipotent adult progenitor cells (MAPC) has been isolated

from the bone marrow of post-natal human and rodents (26–28). These cells can be expanded *in vitro* without senescence. They show clonal *in vitro* differentiation potential to cells of the three germ lineages. When injected into an early blastocyst, single MAPC contribute to most tissues and on transplantation into a non-irradiated host. MAPC engraft and differentiate to the haematopoietic lineage, in addition to the epithelium of liver, lung and gut. Enhanced engraftment is seen in the hematopoietic and gastrointestinal system when MAPC are infused in irradiated recipients (26). Since then, different groups have reported isolation of pluripotent stem cells from human bone marrow (29, 30), umbilical cord blood (31), dermis and skeletal muscle (32).

Although classical MSCs have been shown to differentiate to neurons and hepatocytes, those studies were not done at the single cell level but with bulk populations. Nevertheless, MSCs will be discussed in this article as potential pluripotent stem cells considering that the methods for isolation of pluripotent stem cells from adult bone marrow are based on protocols for isolating MSC by their adherence to tissue culture surfaces.

STEM CELL ISOLATION, INITIAL CULTURE SYSTEMS AND CHARACTERIZATION

The conditions for the isolation of stem cells differ for different stem cell types as they tend to be isolated from different tissues. Even when isolated from the same tissue, different protocols aim to enrich different types of stem cells. As expected, the cultivation conditions also differ for different stem cell types. The cultivation of stem cells generally aim to accomplish three objectives: sustaining the self regenerating stem cell properties, maintaining the capability of differentiation, and enabling cryopreservation for maintaining the established lines. The three aspects are somewhat related. The isolation of different stem cells and their initial cultivation until the establishment of a line is very specific for each type of stem cell. Various protocols exist and are beyond the scope of this article. Readers are referred to general reviews on this subject (33, 34). So are the protocols for cryopreservation of pluripotent stem cells (35, 36).

In the following section, we will first discuss the cultivation method for sustaining the self-regenerating capability of stem cells of different types. To sustain the self-regenerating capability, the first critical issue is the identification of markers associated to the capability of self-regeneration. Without such markers, the alternative will entail various differentiation assays which are tedious and subject to time delay in obtaining the results. We will thus discuss the availability of the markers for different cell types in addition to describing the culture systems.

ES cell isolation, characterization and initial culture systems The mechanism by which ES cells can be expanded while maintaining their tri-lineage differentiation potential in culture (pluripotency) has not been elucidated. Both mouse and hES cells were originally derived using irradiated mouse embryonic fibroblasts (MEFs) as feeder layers in serum-containing medium (2–4). They are also routinely cultivated in the presence of feeder layer after isolation. The ES cells grow on the feeder layer as colonies, as opposed to initially dispersed and then confluent monolayer (Fig. 2). Passaging of mouse and hES cells are somewhat different. Mouse ES cells are normally subcultivated every 2 to 3 d and the colonies are enzymatically dissociated with

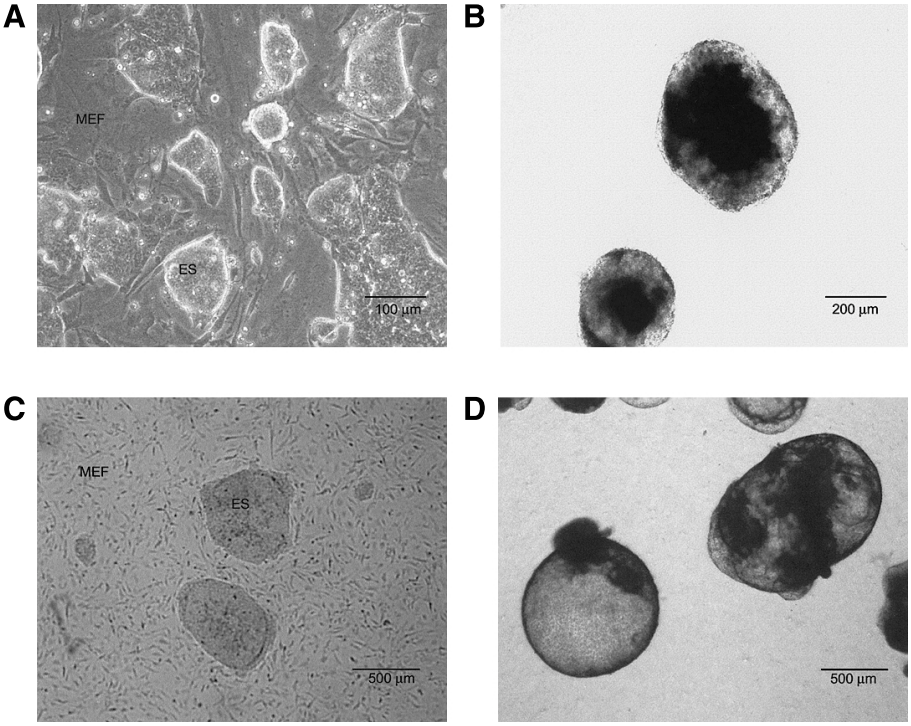

FIG. 2. (A, C) Mouse and human embryonic stem (ES) cells on mouse embryonic fibroblast (MEF) feeder layer. (B, D) Mouse and human embryonic bodies (EBs).

trypsin to obtain single cells which are then plated onto MEFs, whereas hES cells are subcultured every 6–7 d and they are most frequently passaged as small clumps of cells as opposed to single cells. The formation of small hES cells colonies to be passaged can be achieved by different methods including mechanical (manual) dissociation, or enzymatic dissociation using collagenase and trypsin. Maintenance of pluripotency is normally assessed by morphology, where ES cells form compact colonies of cells (Fig. 2) with high nucleus to cytoplasm ratios. Expression of certain markers such as the transcription factors oct-4, nanog and rex-1 and the cell surface markers SSEA-1 in mouse and SSEA-3, SSEA-4, TRA-1-60, TRA-1-81 in humans has also been correlated with pluripotency (37, 38). The identification of these markers has been important for monitoring and quantifying pluripotency of ES cells when testing different culture conditions.

The karyotypic stability of hES cell lines is a major concern for clinical applications. There have been reports that chromosomal abnormalities arise during long term culture of ES cells (39, 40). However, it is still controversial whether hES cells have inherent chromosomal instability over long term culture since no frequent chromosomal abnormalities after prolonged passaging was detected in another study (41). Differences in culture methods among different laboratories may account for the enrichment of cells with abnormal kayotype. Culture conditions appear to play some role as shown in a recent study comparing three passaging methods: manual mechanical dissociation, non-enzymatic cell dissociation buffer and enzymatic dissociation (collagenase/trypsin). The mechanical dissociation allowed passaging of chromosomally normal hES cell lines to a significant higher passage number than the latter two methods (42). If this report is corroborated by other laboratories using more hES cell lines, it will present a challenge for scaling up of undifferentiated hES cell cultures as mechanical dissociation demands intensive manual operation.

Adult stem cells isolation and characterization

Hematopoietic stem cells Bone marrow transplantation has been used for reconstitution of a patient's hematopoietic system after undergoing chemotherapy or radiotherapy to treat cancer and diseases such as aplastic anemia, thalassemia, Gaucher's disease, etc. HSC can be obtained from bone marrow, peripheral blood and umbilical cord blood. *Ex-vivo* expansion of undifferentiated HSCs and HSC that have been directed toward certain speficic lineage of differentiation can potentially shorten the time to repopulating the patient blood and has been long desired. The human HSC has been characterized for cell surface antigen expression $CD34^+$ and $CD38^-$. *Ex-vivo* expansion of human hematopoietic stem and progenitor cells has been described in stroma or stromal free culture systems where limited expansion over a short period of time is obtained mainly by manipulation of the cytokines and growth factors present in the media. For comprehensive reviews on *ex-vivo* expansion of hematopoietic stem and progenitor cells covering bioprocessing aspects, see Mukhopadhyay *et al*. (43) and Noll *et al*. (44).

Mesenchymal stem cells First reported in 1976, Friedenstein showed that bone marrow contains MSC in addition to HSC. MSCs were initially isolated as the plastic adherent fraction of bone marrow (45). The general protocol for isolating MSCs from bone marrow involves isolation of the mononuclear cells (usually by gradient centrifugation) and seeding these cells on tissue culture plates in medium containing fetal bovine serum (FBS). After attachment of the adherent cell fraction, the medium is removed to eliminate non-adherent cells and the adherent cells are expanded for a limited number of passages.

Human MSC (hMSC) express CD105, SH3, Stro-1, and CD13, are $CD45^-$ and differentiate into limb bud mesodermal cell lineages such as adipocytes, chondroblasts, fibroblasts, osteoblasts, and skeletal myoblasts both *in vitro* and *in vivo* (14, 46–48). The molecular mechanisms that control MSC self-renewal and differentiation are not understood in part because of the great variability of the heterogeneous initial population isolated which also vary among different laboratories (34).

A clonal study of hMSCs showed that one third has osteo-, chondro- and adipogenic potential, whereas 60–80% has osteo- and chondrogenic potential. Practically all of the clones displayed osteogenic potential. Upon culture the clones gradually lost adipo- and chondrogenic potential (49).

Bruder *et al*. (50) evaluated the growth kinetics and osteogenic differentiation potential of hMSC for 15 passages, corresponding to 38 population doublings. In the course of passaging, the cells showed marked diminished proliferation potential and change in morphology, although there was not observed effect in their osteogenic potential. In a similar study, Banfi *et al*. (51) reports growth kinetics of bulk and clonal hMSCs for five passages, corresponding to 24 population doublings for the bulk population and 19–23 population doublings for the clones. They found that the doubling time increased from 1.3 d to 16 d during the course of the experiment in the bulk population. They also observed loss of mesenchymal differentiation potential.

The limited life-span of MSCs *in vitro* has been proposed to be due to replicative senescence produced by their lack of telomerase activity, which prevents shortening of telomeres and/or commitment to terminally differentiated cells. A study showed that the telomere shortening in hMSCs during *in vitro* expansion falls within the range of telomere shortening for telomerase-negative somatic cells (50–100 bp/doubling) and the gene expression profile of late passage MSCs revealed a commitment towards the osteogenic lineage (52).

Different strategies have been used to improve isolation and *ex-vivo* expansion of MSCs, including optimization of initial plating density, initial selection of a more purified and homogeneous MSC population based on cell surface markers or cell size and expression of telomerase. Simmons *et al*. first showed that the cell surface marker STRO-1 identifies a population of bone marrow cells that has clonal capacity of forming fibroblast colonies, gives rise to cells with smooth muscle phenotype and has adipogenic and osteogenic potential (53, 54). Cell sorting of human bone marrow mononuclear cells using the cell surface markers STRO-1 and vascular cell adhesion molecule 1 (VCAM-1) allowed purification of a population of cells expressing telomerase

that could be expanded for over 40 population doublings in a serum free media with EGF, PDGF and using fibronectin as extracellular matrix. These cells showed osteogenic, chondrogenic and adipogenic potential. This population showed a 50% cloning efficiency for fibroblast colony. These clones could be expanded for about 20 population doublings and display tri-lineage mesenchymal differentiation potential *in vitro* but heterogenous differentiation capacity *in vivo* (55). The seeding cell density has been correlated with proliferation potential by maintenance of a population of small, rapidly proliferating cells at low cell densities (1–3 cells/cm^2) compared with higher densities (12 cells/cm^2). Cells grown at low cell densities proliferate for 50 population doublings, whereas at higher densities they proliferate only for 15 population doublings (56). Another study examining seeding cell densities in a larger interval (10–1000 cells/cm^2) showed that the lowest density produced the highest fold increase in cell number. Changes in morphology with different cell densities were correlated with differentiation potential. Low density cultures favoured the appearance of thin, spindle shaped cells (called RS-1A) that differentiated into adipocytes. Plating them at higher cell densities promote appearance of wider spindle-shaped cells that could be differentiated into cartilage (57). The same group recently showed that the content of rapidly self-renewing cells in MSCs can be assessed by light scattering with a flow cytometer. The isolated cells showed 90% clonogenic potential and the capacity to differentiate into mineralizing cells or adipocytes (58). Ectopic expression of telomerase allowed expansion of STRO-1 selected MSCs for more that 80 population doublings. These cells showed increased osteogenic and adipogenic potential at higher passages than non-telomerase expressing cells which senesced after 32 population doublings (59). Using the same strategy, a different group has been able to expand hMSC for more than 260 population doublings while maintaining normal differentiation potential (60). Baksh *et al*. (61) proposed a strategy for expansion of MSCs that is not based on adhesion properties. In fact, the cells are not isolated by their adherence to cell culture surfaces but by culturing in stirred suspension bioreactors. The study shows expansion of osteogenic progenitors in suspension cultures supplemented with stem cell factor (SCF) and interleukin 3 (IL-3).

Interestingly, all the optimization studies mentioned above use one or more mesenchymal lineage differentiation potential of the expanded population as objective function; none of the studies has non-mesenchymal differentiation potential after the expansion of cells originally isolated as MSCs has not been yet systematically evaluated.

Pluripotent bone marrow derived adult stem cells

MAPC were first isolated from human bone marrow in an attempt to isolate MSCs and were believed to be mesodermal progenitor cells (27) and subsequently were shown to differentiate into neurons and hepatocytes (62, 63). Isolation of MAPC was achieved by plating bone marrow mononuclear cells depleted of CD45$^+$ and glycophorin-A-positive (GlyA$^+$) in fibronectin coated surfaces and medium containing DMEM-MCDB 201 as basal media with 2% FBS or IGF-1, EGF, PDGF-BB supplemented with dexamethasone, ascorbic acid, insulin and transferrin. MAPC cultured at density between 2000–8000 cells/cm^2 were shown to expand for more than 50 population doublings, maintained their telomere length and retained multilineage differentiation potential. Plating at higher cell densities reduced their proliferation and differentiation potential. This was correlated with acquisition of high levels of CD44 and class I HLA cell surface markers expression (27).

MAPC have also been isolated from mouse and rat in similar culture conditions with the addition of leukemia inhibitory factor (LIF). Cultured at cell densities between 500–1500 cells/cm^2, they were expanded for more than 120 population doublings maintaining unchanged telomere length. Murine MAPC express the ES transcription factors Oct-4 and Rex-1 and were shown to differentiate *in vitro* at the single cell level to hepatocytes, neurons and endothelial cells (26). The requirement for low cell densities to maintain MAPC pluripotency make their expansion for *in vivo* animal studies and clinical applications a challenge (Fig. 3). Gene expression profile comparison of cells maintained at cell densities permissive and non-permissive of pluripotency revealed that the Wnt, TGF-β and Notch signaling pathways might be involved in the change of pluripotent capacity of the cells (unpublished data).

Another population of pluripotent cells from human bone marrow called marrow-isolated adult multilineage inducible (MIAMI) cells has been reported (29). This population was obtained by plating whole bone marrow cells initially in media containing 5% FBS and subsequently maintained in media containing 2% FBS in fibronectin coated dishes, these cultures were maintained in hypoxic conditions (3% oxygen) at cell densities between 1300–1400 cells/cm^2. MIAMI cells express telomerase and the transcription factors Oct-4 and Rex-1 and were expanded for more than 50 population doublings when expanded with 15% MIAMI cells conditioned medium. They were shown to differentiate into mesenchymal lineages as well as neural and pancreatic lineages.

Yoon *et al*. (30) reported the isolation of a pluripotent stem cell (hBMSCs for human bone marrow-derived multipotent stem cells) from human bone marrow with the capacity to differentiate into the three germ layers and regenerate myocardium after myocardial infarction. These population has been expanded for more than 140 population doublings in media containing 17% FBS and maintained at cell densitites ranging from 4000–8000 cells/cm^2. These cells did not express the transcription factor Oct-4 and showed unchanged telomere length after 120 population doublings. Besides differentiation into mesodermal lineages, they showed *in vitro* differentiation potential to neural and endodermal lineages by gene expression and immunocytochemistry.

The relationship between these pluripotent stem cells is not clear neither it is known whether they exist *in vivo* or they arise during culture. However, regardless of their origin, they are extremely valuable as a source of stem cells for cell therapies, drug screening and models of differentiation. They do not have the ethical concern that using hES cells have raised. They show differences in transcription factors and surface marker expression (see Table 1). Since these populations have been just recently reported, there have not been systematic studies to evaluate the effect of culture pa-

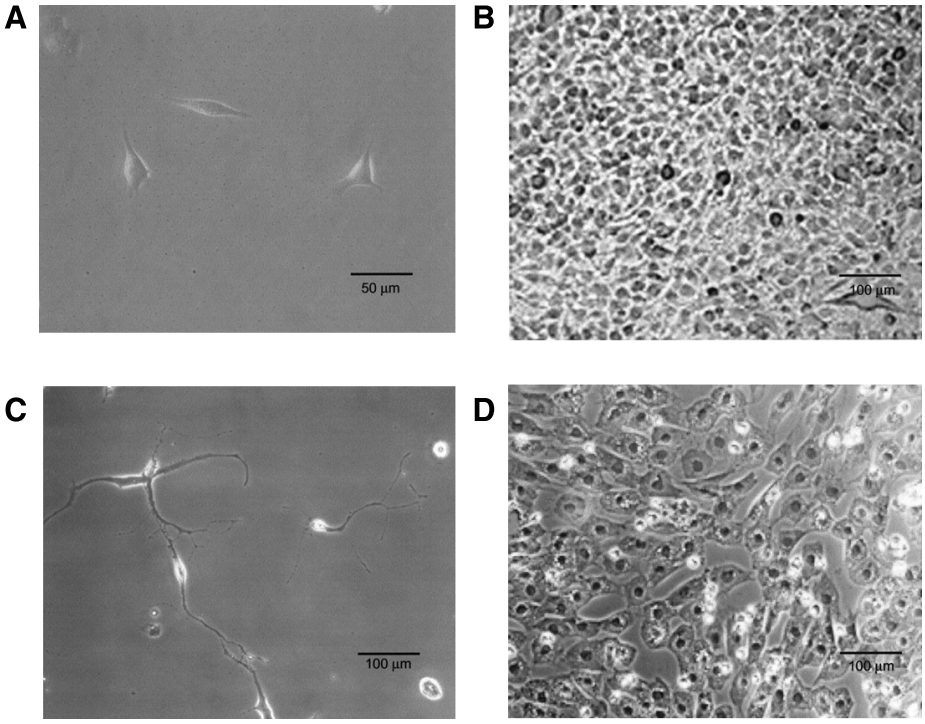

FIG. 3. Undifferentiated MAPC (A), MAPC hepatocyte differentiation (B), MAPC neural differentiation (C), and MAPC endothelial differentiation (D).

rameters (cell density, media components, oxygen concentration, etc.) on the isolation and expansion of these cells which will be required for large scale production for clinical applications.

CULTIVATION METHODS FOR STEM CELL EXPANSION

ES cell expansion and feeder layers As previously mentioned, both mouse and hES cells were originally derived using MEF feeder layers and serum-containing medium (2–4). For mouse ES cells, LIF can substitute for the feeder layer (64, 65). However, this cytokine is not sufficient for maintenance of hES pluripotency (4, 66). The use of MEF layer is not acceptable for clinical applications as the use of mouse cells, albeit irradiated, in contact with cultured human cells poses a risk of the transmission of animal-borne adventitious agents. Much effort has been made in eliminating the use of mouse feeders by substitute them with human derived feeder cells. Most of the studies have tested maintenance of hES cells on human feeders derived from fetal skin, fetal muscle, foreskin cells, adult skin fibroblasts and adult marrow cells (67–69). In addition to maintenance of hES cell lines previously established on MEF, derivation of new hES cell lines using human feeders derived from fetal muscle, fetal skin, adult fallopian tube epithelial cells and uterine endometrial cells has also been demonstrated (70–72).

Despite the progress in expanding hES without the use of MEF it is not clear whether all hES lines can be grown us-

TABLE 1. Characteristics of bone marrow derived pluripotent adult stem cells

Cell type	Surface markers			ES transcription factors	Telomerase	Cloning method	Ref.
	Negative	Low	High				
Human MAPC	CD10, CD31, CD34, CD106, CD117, CD36, HLA-DR, class I HLA	CD44, CD90w, KDR, Flt1	CD13, CD49b	N/A	Positive	10 cells/well retroviral marking	28
Mouse MAPC	CD34, CD44, CD45, CD117, MHC-I, MHC-II	Flk-1, Sca-1, SSEA-1	CD13	Oct-4, Rex-1	Positive	10 cells/well retroviral marking	26
MIAMI cells	CD34, CD36, CD45, CD54, CD56, CD109, CD117, HLA-DR	Flt1, KDR	CD10, CD29, CD44, CD49e, CD90, CD103, c-met	Oct-4, Rex-1	Positive	Cloning ring	29
hBMSC	CD13, CD29, CD31, CD34, CD44, CD45, CD49e, CD73, CD133, KDR, Tie2, HLA-DR, class I HLA	CD90, CD105, CD117		Oct-4 negative	Positive	Limiting dilution	30

ing the same MEF free protocol. The need for continuous derivation and propagation of feeders to support undifferentiated hES cells poses a great obstacle for the large-scale culture needed for clinical applications. hES cells can be maintained without direct contact with MEF when plated on extracellular matrices such as matrigel or laminin and using MEF conditioned media (73). This media contains unidentified factors that are secreted from the feeder layers. The conditioned medium is highly variable and the requirement for growing feeder cells is not completely alleviated.

Serum requirements and toward a defined chemical environment for stem cell culture Both feeder layer and the presence of FBS or other complex medium components present difficulty in elucidating the molecular mechanisms that control hES cell pluripotency. As a first step to move into more defined media formulations, most laboratories have replaced FBS with Serum Replacer, a serum free proprietary formulation of GibcoBRL (Carlsbad, CA, USA) (Patent WO 98/30679, 1998) which still contains animal derived proteins and not fully defined. Amit *et al*. (66) showed that hES cells pluripotency is maintained in media containing Serum Replacer and fibroblast growth factor 2 in the presence of MEF.

As mentioned before, the protocol for isolating MSCs from bone marrow involves plating of the mononuclear cells from bone marrow on tissue culture plates in medium containing FBS. As is well known, subtle variations are inevitable in FBS obtained from different sources and at different time. Variations in FBS appear to have a major effect on MSC culture, affecting the plating efficiency and subsequent expansion. Most of the studies report that selected lots of FBS were used. MAPC isolation and expansion also depends heavily on the FBS lot used and screening of different FBS lots is necessary to achieve optimal results.

There have been efforts to formulate a defined media that maintains pluripotency without the need for feeders or feeder conditioned media. Sato *et al*. (74) used cDNA microarrays to investigate the signaling pathways involved in maintenance of undifferentiated state in hES cells. Not surprisingly FGF, Wnt and TGF-β pathways were found to be transcriptionally active in undifferentiated ES cells. In a subsequent study, the same group found that in the presence of low concentrations of basic fibroblast growth factor (bFGF) (4 ng/ml), activation of the canonical Wnt pathway by using a GSK-3 inhibitor is sufficient to maintain hES cells in the undifferentiated state without feeders or feeder conditioned media (75). The two branches of the TGF-β family of ligands TGF-β/Activin/Nodal and BMP/GDF have been found to have opposite effects on maintenance of undifferentiated ES cells (76). TGF-β was found to promote maintenance of undifferentiated ES cells (76, 77) whereas BMPs have been shown to promote hES cell differentiation (76, 78, 79).

Two recent reports highlighted the need for high concentrations of bFGF to maintain hES cells in feeder free conditions. Wang *et al*. used 26–36 ng/ml of bFGF in media containing serum replacement to maintain undifferentiated hES cells over 30 passages (80). Xu *et al*. (81) showed that unconditioned media containing serum replacement activates BMP signaling activity in hES cells and promotes differentiation at greater extent than MEF conditioned media; this actitivity can be blocked by noggin (BMP inhibitor), high concentrations of bFGF or both. The authors show maintenance of undifferentiated hES cells lines for 18–32 passages in unconditioned media supplemented with 0.5 μg/ml noggin and 40 ng/ml bFGF. It is noteworthy that in all of these studies, the starting ES cells were derived on MEFs and subsequently withdrawn from feeders to test different conditions. Demonstration that these media conditions support derivation of new hES cell lines in feeder free conditions is still necessary.

Studies on the effect of growth factors on MSC expansion have been carried out mostly in FBS containing media. An study on the effect of different growth factors on fibroblast colony formation, proliferation and osteogenic differentiation of hMSC grown in serum containing media concluded that addition of FGF-2 had beneficial effect on proliferation and bone differentiation (82). A more recent study concluded that FGF-2 selects for a population of MSCs with longer telomeres and these cells can be expanded for about 70 doublings *in vitro* while retaining chondrogenic potential for more than 50 doublings (83). Serum free media was used to isolate the STRO-1+ and STRO-1+ VCAM-1+ bone marrow stromal stem cell populations previously described, dexamethasone and L-ascorbate were found to be essential for colony formation whereas EGF and PDGF supported colony growth (53–55). Human MAPC have been isolated in a serum free media containing dexamethasone, ascorbic acid, EGF, PDGF and IGF-1 (27).

Moving towards feeder free and defined media conditions for expansion of stem cells is necessary to avoid variations due to unknown factors produced by feeder cells as well as variability on FBS composition. This will require a thorough understanding of the different pathways involved in maintenance of pluripotency, high throughput gene expression analysis have been used with some success in the hES cell system and should be employed for studying other pluripotent stem cells.

CULTURE METHODS FOR STEM CELL DIFFERENTIATION

In the mouse system, ES cells spontaneously form three dimensional aggregates and differentiate after withdrawal of LIF and transferring to a non-adherent surface. These three dimensional aggregates, called embryoid bodies (EBs), recapitulate early embryological development in the mouse and allow derivatives of the three germ layers to form *in vitro* (Fig. 2). In hES cells spontaneous differentiation towards ectoderm, endoderm and mesoderm has also been reported when cultivated either as EB (84) or as a monolayer at high cell density (8). EB formation in mouse ES cells is normally achieved by dissociating colonies into single cells and promoting agglomeration by seeding at high cell densities in non-adherent petri dishes. Another way to form EB is to suspend cells in small droplets hanging from the underside of a culture plate, often referred to as the hanging drop method (85). For hES cells, EB formation is promoted by detaching small clumps of hES colonies by enzymatic (collagenase/dispase) or chemical dissociation (EDTA), and keeping them in suspension in non-adherent culture dishes.

TABLE 2. Growth factors and FBS requirement for maintenance of undifferentiated stem cells and for their differentiation

Cell type	Lineage	FBS	Growth factors	Ref.
hES	Undifferentiated	None	bFGF 4–40 ng/ml	66, 81
mES	Undifferentiated	15%	LIF 1000 units/ml	64, 65
hMAPC	Undifferentiated	2%	EGF 10 ng/ml, PDGF 10 ng/ml	27
mMAPC	Undifferentiated	2%	EGF 10 ng/ml, PDGF 10 ng/ml, LIF 1000 units/ml	26
hMSC	Undifferentiated	10%	bFGF 1 ng/ml	82
hMSC STRO1+	Undifferentiated	None	EGF 10 ng/ml, PDGF 10 ng/ml	53, 54
hES	Hematopoietic	20%	SCF 300 ng/ml, Flt-3 300 ng/ml, IL-3 10 ng/ml, IL-6 10 ng/ml, GCSF 50 ng/ml, BMP-4 50 ng/ml	87
hES	Neural	None	bFGF 20 ng/ml	96
mES	Hepatocytes	15%	RA 10^{-8} M, aFGF 100 ng/ml, FGF-4 20 ng/ml, HGF 50 ng/ml	103
mMAPC	Neural	None	bFGF 100 ng/ml, FGF-8 10 ng/ml, BDNF 10 ng/ml	26
hMAPC	Endothelium	None	VEGF-B 10 ng/ml	27
h m MAPC	Hepatocytes	None	FGF-4 10 ng/ml, HGF 10 ng/ml	62

h, Human; m, mouse.

Most of the applications for cell replacement therapy using stem cells require the production of specific cell types: *e.g.*, β-cells for diabetes or dopaminergic neurons for Parkinson's disease. Spontaneous differentiation in monolayer or in the EB system is still uncontrolled yielding a mixed population of different types of cells. Therapeutic application of differentiated cell types will require reproducible processes where the differentiated cell type or the progenitor of interest is considerably enriched. Furthermore, the absence of undifferentiated ES cells which upon transplantation may give rise to teratomas has to be assured. Directing ES cell differentiation towards specific lineages is a great challenge and it has been attempted mainly by the use of growth factors that promote differentiation, proliferation or survival of specific cell types (Table 2). The use of growth factors does enrich the desired population; however, a mixed population of differentiated cells is still present. Differentiation of adult pluripotent stem cells is normally achieved by plating them in different extracellular matrices and using growth factors to induce specific lineages. It is most likely that large scale production of a homogeneous differentiated cell population will have to rely on enrichment of a cell type by growth factors in the culture media as well as downstream purification; possibly by cell sorting using specific surface markers or tissue specific reporters, or by selecting for antibiotic resistance genes driven by tissue specific promoters. Selected differentiation processes will be described in the following section.

Mesodermal cell types Hematopoietic cells and cardiomyocytes were among the first specific cell types to be characterized from differentiating EBs formed from mouse ES. In the human system hematopoietic, endothelial and cardiomyocyte differentiation have been reported. Because of the availability of surface markers, hematopoietic differentiation can be tracked by flow cytometry in addition to hematopoietic colony forming assays. The differentiated hematopoietic cells can also be sorted by using the same cell surface markers. Hematopoietic differentiation has been achieved by coculture of hES cells with a murine stromal cell line or a yolk sac endothelial cell line in media containing 20% FBS without addition of cytokines, obtaining 1–2% of $CD34^+$ $CD45^-$ cells (86). EB formation in media with 20% FBS and hematopoietic cytokines plus BMP4 yielded 20% of $CD34^+$ cells and 7% of $CD34^+$ $CD45^+$ cells (87). Serum-free formulations with SCF, Flt-3l, TPO, VEGF and BMP-4 achieved 6% of $CD34^+CD45^-$ cells in the EB system and SCF, TPO and Flt-3l were required for colony forming cells from ES cells differentiated in stromal cell line with serum-free media (88).

Cardiomyocytes were obtained by spontaneous differentiation of hES cells as EBs in media containing 20% FBS for 7–10 d. Upon subsequent plating of the EBs into gelatin coated dishes 8% of the EBs contained clusters of beating cells that expressed cardiac specific proteins (89). Xu *et al.* reported enrichment to 70% of cells expressing cardiomyocytes proteins (cardiac myosin heavy chain and cTnI) by Percoll gradient centrifugation after differentiation of hES cells initiated as EBs and plated in poly-L-lysine coated plates in 20% FBS media (90). In the mouse system, purity of 99% cardiomyocytes has been achieved by driving neomycin resistance under the control of the α-cardiac myosin heavy chain (αMHC) promoter (91).

Cell surface markers for endothelial cells are also well known and can be exploited to generate highly purified populations of cells. Endothelial cells have been derived from hES cells by EB formation. These cells were sorted by flow cytometry using the endothelial marker PECAM-1. Two percent of the cells expressed PECAM-1 at day 13 of EB culture. After sorting and further passaging they were shown to express mature endothelial markers such as von Willebrand factor. They were able to uptake Dill-ac-LDL, to form tube like structures *in vitro* and functional blood-carrying microvessels *in vivo* (92).

Differentiation of pluripotent adult stem cells into mesodermal lineages has been reported with high percentage differentiations. Human MAPC and MIAMI cells differentiate to the mesenchymal lineages osteoblasts, chondroblasts, adipocytes using protocols similar to the ones established for MSCs (27, 29). Induction of MAPC differentiation was achieved by plating the cells at high confluency (greater than 20,000 cells/cm^2) in the presence of specific growth factors or differentiation agents (glycerophosphate, dexamethasone, TGF-β, horse serum). High percentage differentiation was achieved (>80%). Smooth muscle cells were also obtained from human MAPCs and BMSCs. MAPCs required EGF, PDGF-BB, and 5-azacytidine and BMSCs only PDGF (27, 30). MAPC and BMSC also have differentiated into endothelial cells by using VEGF in the culture media,

differentiated MAPCs cells expressed mature endothelial markers as well as functionality *in vitro* such as vascular tube formation and LDL uptake (93).

Ectodermal cell types High percentages (>50%) of cells expressing neural progenitor markers can be obtained from hES cells using growth factors that promote neural fate survival and proliferation; however, the challenge of obtaining specific subtypes of neural cells has proven more difficult. The conditions usually involve multistep protocols with or without EB formation and subsequent plating. Schuldiner cultivated EBs for 21 d in serum replacement media with retinoic acid treatment and subsequently plated them into collagen coated dishes. Fifty percent of the cells expressed neuronal marker neurofilament light chain mRNA (NF-L) that could be increased to 76% after consecutive replatings (94). In another study, 4 d of EB formation with RA, plating into poly-lysine/fibronectin coated plates with N2/B27 media and growth factors yielded cultures with 65% and 56% of cells expressing the neural precursor cell surface markers A2B5 and polysialylated neural cell adhesion molecule (PS-NCAM) respectively. These neural precursors were further purified to 90% using magnetic cell sorting (95). Zhang *et al*. (96) showed that by differential adhesion and enzymatic dissociation with dispase, neural tube-like rosettes with 96% of cells expressing the NSC marker nestin and most of the expressing PS-NCAM and Musashi-1 were obtained using media containing FGF-2, insulin, transferrin, progesterone, putrescine, sodium selenite and heparin. These neural precursor were expanded in suspension culture using conditions similar to the ones used to expand neurospheres and subsequently differentiated into the three neural lineages: neurons, astrocytes and oligodendrocytes.

Spontaneous differentiation of hES cells in monolayer on MEFs for 4 weeks results in a population of differentiated ES cell colonies with 56% of them expressing N-CAM. Manual isolation and further culture in serum free medium with EGF and bFGF allowed expansion of neural progenitors, reaching an almost pure population of cells expressing N-CAM, nestin and A2B5 (99%, 97% and 90%, respectively). These cells could also be differentiated into neurons, astrocytes and oligodendrocytes (97). None of the above reports show significant specific differentiation of the hES derived precursor towards specific neuronal lineages such as dopaminergic neurons (tyrosine hydroxylase positive) which are lost during Parkinson's disease.

Differentiation towards dopaminergic neurons was achieved by co-culturing hES cells with stromal cells and media containing glial-derived neurotrophic factor (GDNF) (98). In another study, stromal cells and media containing sonic hedgehog and FGF8 yielded between 60–80% of tyrosine hydroxylase/Tuj1 positive cells within the neuronal population which comprised 30–50% of the cells (99). Differentiated population with 75% of neurons (45% of cells) tyrosine hydroxylase/B-III-Tubulin were obtained in, suspension culture for 6 weeks in serum-free media containing only DMEM and N2 supplement (100).

Mouse MAPC was reported to differentiate into cells with phenotypic and morphological characteristics of astrocytes, oligodendrocytes and neurons with high differentiation efficiencies (>90%) in monolayer system with media containing bFGF. A sequential protocol with bFGF, FGF-8 and brain derived neurotrophic factor (BDNF) allowed a more mature phenotypes, including 30% of tyrosine hydroxylase positive cells to be obtained (26). Coculture of the neural differentiated MAPCs with astrocytes allowed differentiation into cells with electrophysiological characteristics similar to those of midbrain neurons (101). Similar protocols were used to differentiate hBMSCs and MIAMI cells to neural cells (29, 30).

Endodermal cell types Directed differentiation of ES cells towards cell types of the definitive endodermal lineage such as hepatocytes and pancreatic beta cells has been challenging. The specification and patterning of endoderm in the embryo is not well understood making it difficult to choose growth factors to induce endoderm *in vitro*. Cell surface markers for the different endodermal progenitors have been lacking which impeding their isolation from the mixed population normally obtained after stem cell differentiation. During ES cell differentiation both embryonic and extraembryonic endoderm lineages are seen while the latter also expresses several definitive endoderm markers, it is not known whether they can produce mature definitive endoderm cells. This uncertainty makes using endodermal surface markers even more difficult.

The use of endoderm specific reporters will be helpful to identify the progenitors and define their gene expression profile. It may lead to the identification of useful markers that can be used to purify these cells from a mixed culture. In the mouse ES cell system, Kubo *et al*. (102) used a brachyury-GFP ES cell line to show that definitive endoderm is derived from a mesendoderm progenitor cell expressing brachyury; furthermore, the commitment towards definitive endoderm lineage is dependent on factors contained in FBS that can be replaced by high concentrations (100 ng/ml) of activin A during the first stages of EB formation. Sorted brachyury positive cells could be further cultured and induced towards hepatocyte-like cells expressing mRNA for the mature hepatocyte enzymes tyrosine aminotransferase (TAT) and carbamoyl phosphate synthase I. In a recent report, Teratani *et al*. (103) used a mouse ES cell line with an albumin promoter driven GFP reporter to optimize conditions for hepatocyte differentiation in monolayer culture, that comprises multiple steps with retinoic acid, HGF, FGF-1, FGF-4 and oncostatin M. After 10 d in culture, 30% of cells expressing GFP were obtained when combining the growth factors with collagen as extracellular matrix. The GFP positive cells could be furthered expanded to 80% with subsequent culture in Williams E medium (103).

Hepatocyte differentiation from hES cells has been reported by different groups. Four-day-old EBs formed in presence of 20% of FBS were plated on matrigel as well as a confluent monolayer system. Both cultures were treated with DMSO and sodium butyrate to obtain cultures where 70–80% of cells in both cases express early hepatocyte markers and show glycogen accumulation and inducible cytochrome P450 activity (104). hES cells cultivated as EB for 6 d were plated into collagen I treated dishes using IMDM medium containing dexamethasone, 20% FBS and insulin. The differentiated cells expressed albumin mRNA

at a level 1% of that in adult primary human hepatocytes and produced urea (105).

The differentiation of hES cells towards pancreatic β-cell lineage is probably an even greater challenge. During spontaneous EB differentiation, 1–3% of cells in 60–70% of EBs stained positive for insulin (106). Mouse ES cells were shown to differentiate to insulin producing cells using the ectodermal pathway (107). Segev *et al*. (108) differentiated hES cells towards insulin producing cells by plating EBs in insulin–transferrin–selenium–fibronectin medium, followed by supplementing with N2, B27, and bFGF. Next, the glucose concentration in the medium was reduced, bFGF was withdrawn, and nicotinamide was added. Dissociation of the cells and culturing them in suspension culture that promotes cluster formation increased insulin secretion and the number of insulin (70%), somatostatin (43%) or glucagon (50%) positive cells. Importantly, these cells stained positive for c-peptide which is cleaved upon secretion of insulin provides proofs that insulin was produced by the cells and not uptaken up from the media.

Controversy still persists about the production of insulin secreting cells from ES cells by using the ectodermal pathway. The observed insulin positive cells could be the result of insulin uptake from the media; furthermore, many of the cells that show insulin staining are apoptotic or necrotic (109, 110). None of the papers published on hES cell differentiation towards insulin producing cells report sorting of the cells from the mixed population. From mouse ES cells, β-cells specific promoters driving drug selection genes have been used to produce highly purified populations of insulin producing cells (111, 112).

Endodermal differentiation from adult pluripotent stem cells has been achieved by the combination of growth factors and extracellular matrices. MAPC have been shown to differentiate into cells expressing mature hepatocyte markers and functional characteristics. FGF-4 and HGF in combination with matrigel as extracellular matrix induce MAPC differentiation into epithelioid cells that acquired functional characteristics of hepatocytes: such as urea and albumin secretion, phenobarbital inducibility of cytochrome p450, LDL uptake, and stored glycogen storage. This protocol yields 60% of cells differentiating into albumin, HNF-3β and CK18 expressing cells for mouse and rat MAPCs and 90% for human MAPC (62). hBMSCs were differentiated into cells expressing hepatocyte markers by using a similar protocol with addition of DMSO or sodium butyrate (30). MIAMI cells were shown to differentiate into clusters of cells expressing insulin and glucagon mRNA. The procedure involved sequential steps with bFGF, exendin-4, HGF and Activin A (29).

BIOREACTOR SYSTEMS FOR EXPANSION AND DIFFERENTIATION OF STEM CELLS

The selection of bioreactor system for mammalian cell cultivation beyond bench scale is largely dependent on whether the cells are adherent, suspension grown as single cells or aggregates as depicted in Fig. 4. The principles of selecting bioreactors for stem cell cultivation and differentiation are largely the same as those for mammalian cell culture processing, except that few stem cells grow in suspension other than some in hematopoietic lineage (113). Undifferentiated pluripotent stem cells are anchorage dependent, whereas EBs can be cultivated either stationarily or in suspension. One of the simplest forms of cultivating adherent cells in large quantities, namely roller bottle, is giving

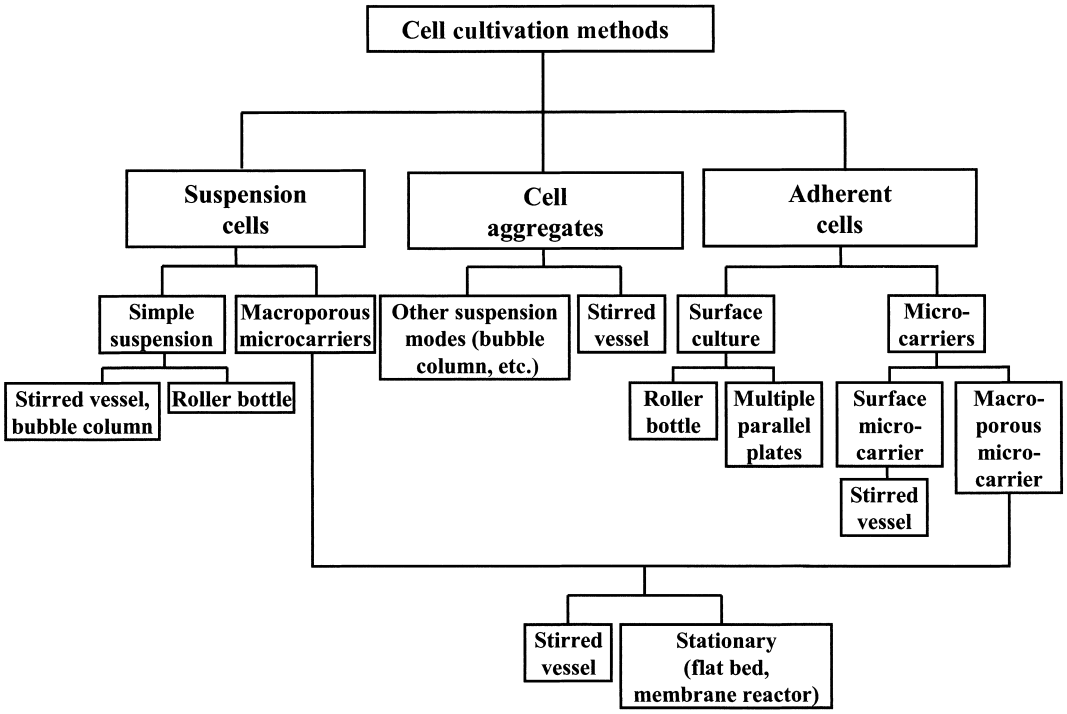

FIG. 4. Cell cultivation methods applicable for expansion and differentiation of stem cells.

way to multiple parallel plates recently. A reasonably large cultivation surface area can be attained with a multiple plate vessel, reducing the labor requirement and the contamination risk significantly as compared to roller bottles. Simple stirred vessels, like spinner flasks and fermentors, are easy to operate and can be readily equipped with on-line monitoring instrumentation for environmental control. However, their use for strictly anchorage dependent cells, such as MSC or MAPC, will require microcarriers to provide attachment surface (114).

Solid microcarriers made of cross-linked dextran, cellulose or polystyrene support cell growth on their external surface. Although those spherical beads have been used in conventional cell culture for vaccine production, their use for stem cell cultivation is still limited. Mechanical agitation may cause subtle effect on cellular membrane and thus affecting their properties. Since cells are directly exposed to the bulk medium, the extensive mixing might decimate any microenvironment that is normally formed by those cells when grown on stationary surface. Macroporous microcarriers, typically made of gelatin, collagen or other soft material with relatively high oxygen diffusion characteristics, have open pores in their interior to provide larger growth surface area (115).

Contrary to common expectation, the rough external surface of most macroporous mirocarriers actually generates more microeddies on their surface than conventional solid microcarriers which have a spherical shape and smooth surface. Cells grown on the external surface are actually often exposed to higher fluid shear force. However, once allowed to enter and grown in the internal pores, cells are protected from the mechanical force generated by fluid flow (116). Macroporous microcarriers may also allow a microenvironment in the vicinity of cell growth to be different from the bulk of the culture vessel. They are not only used in growing adherent cells, but also for suspension cells and have been used in hematopoietic cell cultivation which entails a mixed population of adherent and suspension cells in hematopoietic system (117, 118). In addition to being suspended in stirred vessels, macroporous microcarriers have also been used in membrane bioreactors, either flat bed or in other configurations (119, 120).

Most of the flat bed reactors used consist of membrane separated compartments. In all configurations, a cell chamber is separated by membrane from medium chamber. The mode of oxygen supply, either through an additional gas compartment in contact with the cell chamber, or through the recirculation of medium, further classify the membrane bioreactors into two major categories. In the case that no gas compartment is used, medium becomes the means of oxygen supply. The medium is recirculated at a high rate to provide sufficient supply of oxygen. Two further different operating modes are frequently used: (i) medium is allow to permeate through the membrane from the recirculating loop into the cell chamber and collect at the exit of cell chamber; (ii) nutrients and oxygen are supplied to the cell chamber through diffusion, but not convective flow of medium, across the membrane separating medium and cell chambers. A further variation is one that a slow stream of high molecular weight components is directly supplied at a low rate to the cell chamber as their diffusion through the membrane is rather slow. The inclusion of a gas compartment provides a more uniform oxygen concentration across the cell chamber. It also allows medium (supply of nutrients, autocrine conditioning factors, etc.) and oxygen to be controlled separately; one can operate the reactor at any oxygen concentration independent of medium flow rate.

Aggregates such as EBs, being larger particles than single cells, require somewhat different agitation mechanism than that for single cells. They require more liquid pumping for suspension, thus large impellers and slower agitation rate. However, the tendency of many cell aggregates to agglomerate to become very large particles (thus causing oxygen transfer limitations and necrosis) also requires that sufficiently high agitation rate be used. Because of their tendency to agglomerate, in larger scale operations aggregates are more likely to be cultivated in mixing vessels (stirred vessel, bubble column, or other mixing mechanisms) rather than under stationary conditions. If laid on large flat surface, the fluid movement in the culture, caused by natural convection or other environmental perturbation, will inevitably cause aggregates to agglomerate. Since there is no mixing to break apart the collided aggregates, the particles tend to grow larger over time.

The bioreactors described above are all suitable for the cultivation of stem cells for both propagation and differentiation. However, their main advantage is not merely for generating larger quantity of cells. Their use allows for continuous monitoring and control of the physical and chemical environment that might be crucial for stem cell cultivation. The control is not limited to providing cells with constant and uniform environment. Our ability to supply cells with dynamic profiles of nutrients, oxygen and growth factors over time may change the landscape of stem cell culture. Furthermore, the creation of gradients of environmental conditions is possible. This will allow the potential interaction of cells growing under different conditions in a spatially distributed (*e.g.*, a gradient of oxygen tension) environment to be studied in a single culture. The selection of bioreactors is affected by the cell type, the process and the way the reactor is to be controlled. In the following section, their relevance to stem cell proliferation and differentiation will be described.

Culture systems for stem cell expansion The purpose of stem cell cultivation is to expand stem cells and sustain their pluripotent potential, the resulting cell population needs to be harvested for differentiation or further expansion. This is unlike traditional cell culture processes in which the secreted protein or virus is the product. Therefore, for stem cell propagation, a cultivation method with a readily available means of cell harvesting and passaging is necessary. Furthermore, for stem cell cultivation, in addition to increasing cell number, the retention of the self-regenerating and differentiation capabilities are critical. Therefore, a means of readily examining those characteristics, either basing on surface markers or morphology, is an important consideration in reactor selection. These considerations pose some constrain on the use of membrane reactors and macroporous microcarriers. The long doubling time of undifferentiated hES (~36 h) necessitates a large quantity of

undifferentiated cells to be maintained to give rise to enough cells for differentiation for clinical applications (33). For other stem cells, such as MAPC, the need of maintaining cells at very low density requires a rather frequent passaging to avoid reaching a high density and losing stem cell properties. This necessity certainly limits the choice of reactors when large-scale propagation is needed for clinical trials.

For hES cells, the cultivation is further constrained by the use of feeder cells and the fact that they are passaged as colonies rather than single cells (66). Even if conditioned medium is used (73), the large quantity of conditioned medium needed would almost make the production of conditioned medium a delicate process by itself. Unless the critical components constituting conditioning factors are identified, quality control of the conditioned medium product will be of a great concern. The use of feeder layer or feeder cell conditioned medium most likely will need to be eliminated for large-scale culture. Unless cross-talk between hES and feeder cells is needed (an unlikely event giving some success of replacing feeder cells with conditioned medium), one should be able to eliminate the use of feeder cells and conditioned medium. Paracrines produced by feeder cells and surface ligands of feeder cells that renders the culture conditions permissive for stem cells can both be replaced by defined molecules once they are identified. Until then, flat surface cultivation of hES cells with a direct contact (*i.e.*, growing on) feeder cells or in indirect contact (with communication of diffusible molecules) through a membrane separating the two cell populations is likely to be the choice.

The passaging methods of manually dissecting the colonies will also need to be modified. Replacing them with enzymatic dissociation that could be performed with less labor, or even automated, will be a necessity. The scaled up passaging method will have to be proved to maintain normal karyotype of the cells (42). So far, there has been no report of large-scale expansion of undifferentiated hES cells principally, because the basic biological knowledge of factors that maintain hES pluripotency is still being developed.

Systems for large-scale culture for undifferentiated pluripotent adult stem cells have not been described so far. Basic knowledge on the mechanisms that maintain pluripotency in this system will greatly facilitate the design of large-scale cell culture systems for stem cell applications. These cells have been isolated and expanded as attachment dependant cells (some of them requiring low cell densities). Other strategies such as the recent report from Baksh *et al*. (61), about isolation of MSCs with osteogenic potential in stirred suspension culture could alleviate this problem if the isolated cells are proven to be pluripotent.

Culture systems for stem cell differentiation An ideal differentiation system should be amenable to be scaled-up and allow for environmental control (pH, oxygen, cytokines, nutrients concentration). The differentiation of pluripotent stem cells is typically performed by culturing them as adherent monolayer at high cell densities or by growing them as EBs in the case of ES cells. In general terms, the cell culture bioreactors appropriate for monolayer can be applied to the differentiation processes employing monolayer culture, and those suitable for aggregate cultivation will likely also perform well for EB. The key in the cultivation of pluripotent stem cells for differentiation will thus reside in the control of inductive chemical and physical environment.

The bottleneck in EB cultivation for differentiation is the formation of EBs to start the differentiation process. Some methods more amenable to scale up have been used in EB culture for differentiation of embryonic stem cells. Zandstra *et al*. (121) used spinner flasks to differentiate mouse embryonic stem cells to cardiomyocytes using an ES cell line carrying the neomycin resistance gene driven by the cardiac specific marker αMHC. EBs were first formed in petri dishes to prevent excessive agglomeration of ES cells followed by 18 d culture in spinner flask with 9 d of selection with G418. This process yielded a population consisting of 70% cells expressing sarcomeric myosyin heavy chain by flow cytometry. The initial stages of EB formation in petri dishes pose some challenges, as a high degree of EB agglomeration was seen when the cells were seeded at high density. The resulting large aggregates quickly become necrotic. Formation of excessively large EBs was alleviated by using methylcellulose in the medium, using limited number of cells in small hanging drops of medium, and by alginate entrapment (85, 122). Agarose encapsulation was used to control agglomeration of mouse and hES cells in the initial stages of EB formation, the encapsulated mouse ES cells were then transferred to stirred-cultures. The encapsulation system allowed a 61-fold expansion in the number of cells which was the same as the static control non-stirred culture but significantly higher than stirred non-encapsulated system. Furthermore, the stirred culture system allowed oxygen tension and pH to be controlled for evaluation of differentiation 4% and 20% oxygen concentration (123). Another approach for controlling EB agglomeration employs rotating bioreactors. hES cells were formed small aggregates in a slow turning lateral turning lateral vessel (STLV) only a few large necrotic aggregates formed in a high aspect rotating vessel (HARV). The STLV allowed 70-fold cell number expansion after 28 d of culture and the EBs obtained displayed spontaneous differentiation patterns to the three germ layers (124).

The process of EB formation entails a transition from dispersed single cell stage to semi-organized three dimensional structure. EB formation may take place on a non-adherent surface or in suspension. The process of EB formation, either on a surface or in suspension, is not unlike the formation of hepatocyte spheroids (125–127). In the early stage dispersed cells migrate on surface and move toward each other; the cluster of cells subsequently becomes a focus of agglomeration to engulf other cells which come across its path and establish contacts. In a suspension of cells, the initial clusters are largely formed by collision of particles. This initial cell aggregate later reorganize its structure and establish the characteristic three-dimensional architecture. Although the process has not been examined extensively in EB, a similar process of hepatocyte spheroid formation may hint that after the initial cell agglomeration extensive reorganization of cytoskeleton and cell surface proteins occur to give rise to well organized tissue like structure (128, 129). EBs are not merely an agglomeration of cells, but a mass of cells with relatively regular shape. Many have been

shown to be hollow in its interior (84), while others are solid inside. From the similarity of EB to other three dimensional tissue-resembling structures formed *in vitro*, there are thus reasons to believe that mixing vessel will prove to be efficient for large-scale EB formation and differentiation.

CONCLUDING REMARKS

Both embryonic and adult pluripotent stem cells hold much promise for cell therapies and tissue engineering. The fulfillment of the promise will require further advances in our basic knowledge on the mechanisms that maintain pluripotency and direct differentiation. The inclusion of undefined components in their cultivation for some types of stem cells, such as feeder layers, FBS, pose additional challenges for both clinical application and mechanistic studies. Recent progress toward eliminating those feeder layer requirement and development of better defined medium are encouraging. A variety of culture systems amenable for better environmental control are applicable for stem cell expansion and differentiation. Their use for various biological investigations will greatly aid in the advances in unveiling the regulation of pluripotency and differentiation *in vitro* and facilitate clinical applications.

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REFERENCES

1. **Weissman, I. L.:** Translating stem and progenitor cell biology to the clinic: barriers and opportunities. Science, **287**, 1442–1446 (2000).
2. **Martin, G. R.:** Isolation of a pluripotent cell line from early mouse embryos cultured in medium conditioned by teratocarcinoma stem cells. Proc. Natl. Acad. Sci. USA, **78**, 7634–7638 (1981).
3. **Evans, M. J. and Kaufman, M. H.:** Establishment in culture of pluripotential cells from mouse embryos. Nature, **292**, 154–156 (1981).
4. **Thomson, J. A., Itskovitz-Eldor, J., Shapiro, S. S., Waknitz, M. A., Swiergiel, J. J., Marshall, V. S., and Jones, J. M.:** Embryonic stem cell lines derived from human blastocysts. Science, **282**, 1145–1147 (1998).
5. **Thomson, J., Kalishman, J., Golos, T., Durning, M., Harris, C., Becker, R., and Hearn, J.:** Isolation of a primate embryonic stem cell line. Proc. Natl. Acad. Sci. USA, **92**, 7844–7848 (1995).
6. **Smith, A. G.:** Embryo-derived stem cells: of mice and men. Annu. Rev. Cell Dev. Biol., **17**, 435–462 (2001).
7. **Wobus, A. M., Holzhausen, H., Jakel, P., and Schoneich, J.:** Characterization of a pluripotent stem cell line derived from a mouse embryo. Exp. Cell. Res., **52**, 212–219 (1984).
8. **Reubinoff, B. E., Pera, M. F., Fong, C. Y., Trounson, A., and Bongso, A.:** Embryonic stem cell lines from human blastocysts: somatic differentiation *in vitro*. Nat. Biotechnol., **18**, 399–404 (2000).
9. **Spangrude, G., Heimfeld, S., and Weissman, I.:** Purification and characterization of mouse hematopoietic stem cells. Science, **241**, 58–62 (1988).
10. **Bhatia, M., Wang, J., Knapp, U., Bonnet, D., and Dick, J.:** Purification of primitive human hematopoietic cells capable of repopulating immune-deficient mice. Proc. Natl. Acad. Sci. USA, **94**, 5320–5325 (1997).
11. **Gage, F. H.:** Mammalian neural stem cells. Science, **287**, 1433–1438 (2000).
12. **Pittenger, M. F., Mackay, A. M., Beck, S. C., Jaiswal, R. K., Douglas, R., Mosca, J. D., Moorman, M. A., Simonetti, D. W., Craig, S., and Marshak, D. R.:** Multilineage potential of adult human mesenchymal stem cells. Science, **284**, 143–147 (1999).
13. **Prockop, D.:** Marrow stromal cells as stem cells for nonhematopoietic tissues. Science, **276**, 71–74 (1997).
14. **Fridenshtein, A.:** Stromal bone marrow cells and the hematopoietic microenvironment. Arkh. Patol., **44**, 3–11 (1982).
15. **Potten, C.:** Stem cells in gastrointestinal epithelium: numbers, characteristics and death. Philos. Trans. R. Soc. Lond. B. Biol. Sci., **353**, 821–830 (1998).
16. **Watt, F.:** Epidermal stem cells: markers patterning and the control of stem cell fate. Philos. Trans. R. Soc. Lond. B. Biol. Sci., **353**, 831–837 (1998).
17. **Alison, M. and Sarraf, C.:** Hepatic stem cells. J. Hepatol., **29**, 678–683 (1998).
18. **Krause, D. S., Theise, N. D., Collector, M. I., Henegariu, O., Hwang, S., Gardner, R., Neutzel, S., and Sharkis, S. J.:** Multi-organ, multi-lineage engraftment by a single bone marrow-derived stem cell. Cell, **105**, 369–377 (2001).
19. **Wagers, A. J., Sherwood, R. I., Christensen, J. L., and Weissman, I. L.:** Little evidence for developmental plasticity of adult hematopoietic stem cells. Science, **297**, 2256–2259 (2002).
20. **Murry, C. E., Soonpaa, M. H., Reinecke, H., Nakajima, H., Nakajima, H. O., Rubart, M., Pasumarthi, K. B., Virag, J. I., Bartelmez, S. H., Poppa, V., and other 4 authors:** Haematopoietic stem cells do not transdifferentiate into cardiac myocytes in myocardial infarcts. Nature, **428**, 664–668 (2004).
21. **Bjornson, C. R., Rietze, R. L., Reynolds, B. A., Magli, M. C., and Vescovi, A. L.:** Turning brain into blood: a hematopoietic fate adopted by adult neural stem cells *in vivo*. Science, **283**, 534–537 (1999).
22. **Woodbury, D., Schwarz, E. J., Prockop, D. J., and Black, I. B.:** Adult rat and human bone marrow stromal cells differentiate into neurons. J. Neurosci. Res., **61**, 364–370 (2000).
23. **Lee, K. D., Kuo, T. K., Whang-Peng, J., Chung, Y. F., Lin, C. T., Chou, S. H., Chen, J. R., Chen, Y.P., and Lee, O. K.:** *In vitro* hepatic differentiation of human mesenchymal stem cells. Hepatology, **40**, 1275–1284 (2004).
24. **Wang, X., Willenbring, H., Akkari, Y., Torimaru, Y., Foster, M., Al-Dhalimy, M., Lagasse, E., Finegold, M., Olson, S., and Grompe, M.:** Cell fusion is the principal source of bone-marrow-derived hepatocytes. Nature, **422**, 897–901 (2003).
25. **Lakshmipathy, U. and Verfaillie, C.:** Stem cell plasticity. Blood Rev., **19**, 29–38 (2005).
26. **Jiang, Y., Jahagirdar, B. N., Reinhardt, R. L., Schwartz, R. E., Keene, C. D., Ortiz-Gonzalez, X. R., Reyes, M., Lenvik, T., Lund, T., Blackstad, M., and other 6 authors:** Pluripotency of mesenchymal stem cells derived from adult marrow. Nature, **418**, 41–49 (2002).
27. **Reyes, M., Lund, T., Lenvik, T., Aguiar, D., Koodie, L., and Verfaillie, C. M.:** Purification and *ex vivo* expansion of postnatal human marrow mesodermal progenitor cells. Blood, **98**, 2615–2625 (2001).
28. **Reyes, M. and Verfaillie, C. M.:** Characterization of multipotent adult progenitor cells, a subpopulation of mesenchymal stem cells. Ann. N.Y. Acad. Sci., **938**, 231–233; discussion, 233–235 (2001).
29. **D'Ippolito, G., Diabira, S., Howard, G. A., Menei, P., Roos, B. A., and Schiller, P. C.:** Marrow-isolated adult

multilineage inducible (MIAMI) cells, a unique population of postnatal young and old human cells with extensive expansion and differentiation potential. J. Cell. Sci., **117**, 2971–2981 (2004).
30. **Yoon, Y. S., Wecker, A., Heyd, L., Park, J. S., Tkebuchava, T., Kusano, K., Hanley, A., Scadova, H., Qin, G., Cha, D. H., and other 4 authors:** Clonally expanded novel multipotent stem cells from human bone marrow regenerate myocardium after myocardial infarction. J. Clin. Invest., **115**, 326–338 (2005).
31. **Kogler, G., Sensken, S., Airey, J. A., Trapp, T., Muschen, M., Feldhahn, N., Liedtke, S., Sorg, R. V., Fischer, J., Rosenbaum, C., and other 11 authors:** A new human somatic stem cell from placental cord blood with intrinsic pluripotent differentiation potential. J. Exp. Med., **200**, 123–135 (2004).
32. **Young, H. E.:** Existence of reserve quiescent stem cells in adults, from amphibians to humans. Curr. Top. Microbiol. Immunol., **280**, 71–109 (2004).
33. **Stojkovic, M., Lako, M., Strachan, T., and Murdoch, A.:** Derivation, growth and applications of human embryonic stem cells. Reproduction, **128**, 259–267 (2004).
34. **Baksh, D., Song, L., and Tuan, R. S.:** Adult mesenchymal stem cells: characterization, differentiation, and application in cell and gene therapy. J. Cell. Mol. Med., **8**, 301–316 (2004).
35. **Richards, M., Fong, C. Y., Tan, S., Chan, W. K., and Bongso, A.:** An efficient and safe xeno-free cryopreservation method for the storage of human embryonic stem cells. Stem Cells, **22**, 779–789 (2004).
36. **Heng, B. C., Kuleshova, L. L., Bested, S. M., Liu, H., and Cao, T.:** The cryopreservation of human embryonic stem cells. Biotechnol. Appl. Biochem., **41**, 97–104 (2005).
37. **Henderson, J. K., Draper, J. S., Baillie, H. S., Fishel, S., Thomson, J. A., Moore, H., and Andrews, P. W.:** Preimplantation human embryos and embryonic stem cells show comparable expression of stage-specific embryonic antigens. Stem Cells, **20**, 329–337 (2002).
38. **Carpenter, M. K., Rosler, E., and Rao, M. S.:** Characterization and differentiation of human embryonic stem cells. Cloning Stem Cells, **5**, 79–88 (2003).
39. **Draper, J. S., Smith, K., Gokhale, P., Moore, H. D., Maltby, E., Johnson, J., Meisner, L., Zwaka, T. P., Thomson, J. A., and Andrews, P. W.:** Recurrent gain of chromosomes 17q and 12 in cultured human embryonic stem cells. Nat. Biotechnol., **22**, 53–54 (2004).
40. **Rosler, E. S., Fisk, G. J., Ares, X., Irving, J., Miura, T., Rao, M. S., and Carpenter, M. K.:** Long-term culture of human embryonic stem cells in feeder-free conditions. Dev. Dyn., **229**, 259–274 (2004).
41. **Buzzard, J. J., Gough, N. M., Crook, J. M., and Colman, A.:** Karyotype of human ES cells during extended culture. Nat. Biotechnol., **22**, 381–382 (2004).
42. **Mitalipova, M. M., Rao, R. R., Hoyer, D. M., Johnson, J. A., Meisner, L. F., Jones, K. L., Dalton, S., and Stice, S. L.:** Preserving the genetic integrity of human embryonic stem cells. Nat. Biotechnol., **23**, 19–20 (2005).
43. **Mukhopadhyay, A., Madhusudhan, T., and Kumar, R.:** Hematopoietic stem cells: clinical requirements and developments in *ex-vivo* culture. Adv. Biochem. Eng. Biotechnol., **86**, 215–253 (2004).
44. **Noll, T., Jelinek, N., Schmid, S., Biselli, M., and Wandrey, C.:** Cultivation of hematopoietic stem and progenitor cells: biochemical engineering aspects. Adv. Biochem. Eng. Biotechnol., **74**, 111–128 (2002).
45. **Friedenstein, A. J., Gorskaja, J. F., and Kulagina, N. N.:** Fibroblast precursors in normal and irradiated mouse hematopoietic organs. Exp. Hematol., **4**, 267–274 (1976).
46. **Prockop, D. J.:** Marrow stromal cells as stem cells for nonhematopoietic tissues. Science, **276**, 71–74 (1997).
47. **Haynesworth, S. E., Baber, M. A., and Caplan, A. I.:** Cell surface antigens on human marrow-derived mesenchymal cells are detected by monoclonal antibodies. Bone, **13**, 69–80 (1992).
48. **Gronthos, S. and Simmons, P. J.:** The biology and application of human bone marrow stromal cell precursors. J. Hematother., **5**, 15–23 (1996).
49. **Muraglia, A., Cancedda, R., and Quarto, R.:** Clonal mesenchymal progenitors from human bone marrow differentiate *in vitro* according to a hierarchical model. J. Cell Sci., **113**, 1161–1166 (2000).
50. **Bruder, S. P., Jaiswal, N., and Haynesworth, S. E.:** Growth kinetics, self-renewal, and the osteogenic potential of purified human mesenchymal stem cells during extensive subcultivation and following cryopreservation. J. Cell. Biochem., **64**, 278–294 (1997).
51. **Banfi, A., Muraglia, A., Dozin, B., Mastrogiacomo, M., Cancedda, R., and Quarto, R.:** Proliferation kinetics and differentiation potential of *ex vivo* expanded human bone marrow stromal cells: implications for their use in cell therapy. Exp. Hematol., **28**, 707–715 (2000).
52. **Banfi, A., Bianchi, G., Notaro, R., Luzzatto, L., Cancedda, R., and Quarto, R.:** Replicative aging and gene expression in long-term cultures of human bone marrow stromal cells. Tissue Eng., **8**, 901–910 (2002).
53. **Simmons, P. J. and Torok-Storb, B.:** Identification of stromal cell precursors in human bone marrow by a novel monoclonal antibody, STRO-1. Blood, **78**, 55–62 (1991).
54. **Gronthos, S., Graves, S. E., Ohta, S., and Simmons, P. J.:** The STRO-1+ fraction of adult human bone marrow contains the osteogenic precursors. Blood, **84**, 4164–4173 (1994).
55. **Gronthos, S., Zannettino, A. C., Hay, S. J., Shi, S., Graves, S. E., Kortesidis, A., and Simmons, P. J.:** Molecular and cellular characterisation of highly purified stromal stem cells derived from human bone marrow. J. Cell Sci., **116**, 1827–1835 (2003).
56. **Colter, D. C., Class, R., DiGirolamo, C. M., and Prockop, D. J.:** Rapid expansion of recycling stem cells in cultures of plastic-adherent cells from human bone marrow. Proc. Natl. Acad. Sci. USA, **97**, 3213–3218 (2000).
57. **Sekiya, I., Larson, B. L., Smith, J. R., Pochampally, R., Cui, J. G., and Prockop, D. J.:** Expansion of human adult stem cells from bone marrow stroma: conditions that maximize the yields of early progenitors and evaluate their quality. Stem Cells, **20**, 530–541 (2002).
58. **Smith, J. R., Pochampally, R., Perry, A., Hsu, S. C., and Prockop, D. J.:** Isolation of a highly clonogenic and multipotential subfraction of adult stem cells from bone marrow stroma. Stem Cells, **22**, 823–831 (2004).
59. **Shi, S., Gronthos, S., Chen, S., Reddi, A., Counter, C. M., Robey, P. G., and Wang, C. Y.:** Bone formation by human postnatal bone marrow stromal stem cells is enhanced by telomerase expression. Nat. Biotechnol., **20**, 587–591 (2002).
60. **Simonsen, J. L., Rosada, C., Serakinci, N., Justesen, J., Stenderup, K., Rattan, S. I., Jensen, T. G., and Kassem, M.:** Telomerase expression extends the proliferative lifespan and maintains the osteogenic potential of human bone marrow stromal cells. Nat. Biotechnol., **20**, 592–596 (2002).
61. **Baksh, D., Davies, J. E., and Zandstra, P. W.:** Adult human bone marrow-derived mesenchymal progenitor cells are capable of adhesion-independent survival and expansion. Exp. Hematol., **31**, 723–732 (2003).
62. **Schwartz, R. E., Reyes, M., Koodie, L., Jiang, Y., Blackstad, M., Lund, T., Lenvik, T., Johnson, S., Hu, W. S., and Verfaillie, C. M.:** Multipotent adult progenitor cells from bone marrow differentiate into functional hepatocyte-like cells. J. Clin. Invest., **109**, 1291–1302 (2002).
63. **Zhao, L. R., Duan, W. M., Reyes, M., Keene, C. D., Verfaillie, C. M., and Low, W. C.:** Human bone marrow stem cells exhibit neural phenotypes and ameliorate neuro-

logical deficits after grafting into the ischemic brain of rats. Exp. Neurol., **174**, 11–20 (2002).
64. **Williams, R. L., Hilton, D. J., Pease, S., Willson, T. A., Stewart, C. L., Gearing, D. P., Wagner, E. F., Metcalf, D., Nicola, N. A., and Gough, N. M.:** Myeloid leukaemia inhibitory factor maintains the developmental potential of embryonic stem cells. Nature, **336**, 684–687 (1988).
65. **Smith, A. G., Heath, J. K., Donaldson, D. D., Wong, G. G., Moreau, J., Stahl, M., and Rogers, D.:** Inhibition of pluripotential embryonic stem cell differentiation by purified polypeptides. Nature, **336**, 688–690 (1988).
66. **Amit, M., Carpenter, M. K., Inokuma, M. S., Chiu, C. P., Harris, C. P., Waknitz, M. A., Itskovitz-Eldor, J., and Thomson, J. A.:** Clonally derived human embryonic stem cell lines maintain pluripotency and proliferative potential for prolonged periods of culture. Dev. Biol., **227**, 271–278 (2000).
67. **Amit, M., Margulets, V., Segev, H., Shariki, K., Laevsky, I., Coleman, R., and Itskovitz-Eldor, J.:** Human feeder layers for human embryonic stem cells. Biol. Reprod., **68**, 2150–2156 (2003).
68. **Richards, M., Tan, S., Fong, C. Y., Biswas, A., Chan, W. K., and Bongso, A.:** Comparative evaluation of various human feeders for prolonged undifferentiated growth of human embryonic stem cells. Stem Cells, **21**, 546–556 (2003).
69. **Cheng, L., Hammond, H., Ye, Z., Zhan, X., and Dravid, G.:** Human adult marrow cells support prolonged expansion of human embryonic stem cells in culture. Stem Cells, **21**, 131–142 (2003).
70. **Richards, M., Fong, C. Y., Chan, W. K., Wong, P. C., and Bongso, A.:** Human feeders support prolonged undifferentiated growth of human inner cell masses and embryonic stem cells. Nat. Biotechnol., **20**, 933–936 (2002).
71. **Lee, J. B., Lee, J. E., Park, J. H., Kim, S. J., Kim, M. K., Roh, S. I., and Yoon, H. S.:** Establishment and maintenance of human embryonic stem cell lines on human feeder cells derived from uterine endometrium under serum-free condition. Biol. Reprod., **72**, 42–49 (2005).
72. **Hovatta, O., Mikkola, M., Gertow, K., Stromberg, A. M., Inzunza, J., Hreinsson, J., Rozell, B., Blennow, E., Andang, M., and Ahrlund-Richter, L.:** A culture system using human foreskin fibroblasts as feeder cells allows production of human embryonic stem cells. Hum. Reprod., **18**, 1404–1409 (2003).
73. **Xu, C., Inokuma, M. S., Denham, J., Golds, K., Kundu, P., Gold, J. D., and Carpenter, M. K.:** Feeder-free growth of undifferentiated human embryonic stem cells. Nat. Biotechnol., **19**, 971–974 (2001).
74. **Sato, N., Sanjuan, I. M., Heke, M., Uchida, M., Naef, F., and Brivanlou, A. H.:** Molecular signature of human embryonic stem cells and its comparison with the mouse. Dev. Biol., **260**, 404–413 (2003).
75. **Sato, N., Meijer, L., Skaltsounis, L., Greengard, P., and Brivanlou, A. H.:** Maintenance of pluripotency in human and mouse embryonic stem cells through activation of Wnt signaling by a pharmacological GSK-3-specific inhibitor. Nat. Med., **10**, 55–63 (2004).
76. **James, D., Levine, A. J., Besser, D., and Hemmati-Brivanlou, A.:** TGFbeta/activin/nodal signaling is necessary for the maintenance of pluripotency in human embryonic stem cells. Development, **132**, 1273–1282 (2005).
77. **Amit, M., Shariki, C., Margulets, V., and Itskovitz-Eldor, J.:** Feeder layer- and serum-free culture of human embryonic stem cells. Biol. Reprod., **70**, 837–845 (2004).
78. **Xu, R. H., Chen, X., Li, D. S., Li, R., Addicks, G. C., Glennon, C., Zwaka, T. P., and Thomson, J. A.:** BMP4 initiates human embryonic stem cell differentiation to trophoblast. Nat. Biotechnol., **20**, 1261–1264 (2002).
79. **Pera, M. F., Andrade, J., Houssami, S., Reubinoff, B., Trounson, A., Stanley, E. G., Ward-van Oostwaard, D., and Mummery, C.:** Regulation of human embryonic stem cell differentiation by BMP-2 and its antagonist noggin. J. Cell Sci., **117**, 1269–1280 (2004).
80. **Wang, L., Li, L., Menendez, P., Cerdan, C., and Bhatia, M.:** Human embryonic stem cells maintained in the absence of mouse embryonic fibroblasts or conditioned media are capable of hematopoietic development. Blood, **105**, 4598–4603 (2005).
81. **Xu, R. H., Peck, R. M., Li, D. S., Feng, X., Ludwig, T., and Thomson, J. A.:** Basic FGF and suppression of BMP signaling sustain undifferentiated proliferation of human ES cells. Nat. Methods, **2**, 185–190 (2005).
82. **Martin, I., Muraglia, A., Campanile, G., Cancedda, R., and Quarto, R.:** Fibroblast growth factor-2 supports *ex vivo* expansion and maintenance of osteogenic precursors from human bone marrow. Endocrinology, **138**, 4456–4462 (1997).
83. **Bianchi, G., Banfi, A., Mastrogiacomo, M., Notaro, R., Luzzatto, L., Cancedda, R., and Quarto, R.:** *Ex vivo* enrichment of mesenchymal cell progenitors by fibroblast growth factor 2. Exp. Cell. Res., **287**, 98–105 (2003).
84. **Itskovitz-Eldor, J., Schuldiner, M., Karsenti, D., Eden, A., Yanuka, O., Amit, M., Soreq, H., and Benvenisty, N.:** Differentiation of human embryonic stem cells into embryoid bodies compromising the three embryonic germ layers. Mol. Med., **6**, 88–95 (2000).
85. **Dang, S. M., Kyba, M., Perlingeiro, R., Daley, G. Q., and Zandstra, P. W.:** Efficiency of embryoid body formation and hematopoietic development from embryonic stem cells in different culture systems. Biotechnol. Bioeng., **78**, 442–453 (2002).
86. **Kaufman, D. S., Hanson, E. T., Lewis, R. L., Auerbach, R., and Thomson, J. A.:** Hematopoietic colony-forming cells derived from human embryonic stem cells. Proc. Natl. Acad. Sci. USA, **98**, 10716–10721 (2001).
87. **Chadwick, K., Wang, L., Li, L., Menendez, P., Murdoch, B., Rouleau, A., and Bhatia, M.:** Cytokines and BMP-4 promote hematopoietic differentiation of human embryonic stem cells. Blood, **102**, 906–915 (2003).
88. **Tian, X., Morris, J. K., Linehan, J. L., and Kaufman, D. S.:** Cytokine requirements differ for stroma and embryoid body-mediated hematopoiesis from human embryonic stem cells. Exp. Hematol., **32**, 1000–1009 (2004).
89. **Kehat, I., Kenyagin-Karsenti, D., Snir, M., Segev, H., Amit, M., Gepstein, A., Livne, E., Binah, O., Itskovitz-Eldor, J., and Gepstein, L.:** Human embryonic stem cells can differentiate into myocytes with structural and functional properties of cardiomyocytes. J. Clin. Invest., **108**, 407–414 (2001).
90. **Xu, C., Police, S., Rao, N., and Carpenter, M. K.:** Characterization and enrichment of cardiomyocytes derived from human embryonic stem cells. Circ. Res., **91**, 501–508 (2002).
91. **Zweigerdt, R., Burg, M., Willbold, E., Abts, H., and Ruediger, M.:** Generation of confluent cardiomyocyte monolayers derived from embryonic stem cells in suspension: a cell source for new therapies and screening strategies. Cytotherapy, **5**, 399–413 (2003).
92. **Levenberg, S., Golub, J. S., Amit, M., Itskovitz-Eldor, J., and Langer, R.:** Endothelial cells derived from human embryonic stem cells. Proc. Natl. Acad. Sci. USA, **99**, 4391–4396 (2002).
93. **Reyes, M., Dudek, A., Jahagirdar, B., Koodie, L., Marker, P. H., and Verfaillie, C. M.:** Origin of endothelial progenitors in human postnatal bone marrow. J. Clin. Invest., **109**, 337–346 (2002).
94. **Schuldiner, M., Eiges, R., Eden, A., Yanuka, O., Itskovitz-Eldor, J., Goldstein, R. S., and Benvenisty, N.:** Induced neuronal differentiation of human embryonic stem cells. Brain Res., **913**, 201–205 (2001).
95. **Carpenter, M. K., Inokuma, M. S., Denham, J., Mujtaba, T., Chiu, C. P., and Rao, M. S.:** Enrichment of neurons and

neural precursors from human embryonic stem cells. Exp. Neurol., **172**, 383–397 (2001).
96. **Zhang, S. C., Wernig, M., Duncan, I. D., Brustle, O., and Thomson, J. A.:** *In vitro* differentiation of transplantable neural precursors from human embryonic stem cells. Nat. Biotechnol., **19**, 1129–1133 (2001).
97. **Reubinoff, B. E., Itsykson, P., Turetsky, T., Pera, M. F., Reinhartz, E., Itzik, A., and Ben-Hur, T.:** Neural progenitors from human embryonic stem cells. Nat. Biotechnol., **19**, 1134–1140 (2001).
98. **Buytaert-Hoefen, K. A., Alvarez, E., and Freed, C. R.:** Generation of tyrosine hydroxylase positive neurons from human embryonic stem cells after coculture with cellular substrates and exposure to GDNF. Stem Cells, **22**, 669–674 (2004).
99. **Perrier, A. L., Tabar, V., Barberi, T., Rubio, M. E., Bruses, J., Topf, N., Harrison, N. L., and Studer, L.:** Derivation of midbrain dopamine neurons from human embryonic stem cells. Proc. Natl. Acad. Sci. USA, **101**, 12543–12548 (2004).
100. **Schulz, T. C., Noggle, S. A., Palmarini, G. M., Weiler, D. A., Lyons, I. G., Pensa, K. A., Meedeniya, A. C., Davidson, B. P., Lambert, N. A., and Condie, B. G.:** Differentiation of human embryonic stem cells to dopaminergic neurons in serum-free suspension culture. Stem Cells, **22**, 1218–1238 (2004).
101. **Jiang, Y., Henderson, D., Blackstad, M., Chen, A., Miller, R. F., and Verfaillie, C. M.:** Neuroectodermal differentiation from mouse multipotent adult progenitor cells. Proc. Natl. Acad. Sci. USA, **100**, 11854–11860 (2003).
102. **Kubo, A., Shinozaki, K., Shannon, J. M., Kouskoff, V., Kennedy, M., Woo, S., Fehling, H. J., and Keller, G.:** Development of definitive endoderm from embryonic stem cells in culture. Development, **131**, 1651–1662 (2004).
103. **Teratani, T., Yamamoto, H., Aoyagi, K., Sasaki, H., Asari, A., Quinn, G., Terada, M., and Ochiya, T.:** Direct hepatic fate specification from mouse embryonic stem cells. Hepatology, **41**, 836–846 (2005).
104. **Rambhatla, L., Chiu, C. P., Kundu, P., Peng, Y., and Carpenter, M. K.:** Generation of hepatocyte-like cells from human embryonic stem cells. Cell Transplant., **12**, 1–11 (2003).
105. **Shirahashi, H., Wu, J., Yamamoto, N., Catana, A., Wege, H., Wager, B., Okita, K., and Zern, M. A.:** Differentiation of human and mouse embryonic stem cells along a hepatocyte lineage. Cell Transplant., **13**, 197–211 (2004).
106. **Assady, S., Maor, G., Amit, M., Itskovitz-Eldor, J., Skorecki, K. L., and Tzukerman, M.:** Insulin production by human embryonic stem cells. Diabetes, **50**, 1691–1697 (2001).
107. **Lumelsky, N., Blondel, O., Laeng, P., Velasco, I., Ravin, R., and McKay, R.:** Differentiation of embryonic stem cells to insulin-secreting structures similar to pancreatic islets. Science, **292**, 1389–1394 (2001).
108. **Segev, H., Fishman, B., Ziskind, A., Shulman, M., and Itskovitz-Eldor, J.:** Differentiation of human embryonic stem cells into insulin-producing clusters. Stem Cells, **22**, 265–274 (2004).
109. **Hansson, M., Tonning, A., Frandsen, U., Petri, A., Rajagopal, J., Englund, M. C., Heller, R. S., Hakansson, J., Fleckner, J., Skold, H. N., Melton, D., Semb, H., and Serup, P.:** Artifactual insulin release from differentiated embryonic stem cells. Diabetes, **53**, 2603–2609 (2004).
110. **Rajagopal, J., Anderson, W. J., Kume, S., Martinez, O. I., and Melton, D. A.:** Insulin staining of ES cell progeny from insulin uptake. Science, **299**, 363 (2003).
111. **Leon-Quinto, T., Jones, J., Skoudy, A., Burcin, M., and Soria, B.:** *In vitro* directed differentiation of mouse embryonic stem cells into insulin-producing cells. Diabetologia, **47**, 1442–1451 (2004).
112. **Soria, B., Roche, E., Berna, G., Leon-Quinto, T., Reig, J. A., and Martin, F.:** Insulin-secreting cells derived from embryonic stem cells normalize glycemia in streptozotocin-induced diabetic mice. Diabetes, **49**, 157–162 (2000).
113. **Zandstra, P. W., Eaves, C. J., and Piret, J. M.:** Expansion of hematopoietic progenitor cell populations in stirred suspension bioreactors of normal human bone marrow cells. Biotechnology (NY), **12**, 909–914 (1994).
114. **Peshwa, M. V., Wong, K., and Hu, W.-S.:** Microcarrier culture, p. 1781–1785. *In* Flickinger, M. C. and Drew, S. W. (ed.), Encyclopedia of bioprocess technology: fermentation, biocatalysis, and bioseparation. John Wiley & Sons, New York (1999).
115. **Bancel, S. and Hu, W. S.:** Confocal laser scanning microscopy examination of cell distribution in macroporous microcarriers. Biotechnol. Prog., **12**, 398–402 (1996).
116. **Nikolai, T. J. and Hu, W. S.:** Cultivation of mammalian cells on macroporous microcarriers. Enzyme Microb. Technol., **14**, 203–208 (1992).
117. **Bagley, J., Rosenzweig, M., Marks, D. F., and Pykett, M. J.:** Extended culture of multipotent hematopoietic progenitors without cytokine augmentation in a novel three-dimensional device. Exp. Hematol., **27**, 496–504 (1999).
118. **Banu, N., Rosenzweig, M., Kim, H., Bagley, J., and Pykett, M.:** Cytokine-augmented culture of haematopoietic progenitor cells in a novel three-dimensional cell growth matrix. Cytokine, **13**, 349–358 (2001).
119. **Wang, T. Y., Brennan, J. K., and Wu, J. H.:** Multilineal hematopoiesis in a three-dimensional murine long-term bone marrow culture. Exp. Hematol., **23**, 26–32 (1995).
120. **Mantalaris, A., Keng, P., Bourne, P., Chang, A. Y., and Wu, J. H.:** Engineering a human bone marrow model: a case study on *ex vivo* erythropoiesis. Biotechnol. Prog., **14**, 126–133 (1998).
121. **Zandstra, P. W., Bauwens, C., Yin, T., Liu, Q., Schiller, H., Zweigerdt, R., Pasumarthi, K. B., and Field, L. J.:** Scalable production of embryonic stem cell-derived cardiomyocytes. Tissue Eng., **9**, 767–778 (2003).
122. **Gerecht-Nir, S., Cohen, S., Ziskind, A., and Itskovitz-Eldor, J.:** Three-dimensional porous alginate scaffolds provide a conducive environment for generation of well-vascularized embryoid bodies from human embryonic stem cells. Biotechnol. Bioeng., **88**, 313–320 (2004).
123. **Dang, S. M., Gerecht-Nir, S., Chen, J., Itskovitz-Eldor, J., and Zandstra, P. W.:** Controlled, scalable embryonic stem cell differentiation culture. Stem Cells, **22**, 275–282 (2004).
124. **Gerecht-Nir, S., Cohen, S., and Itskovitz-Eldor, J.:** Bioreactor cultivation enhances the efficiency of human embryoid body (hEB) formation and differentiation. Biotechnol. Bioeng., **86**, 493–502 (2004).
125. **Peshwa, M. V., Wu, F. J., Sharp, H. L., Cerra, F. B., and Hu, W. S.:** Mechanistics of formation and ultrastructural evaluation of hepatocyte spheroids. *In Vitro* Cell Dev. Biol. Anim., **32**, 197–203 (1996).
126. **Peshwa, M. V., Wu, F. J., Follstad, B. D., Cerra, F. B., and Hu, W. S.:** Kinetics of hepatocyte spheroid formation. Biotechnol. Prog., **10**, 460–466 (1994).
127. **Wu, F. J., Friend, J. R., Hsiao, C. C., Zilliox, M. J., Ko, W. J., Cerra, F. B., and Hu, W. S.:** Efficient assembly of rat hepatocyte spheroids for tissue engineering applications. Biotechnol. Bioeng., **50**, 404–415 (1996).
128. **Tzanakakis, E. S., Hansen, L. K., and Hu, W. S.:** The role of actin filaments and microtubules in hepatocyte spheroid self-assembly. Cell Motil. Cytoskeleton, **48**, 175–189 (2001).
129. **Abu-Absi, S. F., Friend, J. R., Hansen, L. K., and Hu, W. S.:** Structural polarity and functional bile canaliculi in rat hepatocyte spheroids. Exp. Cell Res., **274**, 56–67 (2002).