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Stem Cells and Cell Therapy

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Chapter 1
Use of Human Embryonic Stem Cells in Therapy

Ana Maria Fraga, Érica Sara Souza de Araújo, Naja Vergani, Simone A.S. Fonseca, and Lygia V. Pereira

Abstract Pluripotent stem cells (PSCs) are defined by their potential of unlimited self-renewal and the ability to differentiate – both *in vitro* and *in vivo* – into all cell types of endodermal, mesodermal and ectodermal origins, rendering them a promising applicability in cell replacement therapies. These characteristics also make PSCs powerful tools for studying the molecular mechanisms underlying cellular differentiation, as well as for accessing the biological effects of pharmaceutical compounds on the normal embryo development, and also on virtually any differentiated cell type. PSCs can be obtained from early stage embryos – usually from the inner cell mass of blastocysts – and adapted for propagation in culture in the laboratory, thus resulting in the establishment of an Embryonic Stem Cell (ESC) line. They can also be artificially obtained in the laboratory through the use of techniques that induce the reprogramming of somatic, differentiated cell types (i.e. dermal fibroblasts) into undifferentiated, pluripotent stem cells (Induced Pluripotent Stem Cells – iPSCs). In this chapter we discuss the potential advantages and disadvantages of the use of these two PSC types in regenerative medicine, and give an overview of the current status of cell therapy studies and clinical trials in humans. We also comment on a more recently developed alternative source of cells for use in therapy – the direct reprogrammed cells – and finally, we discuss the potential applications of PSCs and directly reprogrammed cells in drug screening assays.

Keywords Cell-based therapy • Regenerative medicine • Human embryonic stem cell • Pluripotent stem cell • Induced pluripotent stem cell

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1.1 Promises of Human ESCs in Cell Therapy

Stem cells have been obtained from distinct sources, and have differential potential to form the innumerous cell types from a multicellular organism. Adult Stem Cells (ASCs) obtained from different types of tissues (bone marrow, dental pulp, neural progenitor cells, umbilical cord blood, amniotic fluid cells, among others) can be differentiated into a variety of cell types. However, the range of differentiation properties of an adult cell is limited, and usually they can only form cell types from the same lineage as the original tissue (multipotent stem cells).

Adult stem cells have been extensively employed for cell-based therapy; several preclinical trials demonstrated that transplantation of autologous bone marrow cells or precursor cells improved cardiac function after myocardial infarction and in chronic coronary heart disease (reviewed in Strauer et al. 2009). In animal models of spinal cord injuries, ASCs provided sensorimotor benefits (reviewed in Thomas and Moon 2011). However, in the vast majority of the cases, positive results could not be reproduced by other laboratories, and, in such models, the mechanism(s) underlying the beneficial outcomes were not well understood. Functional recoveries could be attributed to cell fusion or the release of paracrine factors that could signal the neighboring cells either favoring differentiation (reviewed in Wollert and Drexler 2005) or protecting the cells from apoptosis (Kocher et al. 2001). Only in exceptional cases it has been shown that functional recovery causally depends on the survival and differentiation of the transplanted cells (reviewed in Thomas and Moon 2011).

Several clinical trials using ASCs have been conducted to treat heart diseases (reviewed in Menasche 2007; Meyer et al. 2006; Schuleri et al. 2007) diabetes (Jiang et al. 2011), spinal cord injury (Knoller et al. 2005; Callera and do Nascimento 2006; Lima et al. 2006; Moviglia et al. 2006; Yoon et al. 2007), neurological disorders (Heile and Brinker 2011; Titomanlio et al. 2011; Connick et al. 2012; Mazzini et al. 2012; Savitz 2012), hepatic and intestinal diseases (Burra et al. 2011), retinal injury (Stern and Temple 2011) and muscular dystrophies (Vilquin et al. 2011). In these trials, cells from many distinct origins were employed but, once again, temporary and/or modest improvements in health conditions indicated that the positive outcomes could not be attributed strictly to the differential properties of the injected cells, and anti-inflammatory, paracrine effects were also or mainly taking part in the healing process (Uccelli et al. 2011; Wang et al. 2012).

Human ESCs (hESCs) are derived from the inner cell mass (ICM) of developing blastocyst-stage embryos (Fig. 1.1), are capable of unlimited expansion *in vitro*, and can be maintained in culture indefinitely in their undifferentiated state. In appropriated culture conditions, these cells can spontaneously differentiate into cell types that are representative of the three germ layers: ectoderm, mesoderm and endoderm (reviewed in Trounson 2006). One important characteristic of hESC is their ability to generate teratomas when injected into immunocompromised mice; normally this type of assay is used to demonstrate the pluripotency capability of these cells.

Compared to ASCs, hESC are a more suitable source of cells for therapy since they have unquestioned differentiation potential. The constant interest in these cells can be clearly evidenced by the fact that, since the derivation of the first hESC lines

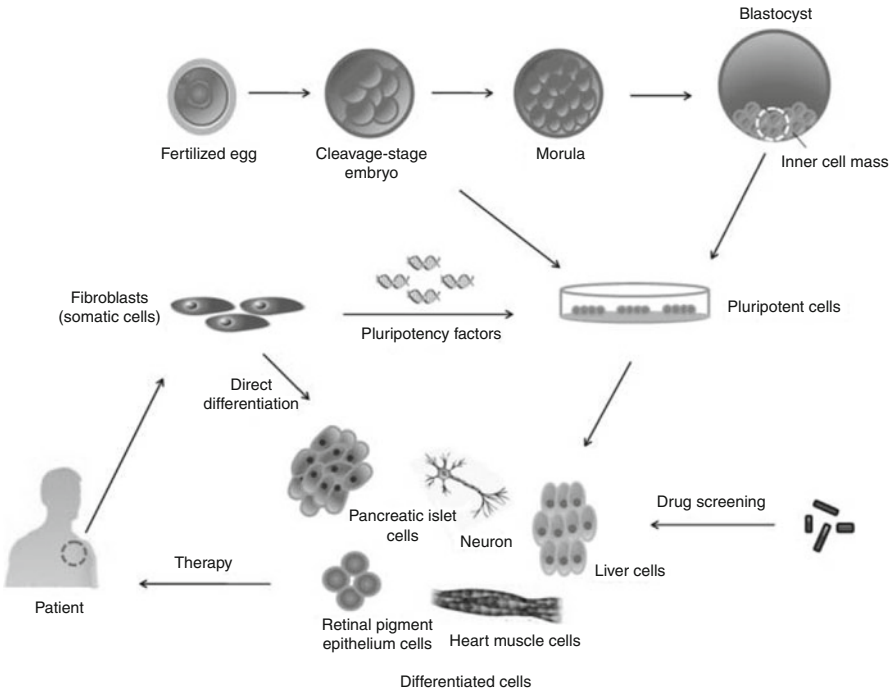

Fig. 1.1 Sources and applications of pluripotent stem cells (PSCs) in therapy and drug screening. Embryonic stem cells are derivated from blastomeres (cleavage-stage embryo) or from the inner cell mass of blastocysts. As an alternative source, PSCs can also be generated from somatic cells by inducing the expression of pluripotency factors. PSCs can be differentiated into the cell type of interest (neurons, pancreatic islet cells) and can be used in cell therapy or drug screening. Direct conversion of somatic cells avoids the reprogramming step to originate PSCs

in 1998 by Thomson's group, the number of different hESC lines registered on the stem cell databases rapidly increased, and more than 800 different hESC lines are now currently available (Fraga et al. 2011a).

Traditionally, the use of hESC in research and cell therapy was controversial due to political, religious and ethical implications about the use of human embryos. Part of the public opinion was against hESC research based on the argument that preimplantation embryos should share the same rights to life as any already-born person, regardless of being in a culture dish or in a woman's body (Hyun 2010). Additionally, there were also concerns about a possible commerce of embryos that could be generated when cell therapy would become a common practice. Nowadays, the ethical controversy over the origin and use of human embryos has been mostly overcome; generally, in permissive countries, surplus embryos from *in vitro* fertilization clinics are used for hESC derivation with parental informed consent. In countries like China and Belgium, that have even more permissive laws, it is also possible to derive new hESCs lines from embryos produced specifically for research purposes (Pennings 2003).

1.1.1 hESC Optimal Conditions for Derivation and Culture

In order to be able to use these cells in therapy, rigorous conditions must be followed during the derivation and culture maintenance of a pluripotent cell line. So far, it is not well established which would be the most effective method for the derivation of new hESC lines (Fraga et al. 2011a). However, thinking about their future application in cell-based therapy, the consensus is that the culture conditions for hESCs should be preferably defined and free of animal-derived contaminants.

The use of animal derived components in hESC cultures has became a major concern after the discovery of the potentially immunogenic nonhuman sialic acid, Neu5Gc, on hESC lines co-cultured with animal cells or in animal serum products (Martin et al. 2005). So, there is a continuous effort to establish non-animal (xeno-free) cellular supports (matrices), completely defined cell culture media, and proper cell culturing procedures (Unger et al. 2008). However, most of the currently available hESCs lines were not derived or maintained under these optimal conditions, as the methodologies employed for the establishment of the majority of these lines were traditionally developed based on mouse ESC (mESC) derivation protocols. It is important to note that the first clinical trial using hESCs for spinal cord injury is being conducted using a hESC line derived under the use of animal components in cell culture (Thomson et al. 1998); this hESC line was further adapted to xeno-free conditions before transplantation into patients, but even so the treatment must be considered as a xeno-transplantation.

Another aspect to consider is that the continuous propagation of PSCs in the laboratory could give rise to abnormal cell populations, as it could progressive select for cells that are most adapted to the employed culture conditions (Baker et al. 2007). It is well documented that the most frequent chromosomal aberrations found in long-term cultured hESC are gains of chromosomes 12 and 17 (Brimble et al. 2004; Baker et al. 2007; Draper et al. 2003). Other authors have also described the gain of one or more X chromosomes in these cell populations (Mitalipova et al. 2005; Ludwig et al. 2006). Recently, the International Stem Cell Initiative published the results from the analysis of 125 hESC lines, where the amplification of the 20q11.21 region was found in more than 20 % of the cell lines (International Stem Cell Initiative 2011). The same findings were previously reported by Lefort and coworkers (2008). The 20q11.21 region contains the *BCL2L1* gene, whose product is an inhibitor of apoptosis, and thus, a strong candidate for driving the adaptation of hESC lines in culture (International Stem Cell Initiative 2011).

1.1.2 hESC-Derived Differentiated Cells

In spite of the continuous improvements observed in the field, there are several distinct protocols available in the literature for the generation of specific differentiated

cell types from hESCs. The great majority of them require extensive manipulation in culture and the use of various reagents (Ramirez et al. 2010), which makes *in vitro* differentiation a laborious procedure. Additionally, many of the available differentiation protocols are not always reproducible, as significant culture-to-culture variability can be observed even when using identical conditions. For these reasons, several groups around the world are currently investing their efforts to develop low-cost, robust, scalable methods for the production of highly homogeneous populations of pre-differentiated and differentiated hESC-derived cells. Indeed, many improvements were achieved in this field in the past 10 years (Ilic et al. 2012; Krawetz and Rancourt 2012).

However, there are some cell types that are still difficult to obtain by current *in vitro* differentiation methodologies, such as pancreatic cells and germinative-derived cells. For instance, the challenge to produce pancreatic cells is due to the fact that they should be able to synthesize, store and acutely secrete insulin when exposed to glucose. Baetge's group developed *in vitro* endocrine cells from hESCs, and, when these cells were injected into mice, they demonstrated the ability to differentiate into glucose-responsive insulin-secreting pancreatic cells (D'Amour et al. 2006; Kroon et al. 2008). Although promising, others investigators failed to reproduce this protocol when using different hESC lines (Cho et al. 2008; Mfopou et al. 2010), and further studies become necessary.

1.1.3 Risk of Teratoma Formation

Perhaps the most important issue concerning hESC-based therapy is related to its clinical safety; more specifically, the intrinsic potential of pluripotent cells to originate teratomas or teratocarcinomas when injected into the patient. On the other hand, this adverse effect could be avoided by pre-differentiating these cells *in vitro* before using them in cell therapy procedures. However, the achievement of a completely homogeneous population of pre-differentiated cells in the laboratory is not such an easy task, as the presence of a small population of non-compromised cells in the material used for injection could potentially lead to the formation of teratomas or teratocarcinomas in the patient.

Nevertheless, preclinical trials have shown that hESC-derived pre-differentiated cells could be safely injected into allogeneic model organisms like rats, mice and guinea pigs without the emergence of teratomas (see Table 1.1), and this counts in favor of hESC-based therapies. On the other hand, Erdö and coworkers (2003) reported that, in animal stroke models, rats that received undifferentiated or differentiated mESCs did not develop tumors while mice did, which demonstrated that different outcomes can be observed in xeno- and homologous transplantations. Thus, extensive clinical trials must be performed with hESCs in order to exclude the possibility of tumor formation in humans.

Table 1.1 Preclinical studies using hESC-derivates in animal models

hESC-derived cell type	Animal	Model	Outcome	Reference
Oligodendrocyte progenitor cells	Rat	Cervical spinal cord injury	Attenuated lesion pathogenesis; improved recovery of forelimb function	Sharp et al. (2010)
	Rat	Spinal cord injury	Enhanced remyelination and improved locomotor ability	Keirstead et al. (2005)
	Mouse	Shiverer model of dysmyelination	Integration, differentiation into oligodendrocytes, and compact myelin formation	Nistor et al. (2005)
Dopaminergic neurons	Rat Mouse	Parkinson's disease	Functional recovery	Kriks et al. (2011)
	Rhsesus monkey		Engrafted cells, dopaminergic neurons survival	
Cardiomyocytes	Guinea pig	Intrinsic heart rate suppressed	Pacemaker activity of the hESC-derived cardiomyocytes	Xue et al. (2005)
	Mouse	Infarcted heart	Improved heart function	Kofidis et al. (2006)
	Rat		Stable cardiomyocyte grafts, attenuation of the remodeling process, and functional benefits	Caspi et al. (2007)
	Rat		Myocardial remuscularization, attenuated heart failure	Laflamme et al. (2007)
Retinal pigmented epithelium	Rat	Retinal degeneration	Improvement in visual performance; photoreceptor integrity for prolonged period	Lund et al. (2006), Lu et al. (2009)
	Mouse	Stargardt's disease	Increase in the visual acuity	Lu et al. (2009)
Keratinocytes	Mouse	Wound healing skin	Construction of a pluristratified epidermis	Guenou et al. (2009)
Osteogenic cells	Rat	Bone regeneration	Extensive bone formation	Kuznetsov et al. (2011)
Lung-epithelial lineage-specific cells	Mouse	Pulmonary fibrosis	Engraftment, reduced fibrosis and lung inflammation	Banerjee et al. (2012)

1.1.4 Preclinical Studies on hESC-Based Therapy

Transplanted organs and cells must find an appropriate environment to engraft, proliferate and successfully improve host's health. So far, results from preclinical studies using hESC-derivatives are exciting, and clinical trials for the treatment of several health conditions and diseases have been progressing (Table 1.1).

Keirstead and coworkers (2005) showed that oligodendrocytes progenitor cells (OPCs) generated from hESCs enhance remyelination and improve motor function after spinal cord injury at the thoracic level in rats. In 2010, Sharp and coworkers published another preclinical trial using OPCs and, this time, the improved recovery was seen in rats with cervical spinal cord injury.

Lu and colleagues (2009) conducted a preclinical study using retinal pigment epithelium cells from purified, spontaneously differentiated hESCs in murine models of macular degeneration. The injected cells were shown to survive more than 8 months without evidence of pathological consequences, and were also able to rescue visual functions.

Additionally, promising results were recently achieved using lung-epithelial lineage-specific cells derived from a hESC line (Banerjee et al. 2012). The authors reported that commercial media used in pulmonary cell culture could successfully induce differentiation of hESCs to a lung phenotype, and the addition of certain molecules in the culture medium could skew differentiation into various proportions of lung cell types. Most importantly, when transplanted into mice previously treated with bleomycin – a drug that causes lung fibrosis -, the hESC-derived pulmonary cells were shown to be able to reverse fibrosis. The authors concluded that transplanted cells may reduce fibrosis either directly by replacing fibrotic tissue or indirectly by paracrine secretion of factors that reduce the deposition of collagen and other components of the extracellular matrix, like laminin and fibronectin.

Woo and coworkers (2012) recently described that hepatocyte-like cells derived from hESCs contributed to the recovery of injured liver tissues in mice, not only by cell replacement but also by delivering trophic factors that support endogenous liver regeneration.

For cardiac regeneration, several reports provide exciting proof-of-principle studies and corroborate with the potential therapeutic use of hESC-derived cardiomyocytes. Laflamme and coworkers (2007) observed consistent formation of myocardial grafts when hESC-derived cardiomyocytes were injected into an infarcted rat model. In addition, infarcted mice transplanted with human cardiomyocytes also had good outcomes (Kofidis et al. 2006), and in guinea pig with suppressed intrinsic heart rates, hESC-derived cardiomyocytes acted as a pacemaker (Xue et al. 2005).

Nevertheless, it is important to note that although humans and mice share 99 % of their genes, it is challenging to correlate phenotypes and treatment responses between these organisms, since there is an intrinsic variation due evolutionary distances between species (Rosenthal and Brown 2007; Schofield et al. 2011). These findings point to a possible limitation in the use of preclinical data for an accurate evaluation of the efficacy of cell therapy in humans. In spite of preclinical

limitations, successful outcomes in animal models justify the implementation of clinical trials in humans. The aforementioned and other preclinical tests are listed in Table 1.1.

1.1.5 HLA Match

The very potent immune response towards foreign transplants occurs most frequently due to recognition of the graft foreign MHC proteins – encoded by the human leukocyte antigen (HLA) – by the T cells of the recipient (reviewed in Wood and Goto 2012).The required level of HLA matches differs among organs or tissues to be transplanted. For example, unrelated donor haemopoietic-cell transplantation requires a stricter matching of HLA (Fleischhauer et al. 2012), whereas liver transplant outcome has not been found to be improved by HLA matching (Muro et al. 2012).

In undifferentiated hESCs, only low levels of MHC-I are expressed, whereas spontaneous differentiation leads to a two to fourfold increase in MHC expression at the embryoid body stage, and a eight to tenfold at the teratoma stage. However, these levels are still lower if compared to the levels observed in somatic cells (Drukker et al. 2002). Currently, it is not clear whether the undifferentiated hESCs and their differentiated derivatives have immunogenic potential (reviewed in Kadereit and Trounson 2011). However, even if they prove to be immunogenic, therapeutic banks of hESC, capable of providing an HLA match for most potential patients, could be generated in order to overcome this issue.

Taylor and coworkers (2005) estimated that 150 hESC lines are needed to cover HLA matching in the UK population. Later studies reported similar results for Japanese (Nakajima et al. 2007) and Chinese (Lin et al. 2009) populations. In Korea, the 29 hESC lines evaluated were shown to be able to serve the most part of the population depending on the level of mismatch permitted (Lee et al. 2010).

It is also worth noting that, one of the implications of the use of supernumerary embryos for the establishment of new lines of hESC is the possible limited genetic diversity of these embryos, that may not attend the whole ethnic admixture of a given population. Some articles have described restricted HLA profiles and genetic backgrounds of the hESC lines established worldwide, which are mainly of European and East-Asian ethnicity (Snyder and Loring 2006; Mosher et al. 2010; Laurent et al. 2010; Fraga et al. 2011b). Thus, it will be important to obtain ethnically diverse embryos for the derivation of new hESC lines with distinct genetic backgrounds and different HLA types. In an HLA-compatibility context, hESC-derived dendritic cells (DCs), the most potent antigen presenting cells, could persuade the immune system of the patients subjected to cell therapy to tolerate allogeneic tissues differentiated from the same hESC line (Silk et al. 2011). This could prolong the acceptance of hESC-derivatives by the treated patient with minimal use of immunosuppressive drugs (Lui et al. 2009).

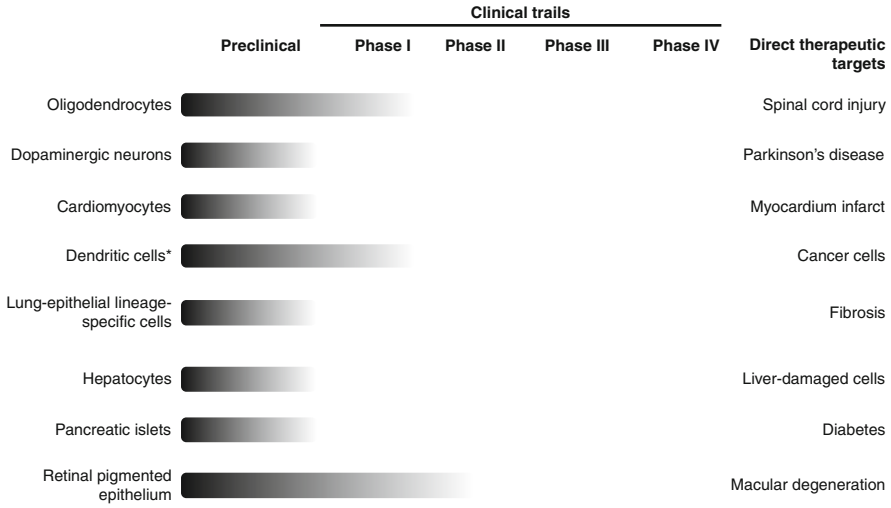

Fig. 1.2 Preclinical and clinical trials using hESCs as therapeutic agents in regenerative medicine. Differentiated cells generated from hESCs are listed on the *left*, and the current stage of the trials is represented by *soft-edges rectangles*. On the *right*, potential therapeutic targets of the hESC-derivated cells. *Dentritic cells are cited here for their potential use as vaccines in cancer treatment

1.1.6 hESC-Based Therapy: Clinical Trials

Until 2012, three health conditions were the main targets of hESC-based therapy trials: spinal cord injury, Stargardt's macular dystrophy and dry-age macular degeneration. In 2009, the American Food and Drug Administration (FDA) approved the first clinical trial using hESC-derived cells, oligodendrocytes, by Geron Corporation. Patients with neurologically complete traumatic spinal cord injuries (between T3 and T11) received a single administration of oligodendrocyte progenitor cells (GRNOPC1) derived from a hESC line, 7–14 days post trauma. The results obtained in the Phase I safety study were presented recently at the "2011 American Congress of Rehabilitation Medicine": administered cells were well tolerated and did not cause any serious adverse effect (www.geron.com). Due to financial issues, in November 2011 Geron interrupted the trials, but the treated patients will be followed up for 15 years.

Advanced Cell Technology (Massachusetts, USA) started clinical studies for dry age-related macular degeneration and Stargardt's macular dystrophy in 2011. The non randomized, sequential, multi center safety and tolerability Phase I/II trial will serve to evaluate the effect of subretinal delivery of hESC-derived retinal pigmented epithelium cells in patients with macular degeneration. Preliminary results from two treated patients have been already reported: no tumor formation was detected during the 4-month follow up period (Schwartz et al. 2012).

Figure 1.2 shows the current scenario of preclinical and clinical trials using hESCs differentiated cells for therapeutic purposes. Most of the studies are still

preclinical and being conducted in animal models, and thus will require further investigation.

ViaCyte Inc is a preclinical therapeutic company focused on cell-based therapy for treatment of diabetes, based on the differentiation of stem cells into pancreatic beta cell precursors. The goal of this type of therapy is to abolish the dependence of insulin in Type I and II diabetes patients on a long term basis, reducing or eliminating hypoglycemia, microvasculature, and weight-related cardiovascular complications through the subcutaneous implantation of pancreatic beta cell precursors in an encapsulation device that protects transplanted cells from the host immune system (www.viacyte.com).

The company has already completed their first Phase I/II clinical trials (2005–2010) using multiple human pancreases isolated from compatible human organ donors. In these first trials, PEG (polyethilenoglicol)-Encapsulated Islet allografts were transplanted into the patients without the requirement of immunosuppressive drugs administration after the implant (Clinical Trials ID: NCT00260234). Currently, the company is developing insulin-producing cells from hESCs, and using in preclinical studies.

1.2 Human iPSC in Cell Therapy: Personalized Medicine

Induced Pluripotent Stem Cells (iPSCs) are generated by techniques that promote the de-differentiation of a somatic cell into a pluripotent state (Fig. 1.1). Besides pluripotency, they share many other features with ESCs, such as cell and colony morphology, unlimited capacity of proliferation, and the ability to form teratomas when injected in immunodepressed mice (Takahashi and Yamanaka 2006), rendering them a great potential for use in cell therapy. However, although iPSCs are very similar to ESCs, they are not identical. Induced PSCs show differences in gene expression and DNA methylation profiles, and present lower efficiencies to differentiate into some specific lineages when compared to ESCs (Chin et al. 2009; Deng et al. 2009; Feng et al. 2009).

Mouse iPSCs were first generated by Takahashi and Yamanaka (2006) through the exogenous expression – driven by retroviral vectors – of four transcription factors, Oct4, Sox2, Klf4 and c-Myc, in both mouse embryonic and adult fibroblasts.

One year after the publication of this work, the first human iPSC lines were established from human adult and fetal fibroblasts through the viral expression of two different sets of transcription factors, respectively: OCT3/4, SOX2, KLF4, c-MYC (Takahashi et al. 2007) and OCT3/4, SOX2, NANOG and LIN28 (Yu et al. 2007). Since then, many other somatic cell types have been proven to be able to undergo reprogramming, and different sets of distinct transcription factors – or even the use of a single one – have been shown to be effective in the generation of iPSC lines.

It is worth noting that both c-Myc and Klf4 are oncogenes, and thus, their use in reprogramming protocols renders to the iPSC a higher potential of tumor formation. In fact, mouse iPSC-derived chimeras have shown to develop tumors as a result of

the reactivation of c-Myc and Klf4 oncogenes (Okita et al. 2007; Markoulaki et al. 2009). Moreover, even if the viral transgenes have been show to undergo nearly complete silencing in the host cells after reprogramming (Yu et al. 2007), the use of retroviral/lentiviral vectors itself represents a higher risk of tumor formation, since the random integration of the viral genetic material into the host cell genome may alter the expression of endogenous genes, making the cells tumorigenic (Hochedlinger et al. 2005). Since the increased risk of tumor development represents a great concern in terms of cell therapy, in order to overcome this problem, many groups worldwide have been directing their efforts to generate human iPSCs without viral integration through the application of transient and/or non-integrative methodologies that use plasmids (Okita et al. 2008), episomal vectors (Yu et al. 2009), adenoviral vectors (Stadtfeld et al. 2008), and, more recently, mRNA (Bosnali and Edenhofer 2008; Kim et al. 2009; Zhou et al. 2009) and Sendai virus vector (Fusaki et al. 2009).

Similarly to hESC, the use of iPSC in cell therapy also confers a risk of teratoma formation if they are injected into the patient in an undifferentiated state. Thus, the *in vitro* pre-differentiation of the human iPSCs before their use in cell therapy is also a requirement. However, their use in regenerative medicine offers an advantage over hESCs in terms of the concern around the use of human embryos. Furthermore, since iPSCs can potentially be generated from any individual – and thus can be generated from somatic cells extracted from the person who is meant to undergo cell therapy – their use in regenerative medicine abolishes the risks of immune rejection. However, this could imply in the need of deriving a new iPSC line for each patient to be treated, turning iPSC-based cell therapy into a much more laborious and an expensive treatment. Although several cell-therapy studies using mouse iPSCs have already been conducted, no clinical trials employing human iPSCs have started yet.

1.3 Perspectives in Cell-Based Therapy

Since the first hESCs lines derivation in 1998, constant work from many laboratories have elucidated many aspects of the molecular events that govern pluripotency, differentiation and cellular plasticity. Next, we present novel strategies in cell-based therapy.

1.3.1 PSCs in Disease Modeling

Since hESCs lines are usually derived from donated embryos from IVF clinics, it is possible to generate *in vitro* disease models from affected embryos diagnosed by preimplantation genetic diagnosis (PGD), a procedure that allows for the detection of a genetic defect at the level of an embryo fertilized *in vitro* (Sermon et al. 2004).

Several articles reported the derivation of hESCs lines carrying causal mutations of Huntington disease (Mateizel et al. 2006; Niclis et al. 2009), Myotonic Dystrophy Type I (Mateizel et al. 2006), Fragile X Syndrome (Urbach et al. 2010), among others (for a detailed review, see Grskovic et al. 2011). Similarly, iPSC lines can also constitute *in vitro* disease models. Indeed, as several hESCs lines, iPSCs lines that manifest cellular phenotypes have been established for numerous monogenic diseases (reviewed in Grskovic et al. 2011). However, given the possibility of being established from adults, iPSCs can likewise be used for phenotype-based drug screens in complex diseases for which the underlying genetic mechanism is unknown.

1.3.2 Drug Screening

Due to its ability to give rise to all cells in the organism, PSCs represent a powerful *in vitro* model for the study of embryonic development; and many aspects of cell differentiation can potentially be clarified through the examination of ESCs' responses to chemical (molecular compounds) and physical (adherence, cell contact, oxygen tension) stimuli. Thus, PSCs-derivatives can serve as tools for drug screening, as well as to evaluate drug toxicity's consequences in cell fate decisions (reviewed in Maury et al. 2012 – Fig. 1.1). Yahata and coworkers (2011), using an iPSC line tested potential drugs for Alzheimer disease in neurons derived from the pluripotent cells. Currently, there are several groups testing drugs using iPSC lines modeling human diseases, mainly in neurological and cardiac disorders (reviewed in Song et al. 2012).

1.3.3 Direct Cell Conversion

Another, more recent approach that can be used to generate cells for cell therapy is the direct conversion of cell types. It consists in the transdifferentiation of a somatic cell into another cell type (i.e. fibroblasts into functional neurons) through the differential expression of defined factors, without passing the cells through a pluripotent state, and thus eliminating the risk of teratoma formation (Fig. 1.1). Since the converted cells can be produced from the patient's own somatic cells, it also avoids the risk of immune rejection, and there is no concern around the use of human embryos. Thus, the direct converted cells potentially represent a new, safer tool for cell therapy compared to hESC and human iPSC.

Although direct conversion of cell types is still a very recent approach, the ability of transdifferentiation of some human cells types has already been documented. In 2010, Szabo and coworkers demonstrated that human dermal fibroblasts could be reprogrammed into progenitors of the myeloid, erythroid and megakaryocytic lineages without establishing pluripotency via lentiviral-mediated overexpression of OCT4 and addition of growth factors and cytokines in the culture medium.

Son and coworkers (2011), reported that the forced expression of a combination of transcription factors could convert mouse and human fibroblasts into functional motor neurons that displayed morphology, gene expression signature, electrophysiology, synaptic functionality, *in vivo* engraftment capacity and sensitivity to degenerative stimuli, similar to motor neurons derived from embryos. The conversion of human fibroblasts into motor neurons was made possible by retroviral transduction of eight distinct transcription factors and addition of GDNF, BDNF and CNTF to the culture media.

Before applying converted cells in regenerative medicine, there are many issues that need to be addressed. It is necessary to assure that the epigenetic changes triggered by the methodology employed are occurring in a precise and controlled manner, and are resulting in the irreversible switch of one cell type to another. Moreover, there should be a concern about the safety of the methodology used, since transient and/or non-integrative methods are preferable. In order to use converted cells for clinical purposes, it is necessary to perform additional experiments to understand the exact molecular mechanisms of these conversions, which could result in highly efficient direct conversion protocols.

1.4 Final Considerations

Donor cells generated from hESCs can act as a source of tissue in regenerative medicine, arise as a solution to the limited number of organ donors, and also to nervous system regeneration and wounded-tissue replacement. Until now, preclinical and clinical trials suggest that these cells present both relevant biological activity and low risk for early or long-term toxicity.

In spite of the promising data, some facts hinder the broad acceptance of hESC in therapy; the most critical is regarding their potential to form teratomas when injected into patients. Other sources of human cells for therapy are currently known – ASCs, iPSCs, and converted cells – but in each case their benefits in regenerative medicine can be balanced by their disadvantages. Currently, no one can state that there is a perfect cell type for the treatment of all health problems; since it certainly depends on the disease to be treated. Therefore, in order to fulfill the many promises of cell therapy, basic and translational research with all types of cells should continue to be promoted.

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Chapter 2
Human Neural Stem Cell-Based Cell- and Gene-Therapy for Neurological Diseases

Seung U. Kim and Hong J. Lee

Abstract Human neurological diseases such as Parkinson's disease (PD), Huntington's disease (HD), amyotrophic lateral sclerosis (ALS), Alzheimer's disease (AD), multiple sclerosis (MS), stroke and spinal cord injury (SCI) are caused by loss of neurons and glia in the brain or spinal cord. Cell replacement therapy and gene transfer to the diseased or injured brain have provided the basis for the development of potentially powerful new therapeutic strategies for a broad spectrum of human neurological diseases. However, the paucity of suitable cell types for cell replacement therapy in patients suffering from neurological disorders has hampered the development of this promising therapeutic approach. In recent years, neurons and glia have successfully been generated from stem cells such as embryonic stem cells (ESCs), induced pluripotent stem cells (iPSCs), mesenchymal stem cells (MSCs) and neural stem cells (NSCs), and extensive efforts by investigators to develop stem cell-based brain transplantation therapies have been carried out. I review here notable experimental and pre-clinical studies previously published involving stem cell-based cell- and gene-therapies for PD, HD, ALS, AD, MS, stroke and SCI, and discuss for future prospect for the stem cell therapy of neurological disorders in clinical setting. There are still many obstacles to be overcome before clinical application of cell- and gene-therapy in neurological disease patients is adopted: (i) it is still uncertain how to generate specific cell types of neurons or glia suitable for cellular grafts in great quantity, (ii) it is required to abate safety concern related to tumor formation following NSC transplantation, and (iii) it needs to be better understood by what mechanism transplantation of NSCs leads to an enhanced functional recovery. Steady and stepwise progress in stem cell research in both basic and pre-clinical settings should support the hope for development of stem

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cell-based therapies for neurodegenerative diseases. This review focuses on the utility of stem cells particularly NSCs as substrates for structural and functional repair of the diseased or injured brain.

Keywords Stem cell • Embryonic stem cell • Induced pluripotent stem cell • Neural stem cell • Mesenchymal stem cell • Cell therapy • Gene therapy • Neurological diseases • Transplantation • Parkinson's disease • Huntington's disease • Amyotrophic lateral sclerosis • Alzheimer's disease • Multiple sclerosis • Stroke • Spinal cord injury

2.1 Introduction

In adult mammalian central nervous system (CNS), injured neurons exhibit low spontaneous capacity for regeneration (Ramon y Cajal 1928). Little progress was seen for more than half century in investigation related to the CNS regeneration until late 1970s. In 1979 two Swedish research groups reported independently that embryonic rat mesencephalic cells transplanted in the brain of Parkinson's disease rat model survived and induced functional recovery (Björklund and Stenevi 1979; Perlow et al. 1979). This is the first time that fetal CNS cell transplants replace lost neuross and restore functional deficits in animal models of neurological disease. Since then, fetal CNS-derived cells were grafted into the brain and spinal cord of animal models of neurological disorders including Parkinson's disease (PD), Huntington's disease (HD), stroke and spinal cord injury (SCI). Starting in late 1980s, transplantation of human fetal ventral mesencephalic tissues into the striatum of PD patients has been adopted as a successful therapy for patients with advanced disease (Lindvall et al. 1990; Olanow et al. 1996; Kordower et al. 1997a, b; Dunnett and Bjorklund 1999). However, this fetal brain tissue transplantation has serious problems including ethical and religious questions and limited supply of fetal tissues. To circumvent these difficulties, utilization of neurons with dopamine (DA) phenotype generated from embryonic stem cells (ESCs), induced pluripotent stem cells (iPSCs), mesenchymal stem cells (MSCs) or neural stem cells (NSCs) could serve as a practical and effective alternative for the fetal brain tissues for brain transplantation.

Stem cells are defined as cells that have the ability to renew themselves continuously and possess pluripotent ability to differentiate into many cell types. Two types of mammalian pluripotent stem cells, ESCs derived from the inner cell mass of blastocysts and embryonic germ cells (EGCs) obtained from post-implantation embryos, have been identified and these stem cells give rise to various organs and tissues (Thompson et al. 1998; Shamblott et al. 1998). Recently there has been an exciting development in generation of a new class of pluripotent stem cells, induced pluripotent stem cells (iPSCs), from adult somatic cells such as skin fibroblasts by introduction of embryogenesis-related genes (Takahashi et al. 2007; Yu et al. 2007; Park et al. 2008). In addition to ESCs and iPS cells, tissue specific stem cells could be isolated from various tissues of more advanced developmental stages such as hematopoietic stem cells (HSCs), bone marrow MSCs, adipose tissue-derived stem cells (ADSCs),

amniotic fluid stem cells and NSCs. Among these, existence of multipotent NSCs has been known in developing or adult rodent brain with properties of indefinite growth and multipotent potential to differentiate into three major cell types of CNS, neurons, astrocytes and oigodendrocytes (McKay 1997; Flax et al. 1998; Gage 2000; Gottlieb 2002; Kim 2004; Lindvall and Kokaia 2006; Kim and deVellis 2009).

In human, existence of NSCs with multipotent differentiation capability has also been reported in embryonic and adult human brain (Flax et al. 1998; Kim 2004; Brustle and McKay 1996; Sah et al. 1997). In a group of cancer patients who had infusion of chemical bromodeoxyuridine (BrdU) for diagnostic purposes and later died, evidence that new neurons are continuously being generated in adult human CNS was demonstrated (Eriksson et al. 1998). Why then there is only limited capacity to repair in adult CNS suffering from injury or diseases? It appears that endogenous brain environment that is responsible for induction of NSC proliferation and consequent NSC differentiation into neurons is not adequate in most of diseased or injured brain.

Recently continuously dividing immortalized cell lines of NSCs have been generated by introduction of oncogenes and these immortalized NSC lines have advantageous characteristics for basic studies on neural development and cell replacement therapy: (i) Stable immortalized NSC cells are homogeneous since they were generated from a single cell, i.e. a single clone; (ii) immortal NSC cells can be expanded readily in large numbers in short time; (iii) stable expression of therapeutic genes can be achieved readily (Flax et al. 1998; Kim 2004; Snyder et al. 1992; Lee et al. 2007a). Immortalized NSCs have emerged as highly effective source for genetic manipulation and gene transfer into the CNS *ex vivo*; immortalized NSCs were genetically manipulated *in vitro*, survive, integrate into host tissues and differentiate into both neurons and glial cells after transplantation to the intact or damaged brain (Fig. 2.1). We have previously generated immortalized cell lines of human NSCs by infecting fetal human brain cells grown in primary culture with a retroviral vector

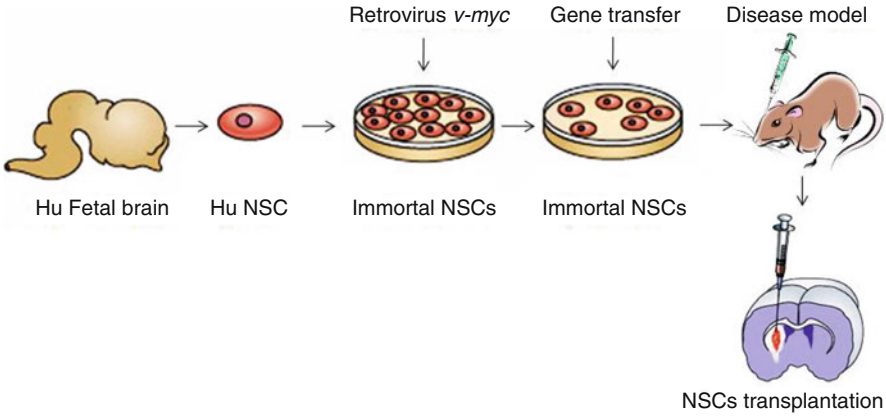

Fig. 2.1 Human neural stem cells (NSCs) isolated from 12 to 15 weeks gestational brain were immortalized by transfection with a retroviral vector encoding v-myc. Immortalized human NSCs were encoded with therapeutic genes such as NGF, BDNF, VEGF, GDNF, Akt-1, tyrosine hydroxylase or choline acetyltransferase, and then transplanted into the brain or spinal cord of neurological disease animal models

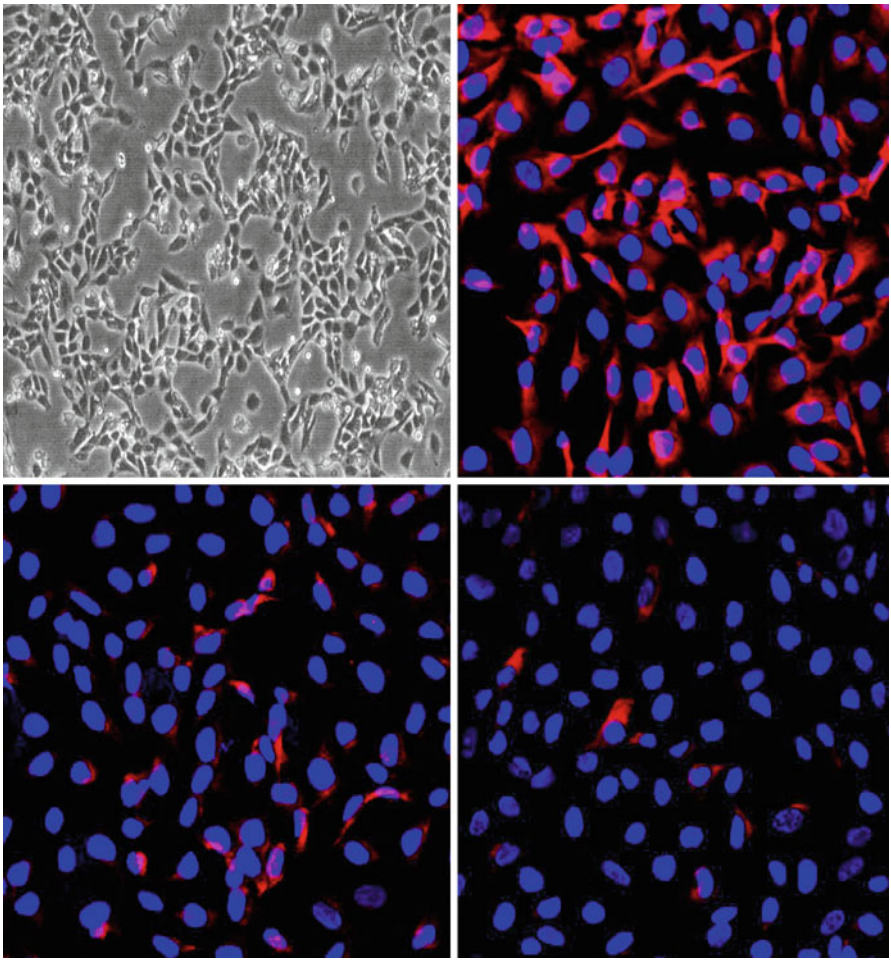

Fig. 2.2 Stable immortalized human neural stem cell line (NSC, F3) was generated by transfection of NSCs isolated from a 15 weeks gestational brain with a retroviral vector encoding v-myc. *Top left*: Phase contrast microscopy of NSCs; *Top right*: NSCs grown in serum-free medium are positive for nestin, a NSC cell type-specific marker; *Bottom left*: NSCs grown in serum-containing medium differentiate into neurons and express neurofilament protein, a neuron-specific marker; *Bottom right*: A small number of NSCs differentiate into astrocyte and express GFAP, an astrocyte cell type-specific marker

carrying v-myc oncogene and selecting continuously dividing NSC clones. Both *in vivo* and *in vitro* these cells were able to differentiate into neurons and glial cells and populate the developing or degenerating CNS (Flax et al. 1998; Kim 2004; Lee et al. 2007a; Kim and de Vellis 2009) (Figs. 2.2 and 2.3).

Stem cell-based cell and gene therapy could serve as potentially powerful new therapeutic strategies for a broad spectrum of human neurological diseases including PD, HD, AD, ALS, MS, stroke, SCI and brain tumors (Brustle and McKay 1996;

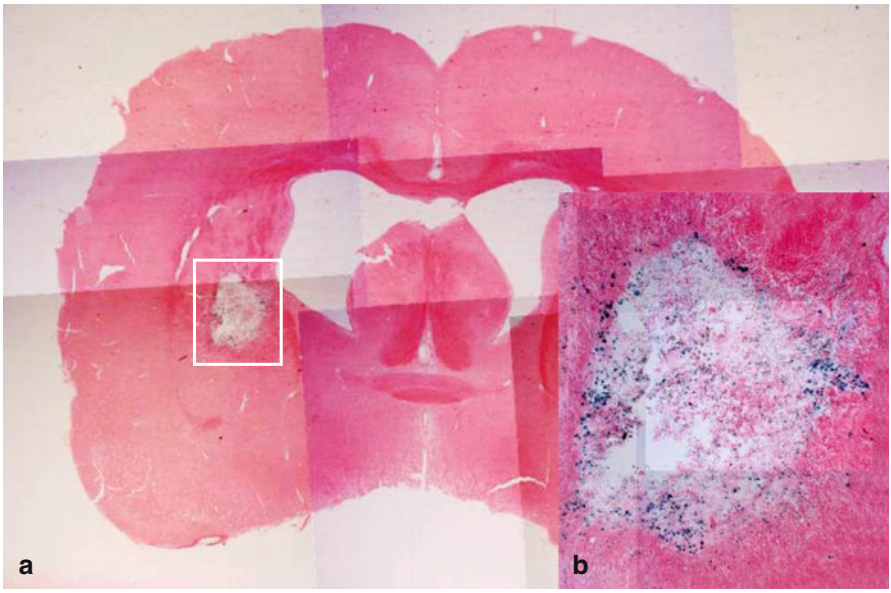

Fig. 2.3 (**a**) F3 human neural stem cells were encoded with **β**-galactosidase (X-gal) and transplanted near collagenase – induced intracerebral hemorrhage stroke lesion site in striatum. Four weeks later, X-gal-labeled human NSCs were found inside and boundary zone of hemorrhage lesion. (**b**) Higher magnification of the boxed area

Flax et al. 1998; Kim 2004; Lindvall et al. 2004; Goldman 2005; Kim and de Vellis 2009). There are still many obstacles to be overcome before clinical application of cell therapy in neurological disease patients is adopted: (i) it is still uncertain how to generate specific cell types of neurons or glia suitable for cellular grafts in great quantity, (ii) it is required to abate safety concern related to tumor formation following NSC transplantation, and (iii) it needs to be better understood by what mechanism transplantation of NSCs leads to an enhanced functional recovery. Steady and stepwise progress in stem cell research in both basic and pre-clinical settings should support the hope for development of stem cell-based therapies for neurodegenerative diseases. This review focuses on the utility of stem cells particularly NSCs and MSCs as substrates for structural and functional repair of the diseased or injured CNS.

2.2 Parkinson's Disease

Parkinson's disease (PD) is characterized by an extensive loss of dopamine neurons (DA) in the substantia nigra pars compacta and their terminals in the striatum (Kish et al. 1988; Agid 1991), and affects more than 500,000 people in the US. While the etiology of idiopathic PD is not known, several predisposing factors for the dopaminergic depletion associated with the disease have been suggested, including

programmed cell death, viral infection, and environmental toxins. As an effective treatment for PD, patients have been given L-dihydroxyphenyl alanine (L-DOPA), a precursor of dopamine, but long-term administration of L-DOPA consequently produces grave side effects (Lang and Lozano 1998a, b). More recently surgical procedure of deep brain stimulation has been adopted as a successful treatment for PD patients (Lyons 2011).

Since late 1980s, transplantation of human fetal ventral mesencephalic tissues into the striatum of PD patients has been adopted as a successful therapy for patients with advanced disease (Lindvall et al. 1990; OLanow et al. 1996; Kordower et al. 1997b; Dunnett and Bjorklund 1999). However, this fetal tissue transplantation has grave problems associated with ethical and religious questions and logistics of acquiring fetal tissues. In addition, recent reports have indicated that the survival of transplanted fetal mesencephalic cells in the patients' brain was very low and it was difficult to obtain enough fetal tissues needed for transplantation (Hagell et al. 1999). To circumvent these difficulties, utilization of neurons with dopamine (DA) phenotype generated from ESCs, iPSCs, MSCs or NSCs could serve as a practical and effective alternative for the fetal brain tissues for transplantation. DA neurons were generated from mouse ESCs after treatment with fibroblast growth factor 8 (FGF8) and sonic hedgehog (Hagell and Brundin 2002), over-expression of Nurr1 (Wagner et al. 1999; Chung et al. 2002; Kim et al. 2003) or Bcl-XL (Shim et al. 2004), or co-culture with a mouse bone marrow stromal cell line (Kawasaki et al. 2000). Neurons with DA phenotype have been generated from monkey ESCs by co-culturing with mouse bone marrow stromal cells and behavioral improvement was seen in MPTP-lesioned monkeys following intra-striatal transplantaiton of these cells (Takagi et al. 2005). DA neurons were also generated from neural progenitor cells derived from fetal brain and induced functional recovery following brain transplantation in parkinsoinian monkeys (Redmond et al. 2007).

Transplantation of NSCs in the brain attenuates anatomic or functional deficits associated with injury or disease in the CNS via cell replacement, the release of specific neurotransmitters, and the production of neurotrophic factors that protect injured neurons and promote neuronal growth. Recently we have generated continuously dividing immortalized cell lines of human NSC from fetal human brain cell culture via a retroviral vector encoding v-*myc* (Kim 2004; Lee et al. 2007a; Kim et al. 2008b) and one of the immortalized NSC lines, HB1.F3, induced functional improvement in rat model of PD following transplantation into the striatum (Yasuhara et al. 2006).

Earlier studies have used gene transfer technology to develop treatment for PD by transferring tyrosine hydroxylase (TH) gene, a rate-limiting step enzyme in catecholamine biosynthesis process, into certain cell types and then implant these cells into the brain of PD animal models (Wolff et al. 1989; Fisher et al. 1991; Jiao et al. 1993; Anton et al. 1994; During et al. 1994). However, gene transfer of TH using genetically modified cells produced only partial restoration of behavioral and biochemical deficits in PD animal models, since the cells utilized did not carry sufficient amount of tetrahydrobiopterin (BH_4), a cofactor to support TH activity (Kang et al. 1993). Therefore, it is necessary to transfer additionally of GTP

cyclohydrolase-1 (GTPCH-1) gene that is the first and rate-limiting enzyme in the BH_4 biosynthetic pathway (Bencsics et al. 1996). Immortalized CNS-derived mouse NSC line C17.2 was transduced to carry tyrosine hydroxylase (TH) gene and GTP cyclohydrorylase-1 (GTPCH-1) gene for production of L-DOPA and following intra-striatal implantation behavioral improvement was seen in 6-hydroxydopamine-lesioned rats (Ryu et al. 2005). We have similarly engineered HB1.F3 human NSC line to produce L-DOPA by double transduction with human TH and GTPCH-1 genes, and following transplantation of these cells in the brain of PD rat model led to enhanced L-DOPA production *in vivo* and induced functional recovery (Kim et al. 2006).

Previous studies have reported that mouse ES cell-derived DA neurons show efficacy in PD animal models (Lee et al. 2000; Kim et al. 2002), whereas DA neurons from human ES cells generally show poor performance. In addition, there are considerable safety concerns for ES cells related to risk of tumor formation and neural overgrowth. More recent studies have indicated that functional human DA neurons could be generated efficiently from human ES cells and upon transplantation in rat PD models ES cell-derived DA neurons induced behavior recovery in the PD animals (Cho et al. 2008; Kirks et al. 2011). These studies indicate that large scale generation of DA neurons is possible from human ES cells as cellular source for cell therapy in PD patients. Human DA neurons derived from iPS cells may provide an ideal cellular source for transplantation therapy for PD. However, developing effective cell therapy approach for PD using iPS cells relies on optimizing *in vitro* production of iPS cell-derived DA neurons and preventing potential risk of teratoma formation *in vivo*. A recent study has reported generation of DA neurons from iPS cells derived from fibroblasts and improved behavior following transplantation of the DA neurons in PD model rats (Werning et al. 2008). Although further research is still required, cell therapy based on DA neurons derived from iPS cells will probably become a promising treatment technique in the coming days.

2.3 Huntington's Disease

Huntington's disease (HD) is an autosomal dominant neurodegenerative disorder characterized by involuntary choreiformic movements, cognitive impairment, and emotional disturbances (Greenamyre and Shoulson 1994; Harper 1996). Despite identification of the HD gene and associated protein, the mechanisms involved in the pathogenesis of HD remain largely unknown and thus hamper effective therapeutic interventions. Transplantation of fetal human brain tissue may serve as a useful strategy in reducing neuronal damage in HD brain and a recent study has documented improvements in motor and cognition performance in HD patients following fetal cell transplantation (Bachoud-Lévi et al. 2000). This trial follows previous reports in experimental animals of HD that striatal grafts could integrate and survive within the progressively degenerated striatum in transgenic HD mouse model (Dunnett et al. 1998). The latter study is consistent with results obtained from

HD patients indicating survival and differentiation of implanted human fetal tissue in the affected regions (Freeman et al. 2000). Cell replacement therapy using human fetal striatal grafts has shown clinical success in HD patients. However, a recent study has reported neural overgrowth of grafted tissue in a HD patient who survived 5 years post-transplantaion (Keene et al. 2009). Overgrown grafts were composed of neurons and glia embedded in disorganized neuropil. This report recalls safety concerns for fetal cell grafts related to potential risk of neural overgrowth following transplantation in HD patients.

Transplantation of NSCs to replace degenerated neurons or genetically modified NSCs producing neurotrophic factors have been used to protect striatal neurons against excitotoxic insults (Bjorklund and Lindvall 2000). At present, little is known regarding whether implantation of NSCs prior to neuropathological damage could alter the progressive degeneration of striatal neurons and motor deficits that occur in HD. This question is important since genetic study of Huntington disease gene mutation (Huntington's Disease Collaborative Research Group 1993) and neuroimaging can provide details on factors involved in the progression of HD (Harris et al. 1999; Thieben et al. 2002) suggesting early intervention using brain transplantation could be effective in "pre-clinical" HD patients carrying mutant HD gene. We have investigated the effectiveness of proactive transplantation of human NSCs into rat striatum of an HD rat prior to lesion formation and.demonstrated significantly improved motor performance and increased resistance to striatal neuron damage compared with control sham injections (Ryu et al. 2004). The neuroprotection provided by the proactive transplantation of human NSCs in the rat model of HD appears to be contributed by brain-derived neurotrophic factor (BDNF) secreted by the transplanted human NSCs.

Rodents and primates with lesions of the striatum induced by excitotoxic kainic acid (KA), or quinolinic acid (QA) have been used to simulate HD in animals and to test efficacy of experimental therapeutics experiments on neural transplantation (DiFiglia 1990). Excitotoxic animal models induced by QA, which stimulates glutamate receptors, resemble the histopathologic characteristics of HD patients, were utilized for cell therapy with mouse embryonic stem cells, mouse neural stem cells, mouse bone marrow mesenchymal stem cells and primary human neural precursor cells and resulted in varying degree of clinical improvement (Kordower et al. 1997b; Armstrong et al. 2000; McBride et al. 2004; Visnyei et al. 2006; Lee et al. 2005, 2006). We have recently injected human NSCs intravenously in QA-HD model rats and demonstrated functional recovery in HD animals (Lee et al. 2005, 2006). The systemic transplantation of NSCs via intravascular route is probably the least invasive method of cell administration (Lee et al. 2006). Neural cell transplantation into striatum requires an invasive surgical technique using a stereotaxic frame. Non-invasive transplantation via intravenous routes, if it may be effective in human, is much more attractive.

Systemic administration of 3-nitropropionic acid (3-NP) in rodents leads to metabolic impairment and gradual neurodegeneration of the basal ganglia with behavioral deficits similar to those associated with HD (Beal et al. 1993; Brouillet et al. 1995), and murine and human NSCs have been transplanted in the brain of

3-NP-HD animal models (Ryu et al. 2004; Roberts et al. 2006). The compound 3-NP is a toxin which inhibits the mitochondrial enzyme succinate dehydrogenase (SDH) and tricarboxylic acid (TCA) cycle thereby interfering with the synthesis of ATP (Alston et al. 1977).

We have investigated the effectiveness of transplantation of human NSCs into adult rat striatum prior to striatal damage induced by 3-NP toxin (Ryu et al. 2004). Animals receiving intrastriatal implantation of human NSCs 1 week prior to 3-NP treatments exhibited significantly improved motor performance and increased resistance to striatal neuron damage compared with control sham injections. The neuroprotection provided by the proactive transplantation of human NSCs in the rat model of HD appears to be contributed by brain-derived neurotrophic factor (BDNF) secreted by the transplanted human NSCs. Previous studies have also demonstrated that BDNF could block neuronal injury under pathological conditions in animal models of HD (Bemelmans et al. 1999; Pérez-Navarro et al. 2000). These findings suggest that proactively transplanted human NSCs were well integrated in the striatum and supported the survival of host striatal neurons against neuronal injury.

Human NSCs derived from ESCs could provide a viable cellular source for cell and therapy in HD, since they can be expanded indefinitely and differentiate into any cell type desired. Three previous studies have shown that neurons expressing striatal markers could be induced from ESCs and brain transplantation of these ESC-derived neurons in QA-lesioned rats leads to behavioral recovery in the animals (Song et al. 2007; Aubry et al. 2008; Vasey et al. 2010).

We have previously written a review that focuses on the stem cell-based therapy for HD and investigators who wish to learn more about the subject are referred to the review article (Kim et al. 2008a).

2.4 Amyotrophic Lateral Sclerosis

Amyotrophic lateral sclerosis (ALS), known as Lou Gehric disease, is a relentlessly progressive, adult onset neurodegenerative disorder characterized by degeneration and loss of motor neurons in the cerebral cortex, brain stem and spinal cord, leading to muscle wasting and weakness, and eventually to death within 5 years after the onset of its clinical symptoms (Hudson 1990; Rowland and Shneider 2001). The proposed pathogenetic mechanisms of ALS, albeit not fully elucidated, include oxidative stress, protein aggregation, mitochondrial dysfunction, impaired axonal transport, glutamate-mediated excitotoxicity, and insufficient production of neurotrophic factors (Boillee et al. 2006). To date there is no effective treatment for patients suffering from ALS.

Recent studies have indicated that it is possible to generate motor neurons in culture from stem cells that include ESCs and NSCs (Wichterle et al. 2002; Harper et al. 2004; Miles et al. 2004; Li et al. 2005). Mouse ESC-derived motor neurons transplanted into motor neuron-injured rat spinal cord survived and extended axons

into ventral root (Miles et al. 2004), and human EGCs transplanted into cerebrospinal fluid of rats with motor neuron injury migrated into spinal cord and led to improved motor function (Kerr et al. 2003). Transplantation of NSCs isolated from fetal spinal cord (Xu et al. 2006) was also effective in delaying disease progression in mouse ALS model. These cell transplantation studies have shown functional improvement in animal models of ALS.

A recent study has reported that iPSCs isolated from an ALS patient were differentiated into motoneurons (Dimos et al. 2008) and these patient-derived neurons could be an ideal cellular source for transplantation. Neurons and glia induced from patient-derived iPSCs are ideal for cell therapy as the iPSC-derived neurons are autologous, easily accessible, without immune rejection and with no ethical problem although there is safety concern of tumor formation following the cell transplantation.

The systemic transplantation of NSCs via intravascular route is probably the least invasive method of cell administration in ALS. Non-invasive transplantation via intravenous routes is much more attractive than surgical technique. Recently rat NSCs labeled with green fluorescent protein were transplanted in rat ALS model via intravenous tail vein injection and 7 days later 13 % of injected cell were found in motor cortex, hippocampus and spinal cord. No improvement in clinical symptoms was reported (Miltrecic et al. 2010).

It is unrealistic to expect the transplantation of stem cells or stem cell-derived motor neurons in ALS patients in a clinical setting replaces lost neurons, integrates into existing neural circuitry and restores motor function. Rather preventing cell death in host motor neurons via provision of neurotrophic factors by transplanted stem cells or stem cell-derived motor neurons is more realistic and achievable approach (Lindvall and Kokaia 2006). Recent studies have shown that the application of an adenoviral vector encoding glial cell line-derived growth factor (GDNF) into injured rat facial motor nucleus rescued motor neurons from cell death (Watabe et al. 2000), and human cortical progenitor cells engineered to express GDNF and transplanted into the spinal cord of ALS rats survived and released the growth factor (Kerr et al. 2003). Several recent studies have also demonstrated that delivery of vascular endothelial cell growth factor (VEGF) significantly delayed disease onset and prolonged the survival of ALS animal models (Klein et al. 2005; Azzouz et al. 2004; Zheng et al. 2004; Storkebaum et al. 2005). VEGF is one of growth factors that can be used in combination with transplanted stem cells to improve therapeutic efficiency of cellular transplantation. VEGF is an angiogenetic growth factor acting as a potent mitogen and survival factor specific to endothelial cells, and also known for neurotrophic and neuroprotective effect against brain injury. Recently we have demonstrated that in transgenic SOD1/G93A mouse model of ALS (Gurney et al. 1994) intrathecal transplantation of human NSCs over-expressing VEGF (HB1.F3.VEGF) induced functional improvement, delayed disease onset for 7 days and extended the survival of animals for 15 days (Hwang et al. 2009b). Immunohistochemical investigation of SOD1/G93A mouse spinal cord demonstrated that the transplanted human NSCs migrated into spinal cord anterior horn and differentiated into motoneurons.

More recently, we generated motoneurons from human NSCs and transplanted these cells into spinal cord of SOD1G93A ALS mouse (Kim et al. 2011). Motoneurons were generated by treatment of human NSCs encoding Olig2 bHLH transcription factor gene (HB1.F3.Olig2) with sonic hedgehog (Shh) protein. HB1.F3.Olig2 NSCs treated with Shh for 4–7 days differentiated not motoneurons expressing motoneuron-specific markers HB-9, Isl-1 and choline acetyltransferase (ChAT) but did not express OLG markers such as O4, galctocerebroside or CNPase. Control HB1.F3.Olig2 NSCs grown in the absence of Shh did not express any of the motor neuron-specific cell type markers. Intrathecal transplantation of motoneuron-committed HB1.F3.Olig2+Shh human NSCs into L5 of spinal cord significantly delayed disease onset (9 days) and expanded the life span (22 days) of SOD1 G93A ALS mice, with 2 out of 6 mice living up to 35 days. Grafted NSCs were found within grey matter and anterior horn of the spinal cord. These results suggest that this treatment modality using genetically modified human NSCs might be of value in the treatment of ALS patients without significant adverse effects (Kim et al. 2011).

2.5 Alzheimer's Disease

Alzheimer's disease (AD) is characterized by degeneration and loss of neurons and synapses through out the brain particularly in basal fore brain, amygdala, hippocampus and cortical area. Memory and cognitive function of patients progressively decline, patients become demented and die prematurely (Whitehouse et al. 1981; Bartus et al. 1982; Coyle et al. 1983). No effective treatment is currently available except for acetylcholinesterase inhibitors which augment cholinergic function but is not curative and only a temporary measure.

As for the pathogenesis of AD, the amyloid cascade hypothesis postulates that memory deficits are caused by increased levels of both soluble and insoluble amyloid β (Aβ) peptides, which are derived from the larger amyloid precursor protein (APP) sequential proteolytic processing (Whitehouse et al. 1981; Bartus et al. 1982; Coyle et al. 1983; Hardy and Selkoe 2002). Recent study has reported that treatment of PDAPP mice, a transgenic mouse model of AD, with anti-Aβ antibody completely restored hippocampal acetylcholine release and high-affinity choline uptake and improved habituation learning (Bales et al. 2006). Based on the study, a clinical trial in AD patients is underway in the US.

Chronically decreasing Aβ levels in brain has been suggested as a possible therapeutic approach for AD, and several experimental evidence indicate that proteinases such as neprilysin (Iwata et al. 2001), insulin degrading enzyme (Farris et al. 2003; Miller et al. 2003), plasmin (Melchor et al. 2003) and cathepsin B (Mueller-Steiner et al. 2006) could be used as therapeutic agents to reduce Aβ levels in AD brain. Recent studies have shown that intracerebral injection of a lentivirus vector expressing human neprilysin in transgenic mouse models of amyloidosis reduced Aβ deposits in the brain and blocked neurodegeneration in the frontal cortex and

hippocampus (Marr et al. 2003), and that intracerebrally injected fibroblasts overexpressing human neprilysin gene were found to significantly reduce amyloid plaque burden in the brain of Aβ transgenic mice (Hemming et al. 2007). These studies support the use of Aβ-degrading proteases as a tool to therapeutically lower Aβ levels and encourage further investigation of *ex vivo* delivery of protease genes using human NSCs for the treatment of AD. We have recently generated a human NSC line encoding human neprylysin gene, transplanted these cells in lateral ventricle of AD transgenic mouse brain, and results are expected some time later.

Earlier studies have indicated that nerve growth factor (NGF) prevent neuronal death and improve memory in animal models of aging, excitotoxicity and amyloid toxicity (Hefti 1986; Fischer et al. 1987; Tuszynski et al. 1990; Emerich et al. 1994; Tuszynski 2002), and could be used for treating neuronal degeneration and cell death in AD brain. However, delivery of NGF into the brain is not possible via peripheral administration. Because of its size and polarity NGF does not cross the blood brain barrier. In order to overcome this difficulty, gene therapy approach could be adopted. Using *ex vivo* gene therapy approach via NGF encoding cells, NGF can be administered directly to the brain and diffuse for distance of 2–5 mm (Tuszynski et al. 1990). A phase 1 clinical trial of *ex vivo* NGF gene delivery was performed in eight mild AD patients by implanting autologous fibroblasts genetically modified to express human NGF into the forebrain. After mean follow-up of 22 months in 6 subjects, long-term adverse effects were not found. Evaluation by MMSE and ADASCS suggested improvement in the rate of cognitive decline. Serial PET scans showed significant increases in cortical fluorodeoxyglucose after treatment (Tuszynski et al. 2005). Since fibroblasts are known for their immobility following brain transplantation (Kang et al. 1993), NSCs with high migratory capacity and pathology-tropic property (Flax et al. 1998; Kim 2004; Lee et al. 2007a; Kim and de Vellis 2009) could be used in place of fibroblasts to deliver NGF into the AD brain. In learning deficit AD model rats induced by okadaic acid injection, transplantation of rat NSCs infected with adenovirus-NGF produced improvement in cognitive performance (Wu et al. 2008).

In a recent study, we used human NSCs in place of rodent NSCs or human fibroblasts to deliver NGF in ibotenic acid-induced learning deficit rats. Intrahippocampal injection of ibotenic acid caused severe neuronal loss, resulting in learning and memory deficit (Lee et al. 2012). NGF protein released by HB1.F3.NGF human NSCs in culture media is tenfold over the control F3 naive NSCs at 1.2 μg/10^6 cells/day. Intra-hippocampal transplantation of HB1.F3.NGF cells was found to express NGF and fully improved the learning and memory function of ibotenic acid-challenged animals. Transplanted HB1.F3.NGF human NSCs were found all over the brain and differentiated into neurons and astrocytes (Lee et al. 2012). In another study, brain derived neurotrophic factor (BDNF), a member of neurotrophin family, secreted by transplanted mouse NSCs was responsible in enhancing cognitive function in triple transgenic mice that express pathogenic forms of myloid precursor protein, presenilin and tau. In these animals cognition was improved without altering Aβ or tau pathology (Blurton-Jones et al. 2009). In other studies in experimental rats with nucleus basalis of meynert (NBM) lesion induced by ibotenic acid,

transplantation of mouse or rat neural precursor cells (NPCs) promoted behavioral recovery (Wang et al. 2006; Moghadam et al. 2009).

In AD patients, dysfunction of the presynaptic cholinergic system is one of the causes of cognitive disorders where decreased activity of choline acetyltransferase (ChAT), which is responsible for acetylcholine (ACh) synthesis, is observed (Terry and Buccafusco 2003). To date, AD therapy has largely been based on small molecules designed to increase ACh concentration by inhibiting acetyl cholinesterase (Musiał et al. 2007). Since therapies with these drugs is only palliative without potential protection against progressive tissue destruction, there is a need for effective therapies for patients with AD, and stem cell-based therapeutic approaches targeting AD should fulfill this requirement. We have recently generated a human neural stem cell (NSC) line over-expressing human choline acetyltransferase (ChAT) gens and these HB1.F3.ChAT NSCs were transplanted into the brain of rat Alzheimer disease (AD) model which was generated by intra-hippocampal injection of kainic acid (KA) in CA3 region which results in severe neuronal loss and profound learning and memory deficit. Intraventricular transplantation of HB1.F3.ChAT human NSCs fully restored learning and memory (Park et al. 2012a). Similarly HB1.F3.ChAT human NSCs were transplanted in AD model rats generated by application of ethylcholine mustard aziridinium ion (AF64A) that specifically denatures chOLinergic nerves and thereby leads to memory deficit as a salient feature of AD (Yamazaki et al. 1991). Transplantation of NB1.F3.ChAT human NSCs in AF64A-treated mice fully restored the learning and memory function of AF64A animals (Park et al. 2012b).

2.6 Multiple Sclerosis

In multiple sclerosis (MS), Oligodendrocytes (OLGs) and myelin are destroyed by inflammation-mediated mechanism (McFarlin and McFarland 1982; Ebers 1988). Although recent advance in treatment using immune moderators such as interferon-β has improved clinical outcome in some patients, functional recovery in most of MS patients is not achieved (Paty and Ebers 1998). Therefore, there is substantial need for effective therapies for MS patients.

Previous studies have reported that OLGs or OLG progenitor cells isolated from mouse or rat brain were transplanted in the brain of dysmyelination mutants or chemically induced demyelination lesions in rats and induced remyeliation in previously dysmyelinated or demyelinated lesion sites (Franklin and Blakemore 1997; Espinosa de los Monteros et al. 1997, 2001; Learish et al. 1999; Zhang et al. 1999; Ben-Hur et al. 2003). Experimental animal models for MS used in transplantation studies include shiverer dymyelination mutant mouse, demyelination lesions induced by ethidium bromide, experimental allergic encephalitis (EAE) or mouse hepatitis virus. Therapeutic approach with myelinating glia could be applied in MS patients by transplantation of human OLGs into demylination lesions. Intact embryonic human brain fragments or OLG progenitors isolated from fetal human brain

have been placed in shiverer mouse brain, a mouse neurological mutant with defect in myelin basic protein gene, and remyelination was confirmed (Lachapelle et al. 1983; Gumpel et al. 1987; Seilhean et al. 1996; Windrem et al. 2004). Transplantation of human OLGs in MS patients to achieve remyelination of previously demyelinated axons, however, has not been undertaken to date. This therapeutic approach of transplantation of human OLGs or OLG progenitors derived from fetal brain is not widely acceptable because of moral, religious and logistic problems associated with tissue collection of human embryonic/fetal brain. In addition, the outcome of graft is not predictable since the implanted embryonic/fetal tissues contain mixed population of neurons, glial cells and CNS progenitor cells, and less than 10 % of cell population for graft expressed O_4, a marker for young OLG (Gumpel et al. 1987). This difficulty can be circumvented by utilization of OLGs or OLG progenitor cells derived from human ES cells or NSCs. Recent studies have reported that OLGs could be generated from mouse and human ES cells (Brüstle et al. 1999; Liu et al. 2000; Glaser et al. 2005; Nistor et al. 2005), bone marrow mesenchymal stem cells (Akiyama et al. 2002) or immortalized mouse NSCs (Yandava et al. 1999). In a mouse EAE, systemically injected mouse neural precursor cells (NPCs) selectively enter the inflamed CNS in EAE model and induce apoptosis of blood-borne CNS-infiltrating encephalitogenic T cells, thus protecting against chronic neural tissue loss. NPCs display immune-like functions that promote neuroprotection in the CN (Pluchino et al. 2005). Similarly human ESC-derived NPCs transplanted into the brain ventricles of EAE mouse reduced clinical signs of EAE and transplanted NPCs were found in the white matter. These results indicate that NPCs act as immune-like cells in the CNS (Aharonowiz et al. 2008).

OLGs could also be generated from stable established cell lines of human NSC and used as cell source of transplantation. Previously we have produced immortalized cell lines of human NSC from human embryonic telencephalon using a retroviral vector encoding myc oncogene (Kim 2004; Lee et al. 2007a; Kim et al. 2008a, b). This human NSC line, HB1.F3 (F3), can be genetically engineered to express foreign transgenes, and following transplantation into brain of animal models of PD, HD and stroke, F3 NSCs survived, differentiate into neurons and astrocytes, and reversed functional deficits (Kim 2004, Lee et al. 2007a; Kim and de Vellis 2009). Most recently we were successful in producing a new F3.Olig2 human NSC line by transduction of F3 with a retroviral vector encoding Olig2 bHLH transcription factor gene. Olig2 is a member of Olig bHLH transcription factor family and plays a crucial role in generation of OLGs and ensuing myelination in the CNS, and also a key factor for generation of spinal motoneurons (Lu et al. 2000; Zhou et al. 2000; Takebayashi et al. 2000; Copray et al. 2006). F3.Olig2 human NSCs, we have generated, express cell type specific markers for OLG progenitors (PDGFRα and NG2), and also cell type specific markers for OLG (O_4, galactocerebroside and CNP). F3.Olig2 NSCs were transplanted in contused rat spinal cord lesion site and at 7 weeks post-transplantation grafted NSCs were found in the white matter and differentiated into mature OLGs. Animals with F3.Olig2 grafts showed an improvement in hindlimbs locomotion (Hwang et al. 2009a).

Following transplantation of HB1.F3.Olig2 cells into the shiverer mouse brain, a mouse neurological mutant with congenital dysmyelination, an extensive

myelination was demonstrated (Kim 2013, Unpublished data). We expect this successful pre-clinical study could lead to cell-based therapy in MS patients, with provision of unlimited number of human OLG/OLG progenitor cells for transplantation from this human cell line.

2.7 Stroke

Stroke represents the second highest among the causes of death in East Asia including China, Japan and Korea, and third highest in US. There are two major types pf stroke and they are ischemia and intracerebral hemorrhage (ICH). Ischemic stroke caused by abrupt and near-total interruption of cerebral blood flow, produces ischemic changes in the striatum and cortex, leading to a long-term sensorimotor deficit. The major cause of ICH is hypertension and less common causes include trauma, infections, tumors, blood clotting deficiencies, and abnormalities in blood vessels such as arteriovenous malformations. Once damage from a stroke occurred, little can be done to restore premorbid functions, and although numerous neuroprotective agents have been clinically tried, no specific agents replaced the lost neurons, improved the deteriorated functions, and reduced the long-term sequelae (Marshall and Thomas 1988). There are numerous previous reports of stem cell transplantation in the stroke animal models (Savitz et al. 2002, 131), and various cellular sources such as human ESC- or iPSC-derived NSCs (Daadi et al. 2010; Kawai et al. 2010), immortalized human NSCs (Chu et al. 2003, 2004; Jeong et al. 2003; Lee et al. 2007a, b, 2009a, b, 2010), mouse neural precursor cells (Veizovic et al. 2001), rodent bone marrow MSCs, human umbilical cord blood cells, human bone marrow MSCs, human teratocarcinoma-derived neurons (Borlongan et al. 1998; Saporta et al. 1999) and were grafted into the ischemic or hemorrhagic rodent brain, and reduced the neurological deficits. An earlier study has reported that in human with ischemic infarct, intracerebral implantation of human teratocarcinoma NT2-derived neurons has resulted in functional improvement (Kondziolka et al. 2000).

Neural stem cells (NSCs) could be isolated from embryonic, fetal or adult CNS tissues of mammals including human. NSCs and neurons could also be derived from ESCs or iPSCs and transplanted in animal models of stroke inducing functional recovery in the animals (Daadi et al. 2010; Seminatore et al. 2010; Kawai et al. 2010). However, risk of tumor formation is a major obstacle to cell therapy based on human ESC- or iPSC-derived cells. Human neural progenitor cells (NPCs) derived from ESCs transplanted into rat brain with MCA lesions, resulted in tumor formation (Seminatore et al. 2010). In another study, transplantation of iPS-derived NSCs into rat ischemic brain also caused tumor formation (Kawai et al. 2010). ESC- and iPSC-derived cells have a promising potential to provide neurons and glia for the cell therapy in stroke. However proper and strict control of tumorigenesis has to be achieved before ESC- or iPSC-based cell therapy becomes a realistic clinical strategy.

We have previously investigated whether conditionally immortalized human NSCs could selectively migrate into lesioned brain sites, differentiate into new

neurons and/or glia, and improve the functional deficits in rat stroke models of focal ischemia (Chu et al. 2003, 2005) and cerebral hemorrhage (Jeong et al. 2003; Lee et al. 2007a, b, 2009a, b, 2010). NSCs can circumvent blood-brain barrier and migrate to the specific pathologic areas of brain with tropism. We introduced immortalized human NSCs intravenously via tail veins or into lesion site and NSCs migrated into the adult rat/mouse brain with transient focal cerebral ischemia or with cerebral hemorrhage. Transplanted human NSCs migrated to the lesion site, differentiated into neurons and astrocytes, and a large number of the grafted human NSCs survived in the lesion sites for up to 12 weeks (Figs. 2.2 and 2.3). Functional improvement was observed in the transplanted animals compared with non-grafted controls on rotarod and turning-in-an-alley tests. Transplantation of NSCs overexpressing neurotrophic factors such as vascular endothelial growth factor (VEGF), glial cell line-derived neurotrophic factor (GDNF) or brain derived growth factor (BDNF) induced good survival and neuroprotection of both host neurons and grafted NSCs in the lesion site and promoted functional improvement in the ICH model animals (Lee et al. 2007a, b, 2009a, b, 2010).

2.8 Spinal Cord Injury

Traumatic spinal cord injury (SCI) results in severe and permanent neurological deficits. However, there is no single effective therapeutic option to improve functional outcome. Intense research efforts, employing a rodent model of contusive injury which closely mimics human SCI, have identified that the pathology in the white matter incurred by injury is closely associated with the extent of functional recovery (Noble and Wrathall 1989; Fehlings and Tator 1995; Basso et al. 1996). One of the important pathological processes in the white matter is a chronic and progressive demyelination of spared axons (Bunge et al. 1993; Reier 2004; Guest et al. 2005), which occurs primarily due to delayed and widespread apoptosis of oligodendrocytes (OLGs) (Crowe et al. 1997; Springer et al. 1999).

Recent advances in stem cell biology have opened up an avenue to therapeutic strategies to replace lost neural cells by transplantation of stem cells in various disorders in the CNS. For spinal cord injury, various cell types such as genetically modified fibroblasts, olfactory ensheathing cells, Schwann cells and stem cells have been used to promote axonal regeneration (Tuszynski et al. 1994; Xu et al. 1995; Li et al. 1997; Liu et al. 1999; Teng et al. 2002). Since an earlier study showing that transplantation of embryonic stem cells (ESCs) promotes functional recovery (McDonald et al. 1999), several studies have reported that various stem or progenitor cells types including ESCs, bone marrow msCs and NSCs induce functional improvement following transplantation into the injured spinal cord (Teng et al. 2002; McDonald et al. 1999; Ogawa et al. 2002; Cao et al. 2005; Cummings et al. 2005; Keirstead et al. 2005; Iwanami et al. 2005; Karimi-Abdolrezaee et al. 2006). However, there are still many obstacles to be overcome before stem cell-based therapy can be adopted for SCI. One of such problems is a massive death of stem cells transplanted into the injured spinal cord tissue. Cell death of transplanted

stem cells arises because the molecular environment in the injured spinal cord tissue promotes apoptotic cell death (Benn and Woolf 2004). Apoptosis is a highly regulated form of cell death involving a cascade of signaling pathways both the extrinsic and intrinsic cell death pathways. It is crucial to develop a strategy to prevent apoptotic death of grafted stem cells for efficient therapeutic application of stem cell transplantation. In a recent study, we have investigated an experimental study to improve survival of grafted neural stem cells by over-expressing anti-apoptotic Bcl-XL gene. Bcl-XL is the most potent anti-apoptotic molecule in the Bcl-2 family, and found to protect various neuronal populations from apoptotic cell death (Yuan and Yankner 2000). Over-expression of Bcl-XL extends cell survival against apoptotic signals induced by a variety of treatments including viral infection, UV and γ-radiation, heat shock, and agents that promote formation of free radicals. We have recently generated a stable cell line of human NSCs over-expressing Bcl-XL gene and this HB1.F3.Bcl-XL cells were transplanted into contused spinal cord lesion in adult rats. As compared with control SCI rats that were implanted with parental human NSCs without Bcl-XL expression, SCI animals grafted with human NSCs over-expressing Bcl-XL demonstrated improved functional recovery. Histological examination of the spinal cord also revealed higher number of surviving cells in the spinal cord in the Bcl-XL group (Lee et al. 2009). It appears that the provision of anti-apoptotic protein to NSCs via gene transfer is one of the ways to extend survival of grafted stem cells in inhospitable environment of SCI lesion site. We have previously established clonal human NSC lines that were immortalized by retroviral transduction of v-myc gene (Flax et al. 1998; Kim 2004; Lee et al. 2007a). The human NSC line is a clonally isolated and can be expanded in a short amount of time. It has the ability to self-renew and differentiate into cells of neural and glial lineage in both *in vivo* and *in vitro*. It has been shown that immortalized NSCs differentiates into neurons, astrocytes and oligodendrocytes *in vivo*, with differentiated neurons forming synapses with other cells, and the immortalized NSCs have advantage of being more amenable to genetic manipulation (Snyder et al. 1992; Snyder 1994; Gage 2000; Kim 2004; Lee et al. 2007a; Kim and de Vellis 2009). We have previously written an extensive review that focuses on the stem cell-based therapy for SCI and investigators who wish to learn more about the subject are referred to the review article (Kim et al. 2007).

2.9 Perspectives

There are a number of issues to be clarified before adoption of stem cells for cell replacement therapy and gene therapy is widely accepted in clinical medicine such as which type of stem cells are most suitable for cell replacement therapy in patients with neurological disorders or brain injury, and safety issues related to the risk of tumorigenesis by grafted stem cells. Since neurons could be derived not only from NSCs, but also from ESCs, EGCs, bone marrow MSCs, umbilical cord blood hematopoietic stem cells and even from iPS cells generated from adult somatic cells, the most pressing question is which cells are best suited for cell replacement therapy.

Since the presence of NSCs in adult CNS is known, it is only a matter of time before neurons and glial cells are cultured from adult CNS tissue samples. There are ongoing debates as to why oocytes, embryonic or fetal materials should be used to generate stem cells when stem cells could be isolated from adult tissues. However, most of research up to now indicates that embryonic or fetal stem cells are significantly more versatile and plastic than adult counterparts.

Previous studies have demonstrated that ESC- or NSC-derived neurons or glial cells could be renewable cell source in cell based therapy for patients suffering from neurological diseases including PD, HD, ALS, AD, MS, stroke and spinal cord injury, however, there exist serious caveats that limit the use of stem cell-derived neurons or glial cells for the purpose. The considerations include (i) the long-term survival and phenotype stability of stem cell-derived neurons or glial cells in the graft following transplantation are not favorable as earlier studies have demonstrated, (ii) highly purified populations of neuronal cell type derived from ESCs or NSCs may contain other neuronal or glial cell types that might produce unpredictable interactions among grafted cells or with host neurons, and (iii) a small number of ESCs or iPSCs that escaped differentiation and selection processes might expand and form tumor in the graft site following transplantation.

Continuously dividing immortalized cell lines of human NSCs as generated by introduction of oncogenes have advantageous features for cell replacement therapy and gene therapy and the features include that human NSCs are homogeneous since they were generated from a single clone, can be expanded to large numbers *in vitro*, and stable expression of therapeutic genes can be achieved readily. Immortalized human NSCs have emerged as highly effective source of cells for genetic manipulation and gene transfer into the CNS *ex vivo* and once transplanted into damaged brain they survive well, integrate into host tissues and differentiate into both neurons and glial cells. It is known that both extrinsic and heritable intrinsic signals play important roles in generating cellular diversity in the CNS. By introducing relevant signal molecules or regulatory genes into the human stem cell line, it is now possible to obtain a large number of selected populations of neurons or glial cells from continuously growing human NSCs. Further studies are needed in order to identify the signals for proliferation, differentiation and integration of NSCs and determine favorable conditions of host brain environment for implanted NSCs to survive, prosper and restore the damaged brain.

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Chapter 3
Vascular Stem Cell Therapy

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Abstract There are numerous diseases associated with a malfunction of the endothelium, including ischemic injuries that follow thrombotic events, visual loss due to a defective cornea endothelium, and endothelial cell (EC) dysfunction in patients with diabetes. Some of these diseases are susceptible of cell therapies that aim to replace the defective endothelium. Thus, there is a need for a robust, clinically suitable source of autologous ECs. In principle, ECs can be obtained from a variety of autologous tissues, including small diameter veins and the microvasculature of tissues such as skin and adipose. However, the clinical use of mature ECs is limited by site morbidity and low cell proliferation potential. These limitations have motivated the search for other sources of ECs with more proliferative and vasculogenic activities such as those derived from embryonic stem cell (ESCs) and induced pluripotent stem cells (iPSCs). However, ethical considerations along with a poor understanding of the mechanisms controlling the differentiation of embryonic stem cells are hurdles that need to be overcome before these cells can be used in a clinical setting. Alternatively, the existence of postnatal endothelial progenitor cells (EPCs) in circulation represents a promising opportunity to non-invasively obtain large quantities of autologous ECs. However, the process for obtaining EPC-derived ECs has not been straightforward in part due to a lack of consensus regarding EPC definition, origin, and function of these cells. As a result, the term EPCs has been used to define two distinct subpopulations of cells, one with hematopoietic (early EPCs)

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and the other with endothelial (late EPCs) characteristics. Despite the often-confusing terminology, the functional distinction between these two very different types of EPCs is increasingly better understood. In particular, the ability of late EPCs to generate large amount of endothelial colony-forming cells (ECFCs) is now well recognized. Blood-derived ECFCs represent a robust population of cells with endothelial phenotype and ability to function as bona fide ECs both in vitro and in vivo, and thus, they constitute a promising source of cells for future vascular cell therapies.

Keywords Vascular therapy • Endothelial progenitor cells • Vasculogenesis • Angiogenesis

3.1 Endothelial Cells for Therapy

Endothelial cells (ECs) line the lumens of all blood vessels. ECs are highly metabolically active and play an important role in many physiological functions, including the control of vasomotor tone, blood cell trafficking, hemostatic balance, permeability, and innate and adaptive immunity (Aird 2007). Thus, it is not surprising to find EC dysfunction responsible for numerous diseases. Consequently, clinical conditions that could potentially benefit from EC therapy are multiple.

The main clinical conditions susceptible to EC therapy are those associated with ischemic injuries that result from the formation of a thrombus (Bagot and Arya 2008; Furie and Furie 2008). The incidence of ischemic injury is vast: myocardial infarction, stroke, and other thrombotic events affect more than 1.3 million individuals each year in the United States alone (www.americanheart.org). Much effort has been focused on delivering angiogenic factors to ischemic tissues to initiate angiogenesis and accelerate revascularization (Cao et al. 2003; Carmeliet 2000; Isner 2002; Khurana and Simons 2003). However, because tissue injury is often associated with a disruptive microenvironment that functions to recruit pre-existing ECs (Rafii and Lyden 2003), exogenous introduction of ECs may facilitate revascularization of the injured, ischemic tissue to restore organ function.

In addition, there are clinical conditions unrelated to thrombosis that may benefit from the development of EC therapies. For example, approximately 38,000 corneal transplants are performed in the United States each year and nearly half of all of these grafts are used to treat vision loss resulting from dysfunction of the corneal endothelium (Fuchs' dystrophy) (Terry 2003). Current treatment options for these conditions include Descemet's Stripping Endothelial Keratoplasty (DSEK), which is a type of partial corneal transplant that involves surgical replacement of the corneal endothelium with a healthy donor endothelium. Thus, availability of healthy autologous ECs may be beneficial in these situations.

EC therapy may also be suitable for tissue engineering of cardiovascular grafts. For example, every year there are approximately 550,000 bypass cases performed (www.americanheart.org) that involve surgical replacement of coronary and

peripheral atherosclerotic vessels. The preferred replacement grafts for small (<5 mm in diameter) diseased segments are autologous vessels, but many patients do not possess suitable vessels (Lloyd-Jones et al. 2010). Alternatively, small-diameter vessels are now being bioengineered following the tissue engineering paradigm in which appropriate biodegradable scaffolds are seeded with autologous cells (Dahl et al. 2011; Kaushal et al. 2001; Niklason et al. 1999). Other cardiovascular structures susceptible to a tissue engineering approach are heart valves (Sacks et al. 2009). Currently, valve replacements utilizing either xenografts, mechanical prostheses, or homografts have certain limitations, including thrombogenicity and inability to grow, repair or develop, which limits their long-term performance, especially in children. Thus, there is great interest in developing a tissue engineered valvular graft as an alternative solution (Gottlieb et al. 2010; Sacks et al. 2009). In both cases, tissue engineered vascular and valvular grafts would benefit from using autologous ECs; ideally, ECs would be seeded onto these engineered grafts to create a non-thrombogenic barrier, thereby improving the biological performance of the grafts in vivo (Kaushal et al. 2001).

Apart from large cardiovascular structures, ECs will likely be used in a broad range of tissue engineering applications that require vascular networks. Most engineered tissues must have a vascular network connected to the host vasculature to guarantee adequate nutrients, gas exchange, and elimination of waste products (Jain et al. 2005). Presently, there are no tissue-engineered (TE) constructs clinically available that incorporate an inherent microvascular bed, and therefore success has been limited to the replacement of relatively thin (skin) or avascular (cartilage) tissues (De Bie 2007; MacNeil 2007). To overcome the problem of vascularization, strategies to promote ingrowth of microvessels by delivery of angiogenic molecules have been proposed (Isner and Asahara 1999; Lee et al. 2002; Li et al. 2005). However, rapid and complete vascularization of thick engineered tissues is likely to require an additional supply of ECs (Jain et al. 2005; Melero-Martin et al. 2008; Rafii and Lyden 2003).

Establishing clinically suitable sources of cells is a priority for the development of EC therapies. ECs may be drawn from a variety of primary tissues. Theoretically, these primary tissues can be xenogeneic (from different species), allogeneic (from different members of the same species), syngeneic (from a genetically identical individual), or autologous (from the same individual). Nevertheless, although animal ECs are a possibility, ensuring their safety remains a concern, as does the high likelihood of their rejection by the host immune system (Eberli and Atala 2006; Langer and Vacanti 1999). Additionally, current clinical use of allogeneic cells is still limited by the need for host immunosuppression. In the future, the emergence of new techniques to render cells immunologically transparent may allow the use of banked allogeneic cells to become a valid option. Until further advances enable the clinical use of other cell sources, autologous cells remain the preferred cells in regenerative medicine, and thus we have focused this review around such cells. Autologous ECs can be obtained from healthy vascular tissues in the patient's own body; upon taking a small biopsy, ECs can be dissociated from the tissue, purified, and culture expanded in the laboratory to obtain sufficient cell number for a given

therapy. While the use of autologous ECs may cause an inflammatory response, it should avoid rejection, and thus the side effects caused by immunosuppressive medications. In addition, the use of autologous primary cells raises no ethical issues and is accepted worldwide (Eberli and Atala 2006).

Beyond primary tissues, another alternative source of autologous ECs could result from therapeutic cloning. In this process patient-specific embryonic stem cells (ESCs) may be derived from pre-implantation stage embryos produced by somatic cell nuclear transfer, therefore obtaining histocompatible cells for engraftment (Han et al. 2007). More recently, the development of techniques that enable the generation of patient-specific induced pluripotent stem cells (iPSCs) from adult primary cells has created another potential means to obtain autologous embryonic-like stem cells (Takahashi and Yamanaka 2006). The theory behind the use of these cell types is that either ESCs or iPSCs could be differentiated into autologous ECs with the potential to replace and regenerate damaged vascular tissues. However, the mechanisms governing their differentiation must be fully understood, and ethical issues surrounding their use must be resolved prior to their implementation in therapeutic strategies.

Finally, we have dedicated a central part of this review to discuss the use of autologous, adult vascular progenitor cells. In particular, the identification of endothelial progenitor cells (EPCs) in peripheral blood during the last decade has presented an opportunity to non-invasively obtain autologous ECs for vascular therapies (Asahara et al. 1997). Despite the potential of EPCs, their clinical use to date has not been realized in part due to a lack of consensus regarding their definition, origin, and function in humans (Fadini et al. 2012; Yoder 2009). Here, we have highlighted some of the critical aspects of EPCs that are now better understood and discussed the clinical potential of these cells in comparison to the other alternative sources of autologous ECs (Fig. 3.1).

3.2 Mature Endothelial Cells

ECs can be obtained from a variety of autologous tissues, including small diameter veins and the microvasculature of tissues such as skin (Davison et al. 1980) and adipose (Kern et al. 1983). In fact, for decades, the study of EC biology has been conducted with cultures of mature ECs that were obtained from living vasculature (Gimbrone et al. 1974; Nachman and Jaffe 2004). The use of mature ECs for vascular therapies is surely a possibility as cultured ECs have been shown to retain their inherent vasculogenic ability in preclinical studies. For example, Schechner et al. demonstrated that human umbilical vein ECs (HUVECs) that were cultured in the laboratory were able to form microvascular networks in collagen/fibronectin gels after implantation into immunodeficient mice (Schechner et al. 2000). Similar results have been reported with human dermal microvascular ECs (HDMECs) seeded on biopolymer matrices, where functional microvessels were evident 7–10 days after implantation into mice (Nör et al. 2001).

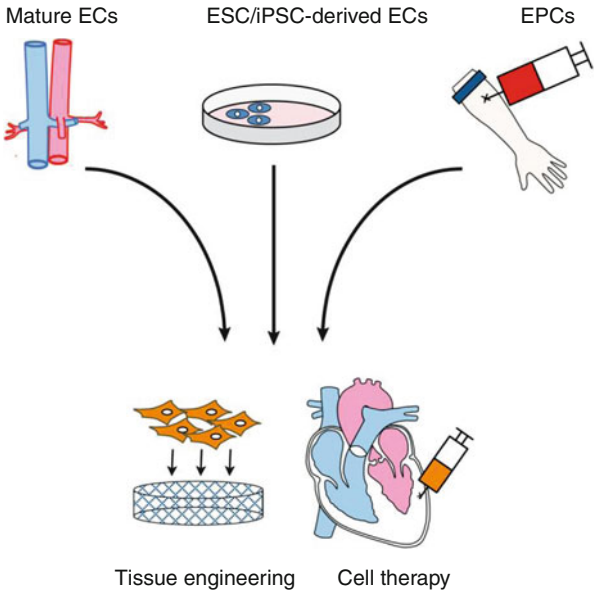

Fig. 3.1 Therapeutic use of endothelial cells (*ECs*). Clinically suitable sources of autologous ECs may include mature vasculature, patient-specific embryonic stem cells, and postnatal endothelial progenitor cells (*EPCs*). Autologous ECs will likely be used in a broad range of tissue engineering applications that require vascular networks as well as in direct vascular cell therapies

Nevertheless, the clinical use of mature ECs derived from autologous vascular tissues presents some important limitations. First, like most human somatic cells, ECs undergo replicative senescence in vitro after a finite number of divisions, which limits the expansion potential of these cells and varies depending on the tissue of origin and age of the subject (Yang et al. 1999). For example, the maximum number of population doublings attained with cultured HUVECs is approximately 50, while foreskin-derived HDMECs can reach 30 population doublings (Yang et al. 1999). In principle, this proliferation potential will allow for a very large number of autologous ECs. However, both HUVECs and HDMECs originate from neonatal tissues and with the exception of research settings, these tissues are not routinely saved for clinical application. Thus, patients in need of autologous ECs would require donation of their aged vascular tissues at the time of therapy, and these ECs would presumably have lower expansion potential than those isolated from neonatal counterpart tissues. For example, ECs derived from adult human saphenous vein (HSVECs) are reported to have approximately 19 population doublings before they reach senescence in culture (Yang et al. 1999). Perhaps a solution to the limited expansion potential could be to develop new clinical services that will enable individuals to routinely cryopreserve neonatal tissues (i.e., umbilical cord blood and foreskin) and cells in anticipation of future clinical uses.

A second limitation of using mature autologous ECs is the site morbidity associated with the harvest of healthy vascular tissue. For example, autologous saphenous veins or an arm vein are currently the vascular tissue of choice for use as a bypass graft in infrainguinal arterial reconstruction for peripheral bypass procedures, and autologous arteries or veins are used in cardiac bypass procedures (Seifalian et al. 2002). However, removing any of these healthy blood vessels for the sole purpose of isolating autologous ECs might be difficult to achieve clinically. Moreover, many

patients may have no suitable vessels available, particularly, if they have previously undergone bypass or their vessels were judged to be of poor quality (Bergan et al. 1982; Veith et al. 1979). Additionally, although in principle the issue of site morbidity could be diminished by smaller biopsies, this practice would in itself augment the problem of limited cellular lifespan, because fewer cells retrieved from those small biopsies would need to be expanded in culture more extensively. Additionally, from a technical standpoint, the procedures to isolate pure populations of ECs from smaller autologous biopsies would be increasingly difficult to perform efficiently. In summary, although healthy vasculature from a given patient is certainly a possible source of autologous ECs, the widespread clinical use of mature ECs will likely depend on our ability to overcome the limitations associated with their limited expansion potential and site morbidity.

3.3 Embryonic Stem Cell-Derived Endothelial Cells

The limitations of mature ECs have instigated the search for other sources of autologous ECs, including those derived from ESCs (Rafii and Lyden 2003). The excitement of using human ESCs in regenerative medicine has existed since they were first isolated in culture from the inner cell mass of human blastocysts (Thomson et al. 1998); ESCs can potentially provide an unlimited number of pluripotent cells, which can subsequently generate sufficient ECs for any vascular cell therapy. In principle, patient-specific ESCs can be derived by therapeutic cloning from preimplantation stage embryos produced by somatic cell nuclear transfer (Wilmut et al. 2002). However, harnessing the full therapeutic potential of ESCs is challenging and would require methodologies for large expansion of ESCs as well as a better understanding of the mechanisms controlling their differentiation. Here, we have summarized the progress of directing human ESCs differentiation into ECs.

3.3.1 Spontaneous Differentiation of Embryoid Bodies into ECs

The first evidence of endothelial differentiation of ESCs came from studies of spontaneous formation of vascular structures in embryoid bodies (EBs) (Wang et al. 1992). EBs are three-dimensional aggregates of pluripotent stem cells, and they are commonly used to achieve spontaneous differentiation of ESCs toward the three germ lineages (Reubinoff et al. 2000). The initial EC differentiation was induced by the low-oxygen environment in the center of the EBs. Levenberg and colleagues enzymatically dissected EBs that were formed by human ESCs into single cells and sorted ECs from the cell mixtures using platelet endothelial cell-adhesion molecule-1 (PECAM1) antibodies and fluorescence-activated cell sorting (FACS) (Levenberg et al. 2002). Subsequent studies that used this EB methodology were also proven

successful: ECs were obtained from ESCs and purified using additional EC markers (vWF and VE-cadherin) and Magnetic-activated cell sorting (MACS) (Cho et al. 2007; Descamps and Emanueli 2012; Wang et al. 2007). From these early studies, we learned that human EB-derived ECs are expandable in culture and display characteristics similar to mature human ECs. These characteristics include expression of EC markers (PECAM1, vWF, VE-cadherin, CD34, eNOS), capacity to uptake acetylated low-density lipoproteins, and ability to form cord-like structures when cultured on Matrigel (Cho et al. 2007). Moreover, implantation of human EB-derived ECs into immunodeficient mice were shown to generate perfused lumenal structures that carried mouse blood, indicating the ability of these ECs to assemble into blood vessels and to form functional anastomoses with living vasculature (Levenberg et al. 2002). In addition, transplantation of EB-derived human ECs into surgically-induced ischemic hindlimb muscles in mice was shown to significantly improve blood reperfusion and limb salvage due to neovascularization (Cho et al. 2007). Collectively, these studies clearly indicated feasibility of obtaining functional ECs from EBs and demonstrated their therapeutic potential.

Despite the feasibility of obtaining ECs from EBs, this approach has several limitations. Firstly, although the three-dimensional nature of EBs may be beneficial to recapitulate the complexity of native vasculature, it also instigates the appearance of cell types from all the three germ layers, which makes obtaining a large number of ECs with high purity and yield much more difficult (Chaudhury et al. 2012). Secondly, methodologies based on EBs are time-consuming, require extensive culture manipulation, and rely on achieving single cells by mechanical or enzymatic means, which can induce undesired damage to the cells (Tatsumi et al. 2011). Thirdly, in contrast to monolayer cultures, where the extracellular microenvironment can be precisely controlled, differentiation of ESCs in three-dimensional EBs poses additional challenges, including inability to control EB size, creation of gradients of morphogens, metabolites, and nutrients, and ultimately variable EC yields and cell viability (Descamps and Emanueli 2012; Kane et al. 2011; Tatsumi et al. 2011).

3.3.2 Induced Differentiation of ESCs into ECs in 2D Culture

In principle, direct induced differentiation of ESCs into ECs in a two-dimensional (2D) culture system would be more advantageous than spontaneous EB-mediated approaches in several aspects. First, 2D culture parameters are easier to control than in 3D EB-based culture systems. Second, the simplicity and the cellular accessibility of the 2D culture systems enable easier implementation of supporting cells as well as uniform ESC exposure to inducing growth factors (Tatsumi et al. 2011). Third, 2D culture system allows for better exposure to specific extracellular matrix (ECM) proteins that are known to induce ESC differentiation (Gerecht-Nir et al. 2003).

Induced differentiation of ESCs into ECs was first reported in mice with a 2D co-culture system that used the bone marrow-derived OP9 mouse stromal cell line (Hirashima et al. 1999). Since then, other murine feeder cells have also been shown

to promote vascular differentiation, including mouse embryonic fibroblasts (MEF) and other stromal cell lines (Descamps and Emanueli 2012). In the murine system, the mechanisms controlling embryonic differentiation are better understood than in other animals. During development, murine ESCs first differentiate to Flk-1$^+$ mesodermal precursor cells (Yamashita et al. 2000), which later give rise to not only ECs, but also hematopoietic cells, vascular smooth muscle cells and cardiomyocytes (Kattman et al. 2006). Commitment of Flk-1$^+$ precursor cells towards EC lineage is promoted by VEGF, bFGF and BMP4 (Blancas et al. 2011; Sone et al. 2007; Yamashita et al. 2000), as well as by interaction with specific ECM proteins such as collagen IV, laminin and fibronectin (Blancas et al. 2011). However, in primates, ESC differentiation into vascular cells follows different steps. For example, unlike murine ESCs, undifferentiated human ESCs already express Flk-1 (also known as VEGF-R2). Thus, human mesodermal precursor cells are not only marked by the expression of Flk-1 but also by the loss of embryonic tumor rejection antigen 1-60 (TRA1-60) (Sone et al. 2007). These human TRA1-60$^-$/Flk-1$^+$ precursors can be efficiently differentiated into VE-cadherin$^+$ ECs and alpha-smooth muscle actin (α-SMA)$^+$ mural cells by exposure to either VEGF or PDGF, respectively (Sone et al. 2007).

In humans, early studies have demonstrated that direct, induced differentiation of ESCs in 2D culture can produce ECs morphologically and functionally indistinguishable to EB-derived human ECs (Sone et al. 2007). However, early co-culture protocols were proven inefficient, with yields of ESC to EC conversion around 1–5 % (Sone et al. 2007; Yamahara et al. 2008). To enhance efficiency, protocols have increasingly incorporated factors that promote vascular differentiation. This includes angiopoietin-1 (Ang1), which is involved in the differentiation of Flk-1$^+$ mesodermal precursors into ECs through angiopoietin receptor TIE-2 signaling (Joo et al. 2011). Another strategy to enhance ESC differentiation into ECs is the approach of inhibiting transforming growth factor (TGF)-β, which has increased the EC yield by tenfold (James et al. 2010). Another technique has been the sequential use of MAPK-MEK-ERK inhibitors and bone morphogenic protein-4 (BMP4), which has been shown to enable differentiation of human ESCs into ECs with 20 % efficiency (Park et al. 2010). Other approaches have included retinoic acid, hypoxia or mechanical force stimulation (Descamps and Emanueli 2012). Collectively, there are increasingly more sophisticated protocols to obtain efficient differentiation of human ESCs into ECs. However, controlling the differentiation of ESCs to achieve a homogenous population of EC, without contamination with other cell types, is still challenging with current protocols.

3.3.3 Differentiation of ESCs into ECs in Chemically Defined Systems

Many of the current protocols for inducing differentiation of ESCs into ECs use supporting feeder cells in addition to chemically undefined animal serum; however, these are drawbacks that will surely limit human clinical trails (Unger et al. 2008).

Thus, in recent years the field has been moving toward feeder-free and chemically-defined, serum-free systems (Yao et al. 2006). For example, Tatsumi and colleagues induced human ESCs to differentiate into ECs using a serum-free medium (StemPro-34 SFM) supplemented with a glycogen synthetase kinase-3b (GSK-3b) inhibitor and recombinant human VEGF165 for 5 days with up to 20 % efficiency (Tatsumi et al. 2011). In this study, the action of GSK-3b inhibitor was found to be critical for the differentiation of ESCs into Flk-1^{+} mesodermal precursors. More recently, Blancas et al. developed several chemically defined formulations to obtain homogeneous cultures of proliferating mouse ECS-derived ECs, achieving a superior differentiation efficiency and purity than previously attainable with serum-containing media (Blancas et al. 2011). Follow up studies with human ESCs are warranted. These are promising examples illustrating how progress in our understanding of the mechanisms controlling ESCs differentiation can be translated into more efficient means to obtain vascular cells with therapeutic potential. However, the future of ESC-derived cells in clinical therapies is still uncertain, and it will likely require overcoming not only technical hurdles, but also ethical considerations.

3.4 Induced Pluripotent Stem Cell-Derived Endothelial Cells

The discovery of methods to convert somatic cells into iPSCs through expression of a set of four transcription factors (Oct4, Sox2, Klf4 and c-Myc) created another possibility of producing patient-specific ECs for regenerative medicine (Takahashi and Yamanaka 2006). As with ESCs, iPSCs can potentially provide an unlimited number of pluripotent cells, which can subsequently generate ECs for vascular therapy. Moreover, patient specific, autologous ECs obtained from iPSCs will avoid allogenic immune rejection, which is one of the main concerns when considering ESCs (Wu and Hochedlinger 2011).

In principle, iPSCs can be obtained from a variety of somatic cell types. For example, iPSCs have been successfully obtained from terminally differentiated hepatocytes, keratinocytes, and B and T cells (Aasen et al. 2008; Aoi et al. 2008; Hanna et al. 2008; Staerk et al. 2010). From a clinical standpoint, the ideal sources of somatic cells for iPSC generation are those that cause minimal patient morbidity, including dermal fibroblasts and cells circulating in peripheral blood. However, multiple factors influence the efficiency by which somatic cells are converted into iPSCs, including tissue of origin, stage of cellular differentiation, and age of the individual donor (Gonzalez et al. 2011). Currently, there are multiple methods for reprogramming somatic cells into iPSCs that are being evaluated, with a focus on enhancing both safety (for example, the use of non-viral means to deliver genes, mRNA, or even proteins into somatic cells (Warren et al. 2010; Zhou et al. 2009)) and efficiency (new sets of crucial transcription factors (Yu et al. 2007); the use of inducing chemicals (Lin et al. 2009)).

The first proof-of-concept regarding generation of human iPSC-derived ECs came from the study of Taura et al. (2009). In this study, iPSCs were generated from

skin fibroblasts transduced with either four (Oct3/4, Sox2, Klf4 and c-Myc) or three (c-Myc was omitted) transcription factors (Taura et al. 2009). Induction of ECs was carried out in a 2D co-culture system that used the bone marrow-derived OP9 mouse stromal cell line and exogenous VEGF, similarly to the method previously described for ESCs (Taura et al. 2009). In fact, the mechanism by which iPSCs differentiate into ECs appears to be identical to that from ESCs, including the intermediate generation of TRA1-60$^-$/Flk-1$^+$ precursors (Taura et al. 2009). With this methodology, the efficiency of obtaining ECs from iPSCs was comparable to that of ESC-derived ECs. Additional studies have also shown that transplantation of iPSC-derived ECs into ischemic hindlimbs of immunodeficient mice were successfully incorporated into the host vasculature and significantly accelerated improvement in local blood flow (Rufaihah et al. 2011). Undoubtedly, there is a lot of excitement surrounding the prospect of obtaining patient-specific ECs from iPSCs. Despite the increasing number of encouraging studies, several hurdles still remain before iPSC-derived cells become a clinical reality, including the uncertainty about their potential tumorigenicity, the long-term consequences of potential genetic and epigenetic alternations, as well as issues regarding their immunogenicity (Barrilleaux and Knoepfler 2011; Wu and Hochedlinger 2011).

3.5 Postnatal Endothelial Progenitor Cells

For clinical applications, the presence of endothelial progenitor cells (EPCs) in circulation represents a promising opportunity to non-invasively obtain the required endothelial population. The presence of circulating ECs was first described five decades ago. Some of the first evidences came from observation of the endothelium in transplanted organs. For instance, Kennedy and colleagues observed that some of the ECs covering the coronary arteries in transplanted human hearts belonged to the recipient and not the donor, which suggested the possibility of these recipient ECs were originated from circulating cells (Kennedy and Weissman 1971). Nevertheless, until approximately 15 years ago, the consensus was that the formation of ECs from mesodermal angioblasts occurs only during embryonic development and not postnatally. This long-held notion began to change in 1997 when Asahara and colleagues described the isolation from peripheral blood of circulating CD34$^+$ progenitor cells with capacity to differentiate ex vivo into ECs (Asahara et al. 1997). These cells were referred to as “endothelial progenitor cells” (EPCs) and they were shown to express a range of EC markers as well as participation into neovascularization processes in models of induced ischemia (Asahara et al. 1999; Isner and Asahara 1999; Kalka et al. 2000; Takahashi et al. 1999). Rafii and colleagues also reported the existence of EPCs in the adult bone marrow and described these cells as a subset of CD34$^+$ cells that differentiate into cells that express von Willibrand factor (vWF) and uptake of Dil-Ac-LDL (Shi et al. 1998). The discovery of EPCs in peripheral blood was exciting because it suggested a promising opportunity to non-invasively obtain large quantities of autologous ECs for either therapeutic

vascularization or tissue engineering, both of which likely require some form of postnatal vasculogenesis. However, the process for obtaining EPC-derived ECs with the ability to form blood vessels in vivo has not been straightforward.

The characterization of EPCs traditionally relied on either the selection of cellular subpopulations from the mononuclear cell (MNC) fraction that circulates in peripheral blood or on the isolation of colony-forming cells culture. The majority of the original studies with circulating EPCs used three cell surface markers: CD34, CD133, and VEGF-R2 (also known as KDR). The first marker used to identify EPCs was CD34; in the context of EPCs, CD34 expression was used to define early stage EPCs because as it is present on hematopoietic cells, whereas CD34 expression is only present in early progenitor and stem cells. CD34 expression was also thought to diminish as EPCs differentiate into mature ECs. CD133 is a transmembrane protein present in 20–60 % of all $CD34^+$ cells in the bone marrow and peripheral blood, was also used to identify early stage EPCs, because this marker is absent in mature ECs. Finally, VEGF-R2, a tyrosine kinase receptor and member of the VEGF receptor family that is known to be expressed during embryonic vasculogenesis as well as by mature ECs, was also proposed as a marker of circulating EPCs. However, it is now known that far from being definitive EPC markers, most of these cellular markers are shared by subpopulations of hematopoietic cells that can be mobilized into circulation from the bone marrow to home sites of neovascularization (Rafii and Lyden 2003; Yoder et al. 2007).

Although the hematopoietic and endothelial cell types are fundamentally different, many studies continued to refer to blood- or bone-marrow–derived adherent cells that express progenitor and endothelial markers such as CD34, CD133 and VEGFR- 2^+ cells as EPCs (Prater et al. 2007). In subsequent years, additional cell markers were proposed to characterize circulating EPCs, including vWF, VE-Cadherin (CD144), and CXCR4. However, to date there is no consensus about a single set of markers that unequivocally identify EPCs in circulation. Thus, it is not surprising to find multiple studies in which EPCs were associated with different cellular subpopulations, including subsets of hematopoietic cells (Richardson and Yoder 2011). Despite this ambiguity, the functional abilities of putative EPCs have been examined over the last few years and are increasingly better understood. For example, Yoder and colleagues demonstrated that most of the cells that were long referred to as EPCs are in fact descendants of hematopoietic stem cells (HSCs); the cells express functional activities of myeloid cells, including paracrine secretion of pro-angiogenic factors, but have no ability to differentiate into functional ECs in perfused blood vessels in vivo (Yoder et al. 2007). Other studies have also shown that fractions of $CD34^+$ and $CD34^+/CD133^+$ cells from adult peripheral blood and umbilical cord blood are enriched by hematopoietic progenitor cells with no potential to participate, structurally, as ECs in postnatal vasculogenic processes (Richardson and Yoder 2011). Case and colleagues described that only circulating cells expressing CD34, CD31, and CD144, in umbilical cord blood were able to generate ECs in culture, but not those expressing CD45 and CD133 (Mund et al. 2012).

In addition to this dispute about surface markers, EPCs with bona fide blood vessel–forming ability seem to comprise a very small population of the circulating

cells. Judging by the number of EC colonies obtained in culture, EPCs are found at a concentration of about 2–5 cells/ml in human umbilical cord blood, and at a concentration of about 0.05–0.2 cells/ml in adult peripheral blood (Ingram et al. 2004). Both the low frequency of EPCs in circulation and the lack of a unique set of distinctive cellular markers have made the isolation of EPCs by flow cytometry or other immunological techniques very challenging. As a result, the most successful methodology for isolating EPC-derived ECs is still based on methods similar to those originally reported for endothelial outgrowth from peripheral blood (Lin et al. 2000). However, some authors still question whether the cells that appear in these colonies from MNC cultures (ex vivo) are indeed related to those circulating EPCs in vivo (Fadini et al. 2012). In any case, based on cell culture assays, there have been two main subpopulations of cells from peripheral blood associated with EPCs: (a) Circulating angiogenic cells or early EPCs, and (b) Endothelial colony-forming cells (ECFCs) or late EPCs.

3.5.1 Circulating Angiogenic Cells or Early EPCs

The most widely studied subpopulation of EPCs, generated by culturing peripheral blood mononuclear cells on fibronectin for 4 days in VEGF-containing medium, do express the pan-hematopoietic marker CD45 as well as myelomonocitic markers such as CD14 and CD11b (Urbich et al. 2003). Over the last decade, these cells with hematopoietic features have been referred to as "colony-forming units-ECs" (CFU-ECs) (Gehling et al. 2000), "circulating angiogenic EPCs" (Rehman et al. 2003), "early EPCs" (Gulati et al. 2003; Hur et al. 2004), and "colony-forming units- Hill (CFU-Hill)" (Hill et al. 2003). Despite expression of hematopoietic markers, early EPCs also co-express EC markers; however, the presence of EC markers has been disputed because it might result from a contamination with microparticles deriving from other elements in the culture (such as platelets). In fact, naming these cells "EPCs" has been often criticized, and many authors now prefer terms such as "circulating angiogenic cells" that reflects their ability to promote angiogenesis in vivo, without assuming endothelial commitment (Fadini et al. 2012).

One of the original goals following the discovery of early EPCs was to evaluate their therapeutic potential (Fadini et al. 2008). In this regard, early EPCs were shown to facilitate the revascularization of ischemic tissues. For example, Isner and colleagues demonstrated that intravenous infusion of cultured human EPCs into athymic nude mice with hindlimb ischemia markedly improved blood flow recovery and capillary density in the ischemic hindlimb, significantly reducing the rate of limb loss (Kalka et al. 2000). Nevertheless, the mechanisms by which revascularization occurred was not fully understood at the time. Potential mechanisms for EPC-induced revascularization included an increased supply of ECs via proliferation and endothelial differentiation of EPCs or an increased supply of growth factors to activate resident mature endothelial cells. Rehman et al., demonstrated that acetylated-LDL^{+}/ulex-lectin^{+} early EPCs do not proliferate but instead release potent

proangiogenic growth factors and that the majority of these EPCs are derived from monocyte/macrophages (Rehman et al. 2003). These findings of low proliferation and endothelial differentiation suggested that the angiogenic effects observed after EPC infusion were most likely mediated by growth factor secretion. Gulati et al. also reported that the vast majority of EPCs arose from a $CD14^+$ subpopulation of peripheral blood MNCs (Gulati et al. 2003). More recently, Yoder et al., demonstrated that early EPCs are in fact descendants of HSCs and express functional activities of myeloid cells, including paracrine secretion of pro-angiogenic factors (Yoder et al. 2007). Collectively, the therapeutic potential of early EPC transplantation in promoting tissue vascularity is well established. However, early EPCs have low proliferation potential and therefore their therapeutic use may be limited by the absolute numbers of EPCs that can be obtained from patients (Murasawa and Asahara 2005). In addition, early EPCs do not give rise to mature ECs with structural lumen-forming capabilities, which limits their use in tissue engineering and other vascular therapies that require functional ECs.

Despite these limitations, circulating levels of early EPCs are considered as biomarkers for coronary and peripheral artery disease. The level of circulating $CD34^+$/KDR^+ EPCs has been shown to predict the occurrence of cardiovascular events and death from cardiovascular causes; thus it may help to identify patients at increased cardiovascular risk (Werner et al. 2005). In fact, there are now multiple studies that have demonstrated that early EPCs are reduced in the presence of classic cardiovascular risk factors, including smoking, hypertension, hypercholesterolemia, obesity, and diabetes (Fadini et al. 2012). Therefore, although early EPCs may not be a source of cells to generate autologous ECs needed for therapy, they appear to provide a useful index of cumulative cardiovascular risk and vascular function (Rosenzweig 2003).

3.5.2 Endothelial Colony-Forming Cells (ECFCs) or Late EPCs

Simultaneously to the identification of early EPCs, additional studies with long-term cultures of blood MNCs in the presence of VEGF yielded outgrowing cells with a more mature EC phenotype (Lin et al. 2000). These cells displayed a different morphology and proliferation pattern than the spindle-shaped early EPC; they formed colonies of cobblestone-shaped endothelial-like cells with a striking proliferative capacity. These cells have been referred to as "late outgrowth ECs" (Lin et al. 2000), EPCs (Kaushal et al. 2001), "late EPCs" (Hur et al. 2004), and "endothelial colony-forming cells" (ECFCs) (Ingram et al. 2004) (We have herein referred to these cells as ECFCs).

ECFCs comprise a very small population of the circulating cells in peripheral blood. Judging by the number of colonies in culture, ECFCs are found at a concentration of about 2–5 cells/ml in human umbilical cord blood, and at a concentration of about 0.05–0.2 cells/ml in adult peripheral blood (Ingram et al. 2004). Both the low frequency of ECFCs in circulation and the lack of a unique set of distinctive

cellular markers have made the isolation of ECFCs by flow cytometry or other immunological techniques very challenging (Mund et al. 2012). As a result, the most successful methodology for isolating ECFCs is based on methods similar to those originally reported for endothelial outgrowth from peripheral blood (Lin et al. 2000). ECFCs are organized in a hierarchy of progenitor stages that vary in proliferative potential and can be identified in clonal plating conditions (Ingram et al. 2004). The expansion potential of ECFCs is enormous although it varies between cord blood and adult blood; while cord blood-derived ECFCs can be routinely expanded in culture for over 70 population doublings, the lifespan of adult blood-derived ECFCs is significantly shorter (20–40 population doublings) (Ingram et al. 2004; Lin et al. 2000; Melero-Martin et al. 2007). The proliferative rate of ECFCs is equally remarkable; for example, we demonstrated that 10^{11} homogeneous ECFCs can be obtained from 25 mL cord blood after just 30 days in culture and 10^{8} ECFCs from 50 mL adult peripheral blood (Melero-Martin et al. 2007). These cell numbers are likely to exceed- in the case of cord blood-and be sufficient- in the case of adult blood- what would be needed for most autologous therapies, confirming peripheral blood as a robust source of ECs. Moreover, the presence of ECFCs in peripheral blood drawn from patients with significant coronary artery disease (CAD) has also been confirmed (Stroncek et al. 2009), although whether these CAD patient-derived ECFCs have the same clinical potential as those obtained from healthy adults remains to be elucidated.

For clinical applications, one critical requisite will be to isolate defined populations of cells. In this regard, the endothelial phenotype of ECFCs has been confirmed in multiple studies (Lin et al. 2011; Melero-Martin et al. 2007; Yoder et al. 2007). Essentially, ECFCs have been shown to express all the markers expected for ECs, including VE-Cadherin, CD31, vWF, CD34, VEGF-R2, and CD105. Moreover, the specific localization of surface markers such as CD31 and VE-cadherin at the cell-cell borders and vWF in a punctuate pattern in the cytoplasm, confirm the endothelial nature of these cells (Melero-Martin et al. 2007). ECFCs also uptake low density lipoproteins (e.g., Ac-LDL) and bind specific lectins (e.g., Ulex europaeus, UEA), as expected from ECs. Additionally, multiple studies have shown that ECFCs are consistently negative for mesenchymal markers such as CD90 and hematopoietic markers such as CD45 and CD14, confirming that these cells are not contaminated with either mesenchymal or hematopoietic cells (Lin et al. 2011; Melero-Martin et al. 2007; Yoder et al. 2007). Furthermore, ECFCs maintain the expression of endothelial markers through prolonged periods in culture (Melero-Martin et al. 2007), indicative of a robust source of ECs.

In addition to a stable phenotype, ECFCs have also been shown to function as ECs. For example, ECFCs respond to EC growth factors (e.g. VEGF, bFGF) by increasing their proliferation and migration in culture (Lin et al. 2011; Melero-Martin et al. 2007). Also, ECFCs can up-regulate leukocyte adhesion molecules and increase the adhesion of leukocytes upon exposure to inflammatory cytokines (e.g., TNF-α), indicating ability to regulate physiologic proinflammatory properties (Lin et al. 2011; Melero-Martin et al. 2007). Finally, ECFCs are able to launch angiogenic sprouts and to assemble into capillary-like structures in 3D cultures (Lin et al.

2012). Collectively, these functional properties of ECFCs are indicative of their bona fide endothelial nature, and can be used to distinguish ECFCs (true ECs) from early EPCs (hematopoietic cells).

The endothelial functionality of ECFCs has also been repeatedly demonstrated in vivo. This includes the feasibility of using ECFCs to endothelialize cardiovascular grafts, which has been shown in multiple animal models. First, autologous ovine ECFCs were expanded ex vivo and seeded on decellularized porcine iliac vessels that were then implanted as a carotid interposition graft in sheep and demonstrated adequate patency and arterial function in vivo for 130 days (Kaushal et al. 2001). Subsequent in vivo studies have further proven the non-thrombogenic properties of ECFCs using a diversity of vascular grafts (Stroncek et al. 2011). More recently, we and others have demonstrated additional vasculogenic properties of ECFCs in vivo (Au et al. 2008; Melero-Martin et al. 2007, 2008; Yoder et al. 2007). For example, we showed that combining both human ECFCs and mesenchymal stem cells (MSCs) results in formation of robust functional microvascular networks in murine models of human cell transplantation (Melero-Martin et al. 2008). Evaluation of implants after 7 days revealed an extensive network of human blood vessels containing erythrocytes, indicating the rapid formation of functional anastomoses within the host murine vasculature. The implanted ECFCs were restricted to the luminal aspect of the vessels, which remained patent after 4 weeks in vivo. This rapid formation of long-lasting microvascular networks by blood-derived ECFCs constitutes an important step forward in the development of clinical strategies for tissue vascularization (Melero-Martin et al. 2008). Similar studies have demonstrated additional characteristics of ECFC-lined microvessels in vivo. For example, Jain and colleagues showed that microvessels that were formed in vivo using cord blood-derived ECFCs were similar to normal vessels in several aspects: they had normal blood flow, selectively regulated permeability of macromolecules, and were able to induce leukocyte-endothelial interactions in response to cytokine activation (Au et al. 2008).

In summary, the existence of postnatal circulating endothelial progenitor cells (late EPCs) in peripheral blood that can give rise to large number of autologous ECs (ECFCs) is now well recognized. Human ECFCs has been extensively interrogated over the last decade in multiple studies that have undoubtedly demonstrated their endothelial phenotype and ability to function as bona fide ECs both in vitro and in vivo. Thus, obtaining ECFCs from peripheral blood constitutes a promising means to generate, in a non invasive manner, autologous ECs for clinical vascular therapies.

3.6 Conclusions

ECs line the lumen of all blood vessels in the body and are central cellular players with multiple physiological functions. For decades the study of EC biology has been conducted with cultures of mature ECs obtained from living vasculature. However, future vascular cell therapies will likely require a large number of

autologous ECs with greater proliferative capacity than mature ECs. Blood-derived ECFCs represent a robust population of cells with an enormous capacity to proliferate, a clear endothelial phenotype, and an ability to function as bona fide ECs both in vitro and in vivo. However, to date, most studies of human ECFC transplantation are preclinical, using a variety of animal models. For years, the lively debate and controversies concerning the exact definition, origin, and function of ECPs has hampered the implementation of ECFCs into clinical trials. Nevertheless, with increasingly more laboratories involved in the study of these cells, the consensus that ECFCs constitute a robust source of autologous ECs is now stronger than ever. Certainly, there is great expectation to see how new vascular cell therapies develop in the next few years, and blood-derived, autologous ECFCs will likely play a role in future clinical applications.

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Chapter 4
Bioprocessing of Human Pluripotent Stem Cells for Cell Therapy Applications

Margarida Serra, Cláudia Correia, Catarina Brito, and Paula M. Alves

Abstract Human pluripotent stem cells (hPSCs), with their unique characteristics for indefinite proliferation and pluripotency, are an appealing source for cell replacement therapies, tissue engineering, drug discovery and *in vitro* toxicology. For the clinical implementation of these cells, there is the need for translating the culture protocols developed at research laboratories into validated bioprocesses that can guarantee reproducibility, scalability, standardization, robustness and safety.

The most attractive strategy for hPSC manufacturing consists in engineering stem cell niches by identifying key factors governing hPSC cell fate and creating culturing approaches that allow for 3D cell organization in a bioreactor-based system where the key environmental conditions are finely controlled. This chapter provides an overview of current bioengineering strategies that could be used to generate large numbers of hPSCs and/or their derivatives with potential application in regenerative medicine and drug discovery.

Keywords Human pluripotent stem cells • Cell therapy • Bioreactors • 3D culturing approaches • Integrated strategies for manufacturing of cell-based products

4.1 Introduction

Advances in stem cell research in recent years have brought stem cell applications in regenerative medicine closer to reality. More specifically, human pluripotent stem cells (hPSCs), including embryonic and induced pluripotent stem cells (hESCs and

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hiPSCs, respectively) have garnered a lot of attention owing to their ability to proliferate indefinitely (self-renewal) and differentiate into all mature cells of the human body (pluripotency). These unique properties have opened opportunities not only for novel stem cell-based regenerative medicines, but also for development of drug discovery platforms and promising *in vitro* model systems for the study of early human development.

With "large-scale" applications of hPSCs in the horizon, strong business opportunities exist today both for companies looking to commercialize hPSC-based products as well as to pursue hPSC-based therapies. However, few clinical trials have been carried out so far. A broad disappointment was faced when Geron aborted the world's first clinical trial testing a hESC-derived therapy targeting spinal cord injury in November 2011 (www.geron.com), 1 year after the launch of the trial. Currently, there are only two ongoing clinical trials being conducted by Advanced Cell Technology, aiming at treating genetic eye disorders (Stargardt's Macular Dystrophy and Advanced Dry Age Related Macular Degeneration) with hESC-derived products. What is still delaying a more rapid implementation and/or investment of/in hPSC-based therapies?

A major challenge in this field is the lack of expertise in product development and specialized cell manufacturing and processing that are imperative to bring hPSC-based products to the clinic/market. This chapter reviews recent progress and challenges in the manufacturing of hPSC-based products for clinical application. Particular focus is given to the identification of essential requirements for the implementation of more robust and integrated hPSC manufacturing platforms. The impact of environmental factors, 3-D culturing approaches and bioreactor-based technologies for controlling bioprocess outcomes and product quality are also discussed.

4.2 Critical Needs in the Manufacturing of Stem Cell-Based Therapies

A critical factor in hPSC bioprocessing is to translate the culture protocols developed in research laboratories into clinically applicable manufacturing designs. These platforms must be affordable, reproducible, predictable and clinically effective, while complying with Good Manufacturing Practice (cGMP) requirements. The major challenges include selection of the cell source (allogenic *versus* autologous), production of the required cell numbers (Scale-up *versus* Scale-out), control of cell differentiation process to generate only the cell population required (purity), while assuring the desired phenotype, potency and function (quality), followed by efficient formulation for application (i.e. storage, delivery and administration). These stringent demands require a close communication between fundamental research (from developmental biology to "omics" technologies and advances in immunology) and existing industrial practice (biologists and bioprocess engineers), essentially on automation, quality assurance and regulation.

4.2.1 *Stem Cell Source: Allogenic* Versus *Autologous*

Today, the industry continues to debate whether allogeneic or autologous therapies will ultimately be most successful. Although both past and ongoing trials have been using hESCs derived from allogenic sources, there are still some concerns that need to be tackled so that their use in the clinic can be perfected. In addition to ethical issues related to the manipulation of human embryos, hESC derivatives present a high risk of being rejected by patient's immune systems. Several strategies have been proposed to reduce or prevent the immune response, including the establishment of large banks of immunophenotyped hESC lines, the creation of an universal donor cell by genetic modification, the induction of tolerance by hematopoietic chimerism as well as cell encapsulation in clinically approved biologically innert biomaterials (Hentze et al. 2007; Lui et al. 2009). The advances in cell reprogramming have also sparked hope that hiPSCs might provide a clinical alternative to hESCs, assuring safer patient-specific (autologous) therapies. However, it is important to highlight that autologous hiPSCs are not suitable for the treatment of genetic diseases, requiring additional genetic manipulation steps to produce gene corrected hiPSCs for the specific therapy (Collin and Lako 2011). Moreover, these personalized hiPSC-based therapies face considerable challenges in what concerns the low efficiency yields of reprogramming process and time and costs associated to the cell manufacturing (Brignier and Gewirtz 2010), i.e. the whole process (isolation, reprogramming, expansion, differentiation and purification) will be too expensive for patients and will require long time for cell production and assessment of their medical stability, safety, and efficacy until further administration. Therefore, the establishment of a MHC-typed bank of hiPSCs for allogenic cell transplantation therapies (Taylor et al. 2005) represents a more realistic clinical scenario in the near future. Recently, researches from the Boston University School of Medicine showed that tissues derived from iPS cells in an experimental model were not rejected when transplanted back into genetically identical recipients (Araki et al. 2013), potentiating the use of hiPSC in the clinic. Overall, each type of therapy (autologous and allogenic) faces unique challenges (Fig. 4.1) and evaluating them prior to manufacturing is critical for decisions regarding the appropriate scale, strategy and methodology for up- and down-stream processing and storage, as discussed in future sections.

4.2.2 *Quantity: Scale-Up Versus Scale-Out*

Determining the final scale of production and manufacturing is related to the type of therapy and clinical phase. The production of allogeneic cell-based products is amenable to scale-up (Fig. 4.1), taking advantage of specific bioprocess technology. Manufacturing of stem cells for allogeneic therapies commonly involves establishing and qualifying master and working cell banks (cryopreserved cells), then producing large lots of product for release testing. As in classical biopharmaceutical

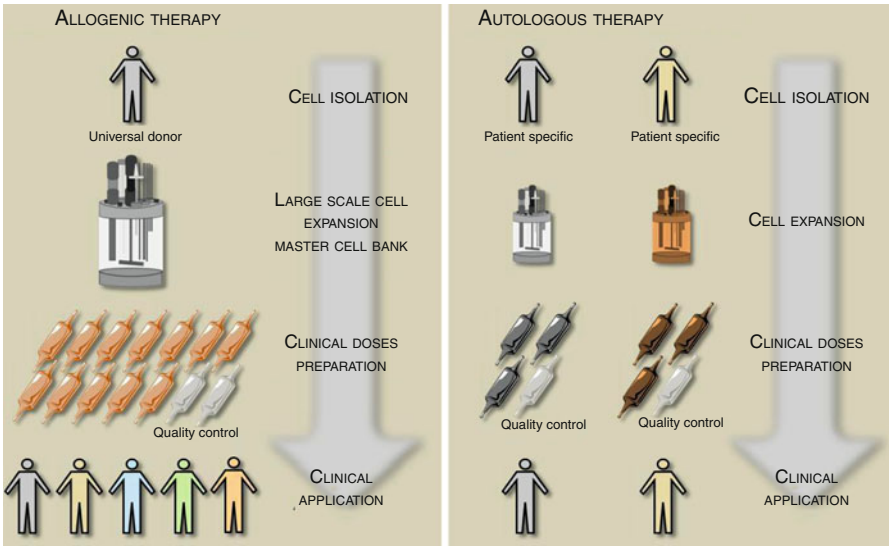


Fig. 4.1 Bioprocessing challenges of allogeneic and autologous therapies. Allogeneic processes generally include master cell banks and large-scale systems and several lots of patient doses. In autologous processing, every lot manufactured is meant to treat only a single patient, and a high portion the final product is lost to quality control tests

manufacturing, the process is scaled up during clinical development, and release testing may be performed on lots that represent hundreds or thousands of product doses (Fig. 4.1). The major challenges in allogeneic manufacturing are (i) maintaining cell product quality and potency while scaling up its production, and (ii) controlling the cost of goods (CoGs). Process scale-up can occur at any point during clinical development stages. However, the regulatory burden incurred when applying such changes increases as a product progresses through the clinical pathway. For example, in early stage trials the regulatory entities may request only comparability studies based on product characterization data; in later stages, clinical bridging studies are required. Therefore, it is imperative to establish and optimize scalable manufacturing processes at early stages, before beginning of phase 3 clinical trials, in order to assure that product quality would not be compromised when transferring to larger scale systems.

On the other hand, production of autologous, patient-specific cell therapy products is not amenable to a scale-up approach, but rather to a scale-out platform in commercial manufacturing (Fig. 4.1). To achieve efficiencies of scale, high-throughput production by small-batch and parallel processing of multiple, separate products in automated and closed systems should be adopted (Brandenberger et al. 2012). Functionally closed process technology provides product and process isolation, maintaining each product entirely within its own separate and sterilized disposable processing set. Such systems are highly amenable for automation and typically associated with extremely low rates of contamination, as described below

(Sect. 4.3.3.4). The major challenge in manufacturing autologous cell therapy products is indeed the logistics associated with handling parallel and similar closed bioprocesses at the same time as well as the greatest potential for product/process variability, since each product is derived and meant to treat only a single patient.

In general, the numbers of hPSCs-derived cells required for effective therapy fall in the range of thousands/millions to few billions, depending on the therapeutic target (Tzanakakis et al. 2000; Lindvall et al. 2004; Lock and Tzanakakis 2007; Jing et al. 2008). For example, for the treatment of Parkinson's disease, it has been proposed that the yield of transplanted cells should allow for at least 1×10^5 grafted dopaminergic neurons to survive (over the long term) in the human putamen (Lindvall et al. 2004). On the other hand, for the replacement of damaged cardiac tissue after myocardial infarction or for the treatment of Type I diabetes, approximately $1–2 \times 10^9$ cells will be required for each patient (50–100 kg) (reviewed in Serra et al. 2012). Since the hPSC volumetric productivities reported by our group (Serra et al. 2010, 2011) and others (reviewed in Serra et al. 2012) fall in the range of $0.1–3 \times 10^6$ cell/mL, the production of cell-based products for autologous and allogeneic cell therapies would require batch sizes with working volumes of 0.1–1 L and 2–20 L respectively, although issues related to the respective efficiencies of differentiation and yields of downstream processing (e.g. for purification and selection of a specific cell type, for cell-microcarrier separation) should be considered as well.

4.2.3 Purity

Unlike traditional pharmaceutical products, strict standards for the purity of cell-based products might not be realistic and might even be undesirable in some cases, where a mixture of several cell types may be necessary to achieve the desired therapeutic effect (Lamba et al. 2010). Thus the first issue is the definition of product purity in a case-by-case approach: which cells "contribute to the beneficial effect" and which cells are "impurities"? Although more insights about the type of cells that "contribute to the beneficial effect" are required, it is well known that undifferentiated hPSCs present tumorogenic potential and are capable to generate tumors in the body after transplantation (Zhao et al. 2011). Therefore, the development of strategies to efficiently eliminate these "impurities" will be essential to allow safe utilization of hPSC derivatives. Recently, efficient methods for the removal of undifferentiated hPSC have been proposed, including the combination of immunodepletion tools (fluorescence-activated cell sorting) with antibodies against SSEA-5 and additional pluripotency surface markers (Tang et al. 2011) and the use of cytotoxic antibodies (Schriebl et al. 2012) or antibodies fragments (Lim et al. 2011) that specifically bind and kill hPSCs in their undifferentiated state.

In addition, the presence of foreign particulate matter, such as microcarriers fines, is unacceptable for injectable products and should be removed prior to administration. These and other major challenges of stem cell purification and downstream processing are discussed in Chap. 8.

4.2.4 Quality

In cell therapy manufacturing it is essential to understand the nature of the product being administered to patients and to ensure that it is the same every time. Essentially, this includes defining the biological aspects (the critical quality attributes- CQAs) of a cell-based product, namely phenotype and potency. Cell genotype and functionality also need to be monitored and controlled throughout the entire bioprocess. Therefore key tests for quality control analysis are necessary at each stage: undifferentiated stem cells have to maintain their pluripotency as well as genetic and epigenetic stability after expansion (as do cells from the master and working cell banks), while stem cell derivatives must express markers of the desired cell lineage and be fully functional after differentiation and purification. Moreover, to develop clinical-grade cells, all procedures (e.g. isolation, reprogramming, expansion, differentiation, purification, cryopreservation) and components (e.g. matrices, culture and cryopreservation media, supplements) must follow the Food and Drug Administration (FDA) and European Medicines Agency (EMA) regulations (Unger et al. 2008). Within this context, six clinical-grade hESC lines were already submitted to the UK Stem Cell Bank, offering optimal defined quality and safety necessary for cell transplantation applications (Crook et al. 2007). For hiPSC lines, there are additional drawbacks associated to the protocols used for reprogramming (usually carried out by genetic modifications using viral vectors). At techniques for the generation of safer iPSC lines are improving including the use of non- integrating viral vectors (e.g. adenovirus or baculovirus), exogenous plasmids, protein factors, small molecules or microRNAs (Selvaraj et al. 2010), the potential for hiPSC-based therapies is rising.

4.3 Process Engineering of hPSCs for Clinical Application

The level of complexity for cell-based products is significantly high, requiring robust bioprocesses that should be designed according to pertinent principles (Fig. 4.2). The most effective strategy for manufacturing of hPSC-based products

Stem cell bioprocessing		
Components	Requirements	Goals
• **Stem cells** • **Environmental factors** • **Culturing approaches** • **Bioreactors**	Scalable; affordable; time-saving; automation; bioprocess characterization monitoring and control; regulated; validated; quality assurance; harvesting, storage and transportation	• **Process** Integration, longevity, reproducibility, efficiency, productivity • **Product** Quantity, quality, purity

Fig. 4.2 Design principles for stem cell bioprocessing (Adapted from Placzek et al. (2009))

consists in identifying key factors governing hPSC fate and engineering culturing approaches that allow for 3D cell organization in a bioreactor-based system where key environmental conditions are monitored and rigorously controlled. In this chapter, the importance of these process components on the design of stem cell manufacturing platforms will be presented, highlighting the main requirements needed to fulfill end products' purity, quality and quantity (Fig. 4.2).

4.3.1 Environmental Factors Controlling Stem Cell Fate and Bioprocess Outcome

The fate of stem cells is highly dependent on cues that lie in their extracellular environment. These cues operate on different temporal and spatial scales, driving specific cellular behaviours, ultimately controlling cell proliferation, differentiation or apoptosis. During the last decade, efforts have been made to identify such *stimuli*; (i) extracellular matrix, (ii) soluble factors, (iii) cell-cell interactions, (iv) physical forces and (v) physiochemical conditions have been pointed as relevant cues impacting on the fate of hPSC and consequently on the outcome of hPSC bioprocesses (Azarin and Palecek 2010).

Extracellular Matrix

Extracellular matrix (ECM) is a key component of the stem cell niche *in vivo* and can influence stem cell fate by mediating cell attachment and migration, presenting chemical and physical cues, as well as binding soluble factors. In a natural setting, this environment encloses a complex and dynamic network of proteins, polysaccharides, proteoglycans and water that provide structural and organizational guides for tissue development. The activation of these signaling pathways through the adhesion of specific components of the ECM to cells via integrins/cadherins/cell surface receptors is not trivial as it is highly dependent on the composition, orientation and structure of the ECM (Lukashev and Werb 1998).

The current gold standard for the culture of hPSCs requires the use of Matrigel or feeder cells (mouse embryonic fibroblasts, human foreskin fibroblasts). However, these matrices are complex, poorly-defined and, in some cases, xenogenic and thus, substantial efforts have focused in developing defined substrates for hPSC cultivation, including human recombinant proteins (Rodin et al. 2010) and synthetic substrates (Kolhar et al. 2010; Melkoumian et al. 2010; Villa-Diaz et al. 2010). At least two matrices composed of well-defined and xeno-free components are commercially available (CELLstart™ from Invitrogen, and StemAdhere™ from Stem Cell Technologies). Research is ongoing into designing surface-engineered substrates based on synthetic materials including Synthemax® (from Corning) (Li et al. 2013; Jin et al. 2012) or UV/ozone radiation (Saha et al. 2011), providing attractive

xeno-free standardized and reproducible cell culture platforms for the scalable production of clinically relevant hPSCs.

Soluble Factors

The outcome of stem cell culture depends also on the presence/concentration of growth/differentiation factors which provide survival, proliferation and differentiation signals to the cells. These regulatory molecules can be either added to the culture or secreted by the cells. Upon diffusion through the medium, these factors are sequestered by the ECM and bind to the cell surface receptors thus activating cellular functions. Although we are moving from the "growth factor lottery" that ruled SC media development in the last decade due to the intensive research on the signaling pathways governing hPSC expansion/differentiation (Azarin and Palecek 2010), the production costs and growth factor low stability in culture media are major hurdles for scale-up.

Potential strategies for reducing the concentration of these compounds without compromising the culture outcome include engineering approaches for the design of more stable molecules together with the development of appropriate culture operation modes such as the adoption of fed-batch and perfusion strategies (Serra et al. 2010). Alternatively, these factors can be immobilized on the surface of biomaterials (Ferreira et al. 2007) or even encapsulated in nanoparticles (Maia et al. 2011) aiming at achieving a better control of the cellular microenvironment. Attempts have also been performed regarding the use of small molecules that can be isolated/synthesized economically; with the advent of high-throughput screening technologies, small molecule libraries have been screened to identify molecular interactions leading to particular stem cell behaviors and specific culture outcomes (Ao et al. 2011; Burdick and Watt 2011).

Cell-Cell Interactions

Cell-to-cell communication, either *in vivo* or *in vitro*, can be established via direct contact (juxtacrine communication) or over distance via the diffusion of soluble signals secreted from closer (paracrine signaling) or distant (endocrine signaling) neighboring cells. While juxtacrine cell-cell communication provides a persistent morphogenic cue, allowing the precise control of cellular responses, paracrine signaling is normally time-constrained. The extent of such limitation is dependent on the spatial distance between proximal population of cells. This occurs because signaling molecules may: (1) degrade very quickly, limiting their effectiveness; (2) be taken by cells rapidly, leaving few to travel further, thus creating a heterogeneous environment where cells are exposed to different concentration gradients; (3) have their diffusion hindered by the ECM. These different cell-cell interactions drive a set of stem cell responses, from the induction of programs of differentiation (Tsai and McKay 2000) to the promotion of proliferation and self-renewal properties

(Purpura et al. 2004). In fact, one of the major requirements for the cultivation of hPSCs is the need for tight controlling of cell-cell interactions. It is well established that large colonies present high levels of spontaneous differentiation while individual hPSCs or small clumps do not grow efficiently (Bauwens et al. 2008). In the last years, scalable propagation of hPSCs has been hindered by poor cell survival after enzymatic dissociation into single cells but efficient strategies have recently been proposed to decrease dissociation-induced apoptosis, including the overexpression of anti-apoptotic proteins (Ardehali et al. 2011; Bai et al. 2012) and the combination of heat shock treatment with the use Y-27632, a specific inhibitor for Rho-dependent protein kinase (ROCK) (Singh et al. 2010). Paracrine factors secreted from other cell types have also been reported to drive a set of stem cell responses, from the promotion of proliferation/self-renewal properties (Lee et al. 2011) to the induction of directed differentiation processes (Ramos-Mejia et al. 2011). Therefore, the combination of inoculation strategies with cell concentration values and/or co-cultivation approaches are important parameters that require accurate optimization, aiming at boosting bioprocess yields.

Physical Forces

A number of *in vivo* and *in vitro* studies have demonstrated that physical forces (e.g. hydrodynamic/hydrostatic, mechanical and electrical) play a key role in the development of tissues and organs during embryogenesis as well as their remodeling and growth in postnatal life. Moreover, it has been found that stem cells are sensitive to fluid flow-induced shear stress, compressive and tensional strains, cyclical stretching hydrostatic pressures (Discher et al. 2009). For example, it has been demonstrated that centrifugal forces up to 1,000 g cause shifts in phenotype and proliferation of ESCs (Veraitch et al. 2008). The results of mechanical stress, caused by stirring, on different hPSC cultures are cell line specific (Leung et al. 2011). Moreover, shear sensitive cell lines spontaneously differentiate even in the presence of cell protective polymers, compromising cell growth (Leung et al. 2011). In another study, the authors reported that the application of shear stress to cells both with and without LIF influenced the expression of murine ESC pluripotency markers including Rex-1, Sox-2 and Nanog (Gareau et al. 2012). Further studies on the impact of these physical forces on cell functionality will be needed. Since scalable culture systems often employ perfusion or mixing that can apply mechanical forces to the cells, detailed characterization of these forces will be very important for the design of efficient and adequate bioreactor-based strategies for the manufacturing of cell-based therapies.

Physiochemical Conditions

Although typically cultivated inside incubators operated at standard values of temperature (37 °C), oxygen (20 %) and pH (7.4), several studies have shown that hPSC

expansion/differentiation potential can be enhanced under different operating conditions. Few studies have been conducted on the effect of temperature and pH in hPSC culture so far. For example, it was shown that the extended exposure of hESC cultures for 1–3 h to ambient conditions (resulted in a rapid drop in temperature and rise in pH) inhibits cell proliferation and reduces Oct-4 expression levels (Veraitch et al. 2008). On the other hand, with hPSC cultures, reducing oxygen concentration towards physiological levels (hypoxia 2–6 % of oxygen) is beneficial for maintaining cell pluripotent status; stem cells self-renewal is supported (Serra et al. 2010), spontaneous differentiation is reduced (Ezashi et al. 2005) and karyotypic integrity is maintained (Forsyth et al. 2006), in contrast with normoxia conditions (20 % oxygen). One major problem in the field is the lack of recognition that the oxygen experienced by the cells (pO_{2cell}) is often different from the oxygen in the gas phase (pO_{2gas}), which makes interpretation of the literature difficult. In fact, pO_{2cell} can differ drastically from pO_{2gas}, as it depends on the cell density, culture system (static, stirred, suspension), cellular oxygen consumption rate and oxygen transfer rate in the culture (reviewed in Millman et al. 2009). Efforts to estimate and control pO_{2cell} (Powers et al. 2010; Serra et al. 2010) are essential for better clarification of the role of oxygen on hPSCs' fate.

4.3.2 Robust Strategies for hPSC Bioprocessing: Moving Stem Cells from 2D Monolayers to 3D Culturing Approaches

hPSCs are traditionally cultured in 2-D systems (e.g. Petri dishes, culture flasks and well plates). These cells are usually propagated as colonies on top of a feeder layer of inactivated fibroblasts. Over the last years, inadequacy of conventional 2-D culture systems in resembling the *in vivo* developmental microenvironment has been observed in both basic biology and tissue engineering studies. In fact, tissue-specific architecture, mechanical and biochemical cues, cell-cell and cell-matrix communications are lost under such simplified and highly biased conditions. The inherent uncontrollability, heterogeneity and low production yields associated with these systems have made them unattractive and unsuitable for clinical and industrial applications.

From an industrial perspective, the most robust and amenable way to produce cell-based products would consist on the cultivation of hPSC as a single cell suspension. Although promising results were reported with murine ESCs (using either gene knockout (Ecad-/) or the neutralizing antibody DECMA-1(EcadAb)) (Mohamet et al. 2010), further improvements are required to increase the viability of hESCs. As mentioned above, one of the main requirements for hPSCs cultivation is the need to maintain cell-cell -matrix interactions. The knowledge accumulated from decades of experience in biopharmaceutical industrial processing, including the establishment of novel suspension culture strategies for mammalian cells, has facilitated the transition of hPSCs from static 2D monolayer cultures to dynamic 3D culturing approaches. Today, a variety of 3D suspension culture strategies are

established for hPSC expansion and/or differentiation: cell aggregates, cell immobilization on microcarrier and cell microencapsulation in hydrogels (Table 4.1). Nonetheless, the cultivation of stem cells in a 3D approach is not straightforward, requiring specific cell culture expertise as well as the implementation of robust and sensitive tools/methodologies for cell culture monitoring and characterization. However, the benefits associated to the implementation of successful and affordable bioprocesses and their translation to novel stem cell-based technologies should more than justify the investment. By providing a cellular context closer to what occurs in a native microenvironment, 3D culture strategies can significantly improve cell viability, functionality and proliferation/differentiation potential, offering a higher degree of efficiency, robustness, and predictability to the resulting hPSC manufacturing process (Cukierman et al. 2002; Pampaloni et al. 2007; Lund et al. 2009). It is important to highlight that these 3D cell culture approaches are also promising tools for drug screening application and disease modeling (Jensen et al. 2009).

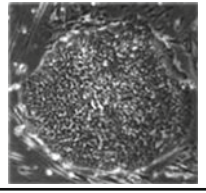
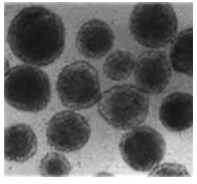
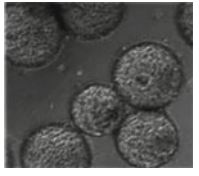
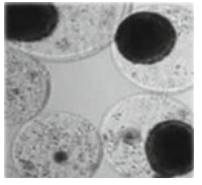
Cell Aggregates

In aggregate cultures, cells can re-establish mutual contacts and recapitulate microenvironments that allow them to express a tissue-like structure, ultimately enhancing cell differentiation and functionality (Burdick and Vunjak-Novakovic 2009; Lund et al. 2009). For hPSCs, this strategy has usually been associated with spontaneous differentiation, promoted by the generation of concentration gradients within the aggregate (Gerecht-Nir et al. 2004; Cameron et al. 2006; Come et al. 2008; Yirme et al. 2008). Knowledge gained from mouse ESC and progresses in stem cell biology has contributed to the design of controlled bioprocesses for hPSC expansion/differentiation as aggregate cultures (Kehoe et al. 2009; Niebruegge et al. 2009; Amit et al. 2010; Krawetz et al. 2010; Olmer et al. 2010; Singh et al. 2010; Zweigerdt et al. 2011). The main limitation of this system is the need to control aggregate size throughout the bioprocess in order to avoid the formation of necrotic centers and/or spontaneous differentiation (Table 4.1). To overcome this drawback suitable culture systems, such us stirred tank bioreactors (Krawetz et al. 2010), must be used and repeated dissociation and re-aggregation steps (Singh et al. 2010) should be integrated in the bioprocess.

Microcarrier Technology

One method for controlling cellular aggregation in suspension conditions is to use microcarriers. A microcarrier is a support matrix that allows the growth of anchorage-dependent cells in suspension systems. A wide range of microcarrier types have been proposed for the cultivation of hPSCs. These supports can be porous or non-porous, composed by gelatin, glass, collagen, cellulose, presenting dimensions within the range of 10–200 μm (Phillips et al. 2008; Fernandes et al.

Table 4.1 Advantages and disadvantages of different culture systems for stem cell bioprocessing

Culture strategy	**2D cultures**	**3D cultures**		
	Colony culture	**Cell aggregates**	**Microcarrier technology**	**Cell microencapsulation**
				
Advantages	Easy visualization/ monitoring	Easy handling	**Non-porous**	Scalable
	Affordable	Scalable	Easy handling	High reproducibility
	Suitable for small-scale studies	High reproducibility	Scalable	High surface to volume ratio
		Preservation of 3D cell-cell contacts	High reproducibility	High cell production yields
		Can mimic stem cells' native microenvironment	Easy visualization/monitoring	Protection from physical forces
		High differentiation efficiency	No limitations in mass and gas diffusion	Preservation of 3D cell-cell and cell-matrix contacts
		High cell production yields	High surface to volume ratio	Flexibility for engineering approaches to improve cell culture performance
			High cell production yields	Process integration in transplantation studies
			Porous	
			Easy handling	
			Scalable	
			High reproducibility	
			High surface to volume ratio	
			High cell production yields	
			Protection from physical forces	

Disadvantages	Low reproducibility	Difficult to control culture outcome	**Non-porous**	Difficulty in culture visualization/ monitorization
	Low scalability	Control of aggregate size	Controlling microcarrier agglomeration/clumping	Limited mass and gas diffusion inside the pores
	Difficult to control culture parameters and diffusion gradients	Single cell harvesting (difficult to dissociate aggregates without compromising cell viability)	Cell-bead separation step required	Cell harvesting (decapsulation step required)
	Low cell production yields	Cell damage due to physical forces	Cell damage due to physical forces (hydrodynamic shear, perfusion flow)	Material costs associated with encapsulation equipment/process and biomaterials
	Limited resemblance to *in vivo* tissues		Material costs (microcarrier)	
			Porous	
			Difficulty in culture visualization/ monitoring	
			Limited mass and gas diffusion inside the pores	
			Cell-bead separation step required (except for biodegradable supports)	
			Material costs (microcarrier)	

Adapted from Serra et al. (2012)

2009; Kehoe et al. 2009; Lock and Tzanakakis 2009; Nie et al. 2009; Oh et al. 2009; Chen et al. 2010, 2011; Lecina et al. 2010; Serra et al. 2010; Storm et al. 2010; Heng et al. 2012; Leung et al. 2011). The microcarrier type should be selected according to stem cell type/characteristics (size, morphology, clonal efficiency) and process requirements (expansion, differentiation, cell harvesting). It is important to highlight that, regarding the culture of hPSCs, these microcarriers should be further functionalized with different coating materials (e.g. ECM proteins) in order to improve cell culture performance namely, cell attachment, growth and differentiation, without compromising their characteristics (Chen et al. 2011). The development of defined, GMP compliant and xeno-free microcarriers has long been awaited.

Another advantage of microcarrier technology in stem cell expansion processes is the flexibility to easily adjust the area available for cell growth, further facilitating the process scale-up. From clinical/industrial perspectives, this attribute has a tremendous impact on reducing the costs of cell manufacturing by reducing the amount of culture media, growth factors and other costly supplements required in hPSC cultivation (Fernandes et al. 2009). However, this approach also has some disadvantages, including potential harmful effects of shear stress (Leung et al. 2011) and microcarrier clumping (Serra et al. 2011) as well as increased operating costs associated with the incorporation of an additional downstream step for cell-bead separation and microcarrier removal (Table 4.1). To overcome this last limitation, clinically approved biodegradable microcarriers are promising approaches. Indeed, gelatin and pharmacologically active microcarriers (PAMs) have been used successfully in adult stem cell-based therapy, showing enhanced cell survival, differentiation and graft integration (Delcroix et al. 2010; Hernandez et al. 2010).

Cell Microencapsulation

The main benefit of cell microencapsulation technology is the possibility of designing the scaffold environment with specific biomaterials selected from the wide range available with different mechanical/chemical properties, correlating to the properties of native tissues (Burdick and Vunjak-Novakovic 2009; Lund et al. 2009). Such tailored microenvironments may be more suitable for the self-renewal of stem cells, for directing their differentiation into specialized cell types, for promoting the organization of cells in 3-D configurations similar to those established *in vivo*. Several scaffolds/biomaterials have been used to enhance the culture of hPSCs including alginate (Siti-Ismail et al. 2008; Serra et al. 2011), hydrogels of agarose (Dang et al. 2004), chitosan (Li et al. 2013) and hyaluronic acid (Gerecht et al. 2007). It is important to highlight that microencapsulation technology potentially contributes for the success of transplantation experiments (Murua et al. 2008). In contrast to cells in suspension, encapsulated tissue constructs are less susceptible to immune rejection, their delivery is better targeted and the *in vivo* degradation kinetics can be tuned allowing a more efficient and functional integration of cells in the host organ (Delcroix et al. 2010; Murua et al. 2008). Thus, the source and properties

of the encapsulation material (i.e. elasticity, stability, permeability, biocompatibility and biosafety) should be selected taking into account the culture outcome, the final application and safety issues.

Cell microencapsulation in hydrogels also ensures a shear stress-free microenvironment while avoiding excessive clumping of microcarriers or aggregates in culture (Siti-Ismail et al. 2008; Chayosumrit et al. 2010; Jing et al. 2010; Serra et al. 2011). This 3D strategy has been shown to be extremely attractive for use in large-scale bioprocesses, enabling tighter control of the culture and higher cell yields than non-encapsulated cultures (Serra et al. 2011) (Table 4.1).

4.3.3 Bioreactors for hPSC Cultivation

Bioreactors have been, and still are, extensively used in chemical/biological industries for the production of antibodies and recombinant proteins amongst many other products. The knowledge accumulated from recent years has facilitated their transition to stem cell bioengineering, in which the cells are the products. In particular, bioreactors for stem cell bioprocessing should be designed to accurately control/regulate the cellular microenvironment that supports cell viability and provides spatial and temporal control of signalling. These environmentally controlled bioreactors should guarantee rapid and controlled cell expansion/differentiation, the efficient local exchange of gases (e.g. oxygen), nutrients, metabolites and growth factors as well as the provision of physiological stimuli (Fig. 4.3). In the end, by generating and maintaining a controlled culture environment, stem cells bioreactors represent a key element for the development of automated, standardized, traceable, cost-effective, and safe manufacturing processes for stem cell-based products. At present, there is a large range of bioreactor types available for hPSC bioprocessing (Placzek et al. 2009) (summarized in Table 4.2): microfluidic devices, rotary cell culture systems, stirred culture vessels and disposable bioreactors have been the main bioreactors explored to date.

Microfluidic Culture Systems

Microfluidic devices, or micro-bioreactors, are efficient small-scale systems mainly used for the optimization of cell expansion and differentiation culture conditions while also providing the precise control over the cell microenvironment (Azarin and Palecek 2010; Placzek et al. 2009). Arrays of micro-bioreactors have been developed to study growth and differentiation of hESC and ASC in a 3-D perfusion system (Cimetta et al. 2009; Fong et al. 2005; Zhao et al. 2009). The microenvironment can be controlled by adjusting operating parameters such as the perfusion rate, resulting in a high-throughput system for evaluating the effects of concentration gradients of soluble factors on various cell processes. However, the main limitations of these culture systems are low scalability and the drawbacks

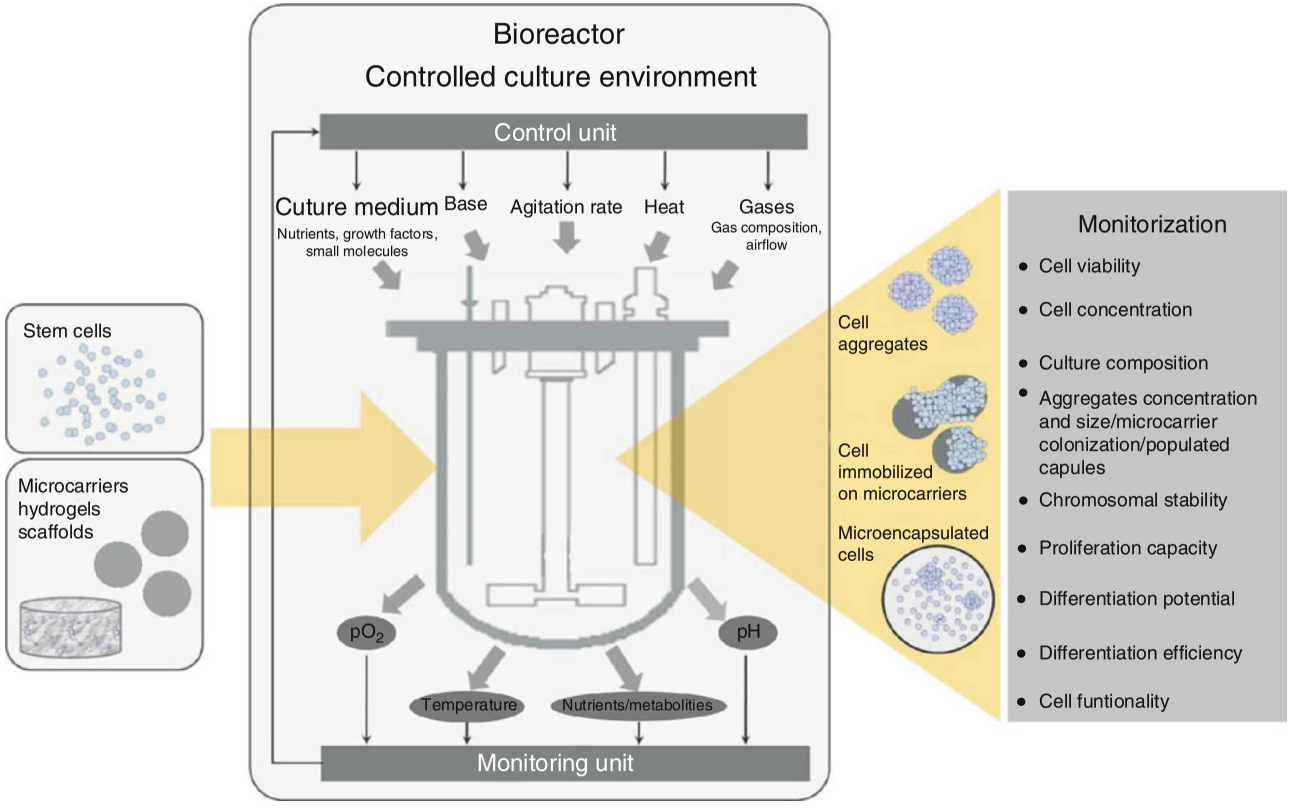


Fig. 4.3 Schematic representation of stirred tank bioreactor system for stem cell cultivation. Fully controlled stirred tank bioreactors provide an automated control of the environment (temperature, pH, pO_2 as well as nutrients/metabolites) mandatory for reproducible stem cell cultivation. Stirred culture vessels are scalable and hydrodynamically well characterized and enable easy non-destructive sampling for continuous monitorization of culture status (e.g. cell concentration/viability, differentiation potential, functionality). These bioreactors provide the operator with the flexibility of culturing hPSCs in different 3D approaches including the cultivation of cell aggregates, cells immobilized on microcarriers or entrapped in microcapsules

Table 4.2 Culture systems for stem cell expansion and differentiation

Bioreactor type	Culture surface area ($\times 10^3$ cm^2/L)	Production ($\times 10^6$ cell/mL)	Easy of sampling	Non-destructive sampling	Monit. & control	Mass transfer	Easy to scale-up	Shear stress (dyn/cm^2)	Operation mode		Culture approach	
									Batch	Perf	2D	3D
Static systems (T-flasks, dishes)	0.29	0.1	High	No	Low	Low	Low	0	Yes	No	Yes	No
Stirred culture systems	2.8	1–10	High	Yes	High	High	High	2–40	Yes	Yes	Yes	Yes
Perfusion chamber	18	10–100	Medium	No	Medium	Medium	Medium	1–5	No	Yes	No	Yes
Perfusion hollow fibre	100–200	100–200	Low	No	Low	Medium	Medium	0	No	Yes	Yes	Yes
Rotating wall vessel	18–22	n.a.	Medium	No	Medium	Medium	Low	0.5–2	No	Yes	Yes	Yes
Grooved bioreactor	18–20	10–100	Medium	No	Medium	Medium	Medium	0.1–0.5	No	Yes	No	Yes
Packed bed (Bellocel)	18	1.5–200	Medium	No	Low	Medium	High	1–5	No	Yes	Yes	Yes
Airlift	2,800	0.5	Medium	Yes	High	High	Medium	10–30	Yes	Yes	Yes	Yes
Disposable bag bioreactors (wave bioreactor)	not available	10–20	High	Yes	High	Medium	High	0.1–0.5	Yes	Yes	Yes	Yes

Adapted from Placzek et al. (2009) and Azarin and Palecek (2009)
n.a. not available

associated to perfusion including high shear stress and continuous removal of important factors secreted by the cells that could ultimately compromise stem cell performance.

Rotary Cell Culture Systems

Developed by NASA, Rotary cell culture (RCC) bioreactors (which includes STLV-slow turning lateral vessel and HARV- high aspect rotating vessel) are composed by a rotating 3-D chamber in which cells remain suspended in near free-fall, simulating microgravity conditions. These low shear stress bioreactors can provide a well mixed environment for cell growth as well as efficient gas transfer through a silicon membrane. Rotary cell culture systems have been used for expansion of cells as of human EBs, and for multiple ASC using scaffolds (Come et al. 2008; Gerecht-Nir et al. 2004; King and Miller 2007).

Amongst the main disadvantages associated to the use of RCC are the limited control of (i) aggregate size and (ii) nutrient/gas concentrations throughout the vessel. This may result in the formation of necrotic centers, leading to cell death inside the aggregates, and uncontrolled microenvironments, caused by the concentration gradients resulted from mass transfer limitations. In addition the working volume of these bioreactors is still low, thus limiting their use in larger scale bioprocesses.

Stirred Culture Vessels

Stirred culture vessels, including spinner vessels and stirred tank, are scalable and hydrodynamically well characterized systems with simple design and operation. The main characteristic of these bioreactors is the possibility of culturing cells in a dynamic stirred environment, overcoming the mass transport and gas transfer limitations of static and other bioreactor systems (Table 4.2). Here, the impeller design and ranges of stirring rate should be selected specifically for each case study since each stem cell type presents different sensitivities in terms of the shear stress. Another important feature of these bioreactors is the feasibility to perform non-destructive sampling thus enabling the continuous monitorization/characterization of the stem cell culture status/performance which is critical for process optimization (Fig. 4.3).

In particular, fully controlled stirred tank bioreactors provide an automated control of the environment, allowing the on-line monitoring and control of specific culture variables (temperature, pH, dissolved oxygen, nutrients) that can affect stem cell self-renewal and directed differentiation, ultimately improving culture outcome and ensuring reproducibility. These bioreactors are highly flexible as they can operate in different culture operation modes (batch, perfusion), can be adapted to different type of bioprocesses (stem cell expansion and/or differentiation) and can be accommodated to different 3-D culture strategies (cell aggregates, microcarriers, microencapsulated cells) (Table 4.2 and Fig. 4.3), presenting widespread potential

in stem cell bioengineering (Jing et al. 2010; Niebruegge et al. 2009). One of the main limitations of stirred culture vessels is the hydrodynamic stress promoted by stirring. Up to now, the minimal volume required to set up the experiments was very high (approximately 50 mL), which compromised the use of stirred bioreactors for high-throughput applications by demanding higher starting cell numbers and increasing the costs associated to optimization studies. Recently large efforts have been made towards the development of smaller scale systems (working volume 10–15 mL) and at least two options are available today including the ambr® systems (from TAP Biosystems) and the low volume spinner flasks (from HexaScreen).

Disposable Technology: Single-Use Bioreactors

The single-use bioreactor concept was introduced by the wave bioreactor bag (now from GE Healthcare) almost 15 years ago. Aiming at establishing larger scale suspension culture of stem cells, numerous disposable bioreactors have been developed: the BIOSTAT CultiBag from Sartorious-Stedim, Air-Wheel® Bioreactor Systems from PBS Biotech, PadReactor from ATMI, and the Mobius® CellReady Single Use Bioreactor from Merck Millipore are some examples. All these single use devices are mechanically agitated aiming at assuring efficient gas and mass transfer within the culture. Conveniently, all come as "single-use", presterilized bioreactors. Although successfully used in multiple biomanufacturing applications, the translation and feasibility of these platforms for hPSCs bioprocessing has not been reported so far.

4.3.4 Integrated Bioprocesses and Tools for hPSC Manufacturing

Optimal hPSC bioprocess should yield large cell quantities, not by embracing traditional scale-up principles (for example, by the use of industrial-scale bioreactors) but through process intensification and integration. Within this context, the establishment of platforms capable of integrating hPSC isolation/reprogramming, inoculation, expansion, differentiation, harvesting and purification would ultimately result in the scale-up of differentiated cells to relevant numbers to satisfy the clinical demands. During the last years, several bioprocesses that combine hPSC expansion and differentiation steps have been reported aiming at providing robust strategies for the scalable production of hPSC derivatives (reviewed in Serra et al. 2012). Another major challenge is the production of master/working cell banks of well-characterized cells in parallel to the manufacturing platform. Although important developments regarding integrated bioprocesses capable of guaranteeing efficient cell cryopreservation after large-scale expansion have recently been achieved (Nie et al. 2009; Serra et al. 2011), further research is necessary. Indeed, the establishment of fully integrated bioprocess for the expansion, differentiation, purification

and cryopreservation of stem cell derivatives will clearly support both autologous and allogenic stem cell therapies where it is often difficult to predict the recovery/availability of patients for transplantation.

From an engineering perspective, the development of a fully automated production platform requires the integration of novel technologies to monitor/control not only a set of process parameters (e.g. pH, pO_2, temperature) but also cell culture status over time including cell viability, phenotype and functionality. Significant benefits would derive from implementing sophisticated sensing/monitoring tools and devices within the manufacturing platform. The traceability, efficacy, safety and quality of the bioprocess would be highly improved, for sure, creating defined and robust GMP platforms to deliver safe and efficacious cell-based products.

4.4 Looking Ahead

The implementation of novel high-throughput methods allowing for a better characterization of cell genetic, epigenetic, proteomic and metabolic status and further understanding of cell biology is ultimately needed. The lack of available data in this field strongly compromises and limits the application of worldwide recognized tools for bioprocess description and prediction - mechanistic knowledge - that would be extremely useful for understanding how hPSCs respond to specific *stimuli* with the ultimate goal of predicting key molecular interactions that impact cell fate (Kirouac et al. 2010).

The development of novel 3D culture strategies and robust bioreactor-based systems is now, more than ever, bringing hPSCs closer to clinical/industrial applications. Recent advances in this field show that there is not an ideal hPSC-based bioprocess capable of embracing all type of therapies and applications. Nonetheless, the knowledge gained during the last years, including the impact of specific environmental factors on hPSC culture combined with the development of robust bioreactors, provides important insights for the implementation of more "universal" hPSC manufacturing platforms. Indeed, the complexity involved in the 3D cultivation of hPSC in a dynamic environment requires a multidisciplinary approach. By combining biology, immunology, engineering, physics, chemistry, and material sciences, stem cells-based products will be more accessible in the near future. Also important is the development of mathematical models and the integration of biostatistics tools capable of predicting the outcome of stem cell bioprocesses (e.g. yields of stem cell expansion/differentiation, cell purity, percentage of cell contaminants, etc.) as well as to provide insights into how the quality, purity and potency of cell-based products would ultimately impact on the efficacy of stem cell transplantation. These advances will for sure contribute to the implementation of robust manufacturing platforms and potentiate the development of novel cell therapies, fulfilling, at last, the high expectations posed by hPSCs.

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Chapter 5
Blood Cell Bioprocessing: The Haematopoietic System and Current Status of *In-Vitro* Production of Red Blood Cells

Susan M. Browne and Mohamed Al-Rubeai

Abstract Haematopoietic stem cells and a number of cell types derived from them are routinely transfused clinically. Haematopoietic lineage cells used for transfusion are predominantly red cells, followed by platelets and granulocytes. However, although blood transfusion has revolutionized modern medicine over the past century, many issues still exist with supply, particularly in developing nations, and adverse reactions and infections are still commonplace raising concerns for both donor and recipient safety. The production of hematopoietic lineage cells *in vitro* would assuage safety concerns and maintain supply. It would also resolve issues such as blood cell shelf-life and ageing by providing homogenous populations of cells; allow for production of universal/rare blood types on demand; and could provide a therapeutic solution in the case of conditions such as haemoglobinopathies. Theoretically it is possible to generate functional, mature cells of all hematopoietic lineages *in vitro*, however, the sheer volumes of blood cells required to meet current transfusion demand (or even demand for rare blood types), and production-associated costs means that, at present, this process is not feasible for routine treatment. Here we review the current state of *in vitro* blood cell production with particular emphasis on the generation of red blood cells/erythrocytes; and explore the technical issues associated with medium-to-large scale manufacturing of these cells, and the likelihood of commercial production and routine clinical use in the future.

Keywords Blood transfusion • Haematopoietic stem cells • *In-vitro* erythropoiesis • Stem cell therapeutics

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5.1 Introduction

The first successful human-human blood transfusion was carried out in 1818 by James Blundell, however, red blood cell donation and transfusion did not became routine until the early twentieth century when the discovery of ABO blood groups and the use of anticoagulants increased safety and allowed blood to be collected, stored and distributed in an organised way. Around the same time the efficacy of platelet transfusion to reduce the risk of bleeding in thrombocytopenic patients was also recognised, however, at this point platelet numbers were increased through the administration of large amounts of whole blood and it wasn't until the 1970s that transfusion of platelet concentrates became routine.

The Cook County Hospital in Chicago last year celebrated the 75th anniversary of the opening of the first hospital blood bank and in the relatively short period since then the field of blood transfusion has grown to the extent that it is now estimated some 92 million whole blood donations are collected throughout the world annually (World Health Organization 2011). In addition to red blood cells, platelets are routinely transfused for the treatment of thrombocytopenia and platelet dysfunction, and, though demand has traditionally been low due to problems collecting sufficient quantities and maintaining functionality, there has been a resurgence of interest in granulocyte transfusion for the treatment of neutropenia. Platelets for transfusion are derived either through the separation of whole blood units into components – concentrated red cells, platelets, fresh-frozen plasma, and cryoprecipitate – with concentrates from four to five donors combined to generate a platelet transfusion unit; or a single unit can be isolated per donor via platelet aphaeresis (a safer method as it limits the likelihood of recipient infection). Granulocytes can also be collected by aphaeresis; however, the administration of steroids/growth factors is generally required to mobilize sufficient quantities to the peripheral circulation and thus granulocyte donation and transfusion remains limited.

The development of organised systems for blood component donation and transfusion has revolutionised health care, however, issues still exist relating to supply and safety, particularly in developing nations. Of the 92 million annual whole blood donations, approximately half are collected in high-income countries even though these countries only represent about 15 % of the global population. In its most recent worldwide survey of blood donation and utilization patterns the World Health Organization proposed that somewhere between 30 and 50 donations per 1,000 population per year would to be sufficient to maintain an adequate national blood supply. However, while in high-income countries the median whole-blood donation rate is roughly 36 donations/1,000 population, in middle-income nations it is 12 donations/1,000 population and for low income nations only 3 donations/1,000 population; and half of all countries surveyed report less than 10 donations/1,000 population (Fig. 5.1) (World Health Organization 2011). Even in high-income nations localised shortages are often reported (US Department of Health and Human Services 2011) and even some EU member states report sizeable shortages year-on-year (European Directorate for the Quality of Medicines and Healthcare 2009). It is also believed that the ageing demographic of many high-income countries will lead

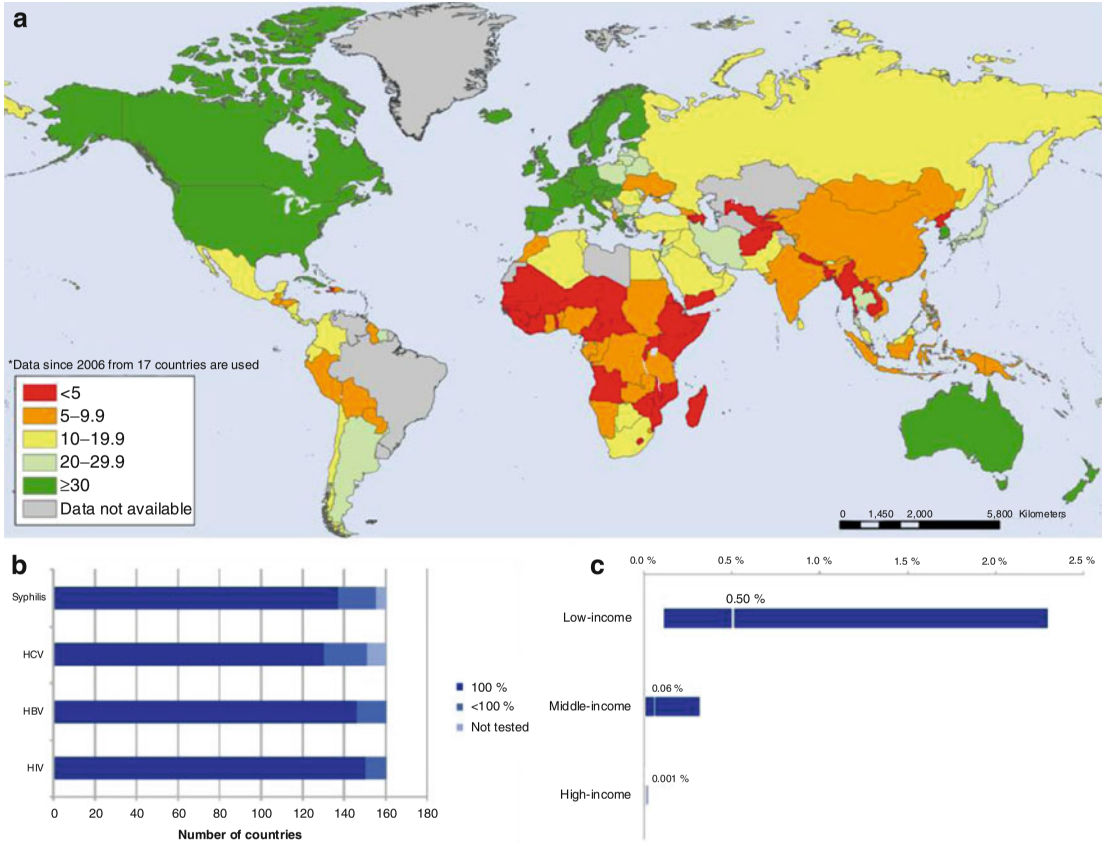


Fig. 5.1 Worldwide blood donation levels and blood product screening. (**a**) Blood donation levels per 1,000 population. (**b**) Level of laboratory screening for transfusion transmissible infections. (**c**) Prevalence of HIV in donated blood by country income group; numbers shown indicate median value while *left* and *right bars* show lower and upper quartiles respectively (Adapted from WHO Global Database on Blood Safety (2011))

to shortages of supply as the size of the donor base falls and the proportion of the population over the age of 65 (by far the largest recipient group in high-income countries) expands.

In addition to supply issues there are still safety problems associated with both blood donation and transfusion – primary among these are sepsis, haemolysis, allergic reactions and anaphylaxis, TRALI (transfusion related acute lung injury), TACO (Transfusion associated circulatory overload) and transfusion associated HCV in blood component recipients; and loss of consciousness, nerve irritation, and induction of anaemia in donors. Although the WHO recommends that all blood components should be screened for evidence of infection prior to release, and states that screening should be mandatory for HIV, Hepatitis B, Hepatitis C, and syphilis, almost a quarter of countries (39 out of 164) surveyed stated that blood donations are not routinely tested for TTIs (transfusion transmissible infections) (Fig. 5.1), with irregular supply of test kits cited as one of the most common obstacles to testing (World Health Organization 2011). Low income countries have the lowest likelihood of testing in spite of the highest prevalence of TTIs and the highest percentage of infections in those units that are tested. In addition to these challenges, blood centres throughout the world are also struggling to develop guidelines to deal with new and emerging diseases such as variant CJD or as yet unidentified threats.

Another safety issue is the age/storage duration of transfused blood cells – red cells undergo biochemical changes with time in storage such as loss of ATP and 2,3-DPG and suffer membrane damage, thus the cut-off for storage is 42 days; platelets and granulocytes meanwhile have very short storage times and must be used as soon as possible (platelets have a maximum storage time of 5 days and granulocytes have a maximum storage of 2 days) as functionality is quickly lost. Platelets also have an increased likelihood of bacterial contamination due to their storage at 22 °C.

A major issue is that blood cells are not produced for market like other commodities – their supply relies on the altruism of donors, and they are also highly heterogeneous with respect to blood group antigens, donor age, infection status, and storage time. With this in mind many are looking towards *in-vitro* manufacturing of blood cells to achieve a reliable and well characterised blood supply. In this chapter we will summarise the achievements to date in the field of *in-vitro* blood cell production and examine the challenges to be overcome if this system is to become a routine, large-scale alternative to donor blood cell collection. The ultimate aim is to achieve the routine production of safe, matched, well characterised, pathogen-free blood products in sufficient amounts to maintain a constant supply, all at a realistic cost. We have reached the stage where it is technically possible to generate all types of hematopoietic cells *in-vitro* from hematopoietic stem cells (derived from various sources), although, how far we are from reaching the point where it is possible to generate these cells in sufficient quantities to meet market demands at acceptable costs is debatable. Here we summarise research to date on the *in-vitro* production of cells of hematopoietic lineages, and discuss some of the challenges associated with the transfer of this technology from the lab towards large scale production at sufficient levels to supply clinical demand.

5.2 The Haematopoietic System and Haematopoiesis

The hematopoietic system comprises all blood cells and blood forming tissues. In the average human adult this consists of between 4.5 and 5 l of blood, approximately 7 % of body weight, and of bone marrow, the major site of blood cell production in the adult, which makes up a further 4.5 % body weight (Stiene-Martin et al. 1998). The blood is made up of the various blood cells (red (erythrocytes) and white (leukocytes) blood cells) and platelets (~45 % total volume) suspended in liquid plasma (~55 % total volume) and has a range of functions in the body including regulation of temperature, pH and osmolarity, nutrient and hormone transport and waste removal, gas exchange, coagulation, and the various specific and non-specific immune functions. RBCs (red blood cells) compose by far the largest fraction of blood cells, and distribute oxygen throughout the body; platelets (cell fragments that are sloughed from large megakaryocyte cells), although not technically cells, represent the second largest fraction, and are responsible for clotting; while WBCs (white blood cells) can be divided into monocytes/macrophages which phagocytose pathogens and dead/apoptotic cells; granulocytes – granular cells responsible for non-specific immunity against pathogens; and lymphocytes – specific immune cells involved in antigen presentation, and antibody-mediated immune responses. See Table 5.1 for a list of blood components and corresponding functions (although volumes for leukocytes are given per L of peripheral blood, they are mostly situated/active within the various other tissues, and thus these figures may not represent their full quotient). The bone marrow is encased in the endosteum (a membrane lining the marrow cavity), and contains vessels and nerves, hematopoietic cells at progressive stages of differentiation, stromal cells and an extracellular matrix (ECM) made up of fibronectin, collagen, and various proteoglycans all arranged in a highly organized 3-D structure.

The hematopoietic system is in constant turnover and is maintained throughout the lifetime of an organism by the generation of new blood cells from hematopoietic stem cells (HSCs) – primitive, undifferentiated, multipotent cells whose function is divided between either self-renewal, or expansion and differentiation into the mature blood lineages. Vertebrate haematopoiesis occurs in two successive and overlapping waves – primitive and definitive – which differ in anatomic sites and in the types of cells produced. Initially during fetal development primitive haematopoiesis occurs in the extra-embryonic yolk-sac blood islands generating primitive erythroblasts and macrophages, allowing early oxygen delivery and protection from pathogens in the embryo. Definitive haematopoiesis then proceeds in the yolk-sac and foetal liver to produce a transient population of erythroid-myeloid progenitors (EMPs), and in the AGM (aorta-gonad-mesonephros) region of the embryo producing HSCs from the hemogenic endothelium (Sood and Liu 2012; Baron et al. 2012). HSCs then move to the fetal liver, followed by the fetal spleen and eventually the bone marrow once this is sufficiently developed to support haematopoiesis. The pool of HSCs that is generated in the hemogenic endothelium during embryogenesis is sufficient to reconstitute the haematopoietic system throughout the lifetime of the organism.

Table 5.1 Blood components

Component		Composition	Function	Volume
Plasma		≥90 % water & dissolved proteins e.g. Albumin; globulins; fibrinogen	Carries glucose, nutrients, hormones, growth factors, electrolytes, clotting factors etc. Maintains pH, osmolarity.	Approx. 55 % of blood volume 2.3–2.8 L in average adult
Erythrocytes		Biconcave disc approx. 7 μm High haemoglobin content Lacking nuclei and organelles	Transportation of O_2	5×10^{12} cells/L
Monocyte/ macrophages		Contain lysosomal enzymes Mononuclear cells 12–22 μm	Phagocytosis of microorganisms; foreign bodies; apoptotic cells; erythroid nuclei Antigen presentation	4×10^{8} cells/L
Megakaryocytes/ platelets		Megakaryocytes are large cells with multi-lobed polyploid nuclei Develop long, cytoplasmic processes that fragment into platelets	Platelet production Clotting	3×10^{11} platelets/L
Granulocytes	Neutrophils	Distinct granules in cytoplasm which contain enzymes such as peroxidases and proteases Polymorphonuclear-multi-lobed, segmented nuclei 10–15 μm	Phagocytosis of bacteria and fungi	5×10^{9} cells/L
	Eosinphils		Destroys larger parasites and modulates allergic inflammatory responses	2×10^{8} cells/L
	Basophils		Releases histamine in certain immune reactions	$2–5 \times 10^{7}$ cells/L
Lymphocytes	T-Cells	No obvious granules in cytoplasm Mononuclear – nucleus may vary in shape, but is a single mass 6–8 μm	Kill virus-infected cells and regulate activities of other leukocytes	1×10^{9} cells/L
	B-Cells		Produces antibodies	2×10^{9} cells/L
	NK cells		Kill virus-infected cells and some tumour cells	1×10^{8} cells/L

After birth haematopoiesis occurs primarily in the bone marrow – occurring throughout the skeleton, including the long-bones, during childhood; and then decreasing in the number of sites with maturity so that in adults it is mainly confined to the sternum, cranium, ribs, vertebrae, pelvis, and just the proximal regions of the long bones; although extramedullary haematopoiesis can take place in the spleen, liver and lymph tissues in times of hematopoietic stress (O'Malley 2007).

HSCs were the first identified stem cell (a primitive cell capable of self-renewal and multi-lineage differentiation) (Till and McCulloch 1961) and remain the best

characterized to date. As well as self-renewal the HSC is multipotent and generates all cell types of hematopoietic lineage. The decision between self-renewal and differentiation of HSCs into the various blood cells is regulated by cues from their surrounding microenvironment, or "niche".

The balance between self-renewal and differentiation is vital – sufficient self-renewal is required to maintain an infinite supply of HSCs for reconstitution throughout life, and adequate levels of blood cells (more than 10^{11} cells/day in the average adult) need to be produced to prevent cytopenia, while at the same time regulating proliferation to prevent overproduction – as is the case with leukemia or polycythemia. In addition, different hematopoietic cell types have different rates of turnover in the body, so a complex regulatory system is required.

The concept of the HSC niche, first proposed by Schofield in 1978, hypothesizes that the self-renewing stem cell is associated with a cohort of cells (the niche) that prevent its maturation, while differentiation is triggered in its progeny if they cannot occupy this same niche (Schofield 1978). Although the HSC niche has been extensively studied its precise composition is still open to debate. It is generally believed that alternate niche conditions maintain HSCs in either a quiescent/G0 state or an active proliferating state. Thus, an adequate pool of primitive cells is maintained, while a constant supply of cells is provided for reconstitution. Two main "niches" have been proposed – an osteoblastic/endosteal niche near the trabecular bone surface, where quiescent HSCs have been shown to localize, and a sinusoidal/endothelial niche near the vascular interface which maintains active HSCs – however, consensus has not been reached as to whether these sites represent two distinct niches, two overlapping niches, or whether the HSCs represented in each are actually discrete stem cell sub-types (Wang and Wagers 2011) (Fig. 5.2).

Evidence of an osteoblastic niche was provided by studies which showed that HSCs in the bone marrow were localized in close proximity to osteoblasts at the endosteum and that a correlation existed between the number of quiescent HSCs and the number of osteoblasts. In addition, osteoblasts are one of the main sources of CXCL12 (chemokine stromal-derived factor-1), which plays an important role in HSC homing, and binds to the CXCR4 and CXCR7 receptors on HSCs, and physiological hypoxia in the osteoblastic/endosteal environment has been shown to both increase CXCL12 expression and to be involved in maintaining self-renewal of HSCs (Shiozawa and Taichman 2012).

The profile of the proposed vascular niche still remains controversial; however vascular endothelial cells have also been shown to express CXCL12, and oxidative stress (typical of the higher oxygen tension at the vascular sinusoids) has been shown to trigger an exit of quiescent HSCs from dormancy supporting the notion of separate quiescent and active niches (Shiozawa and Taichman 2012).

Some interesting studies over the past few years have also shown effects of circadian rhythms on the HSCs and their niche. Noradrenalin secreted by sympathetic nerves in the bone marrow has been shown to inhibit CXCL12 expression, while the sympathetic nervous system has also been shown to inhibit osteoblast production, and increase osteoclast activity (Shiozawa and Taichman 2012).

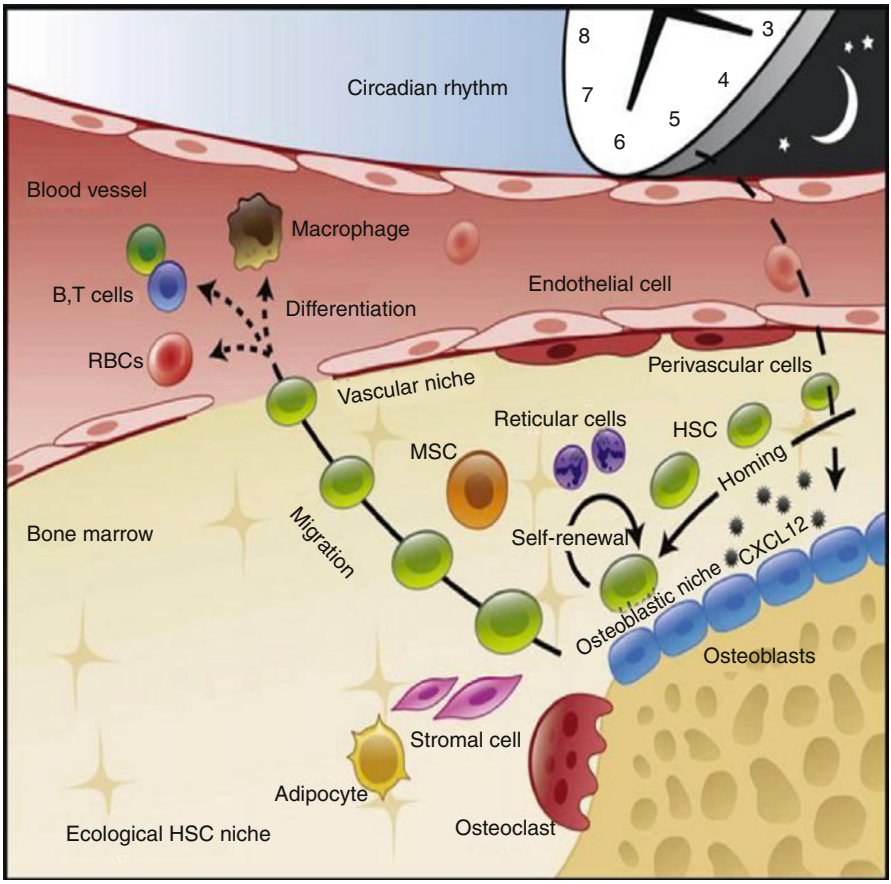


Fig. 5.2 Schematic of proposed stem cell niche environments (Shiozawa and Taichman 2012)

5.3 Expansion and Differentiation of Blood Cell Lineages

Once the HSC leaves the niche the formation of mature blood cells proceeds through a hierarchical format – LT-HSCs (long-term repopulating haematopoietic stem cells) give rise to ST-HSCs (short-term HSC), which give rise to multipotent progenitors (MPPs) which, although they can generate a number of different cell types, can no longer self-renew. MPPs then generate progenitor cells that are restricted to either lymphoid or myeloid differentiation – the common lymphoid progenitor (CLP), or the common myeloid progenitor (CMP) – which in turn generate megakaryocyte/erythrocyte progenitors (MEPs), or granulocyte/monocyte progenitors (GMPs) respectively (Fig. 5.3). Classification of these groups is based on temporal expression of particular combinations of cell surface antigens (Sigvardsson 2009).

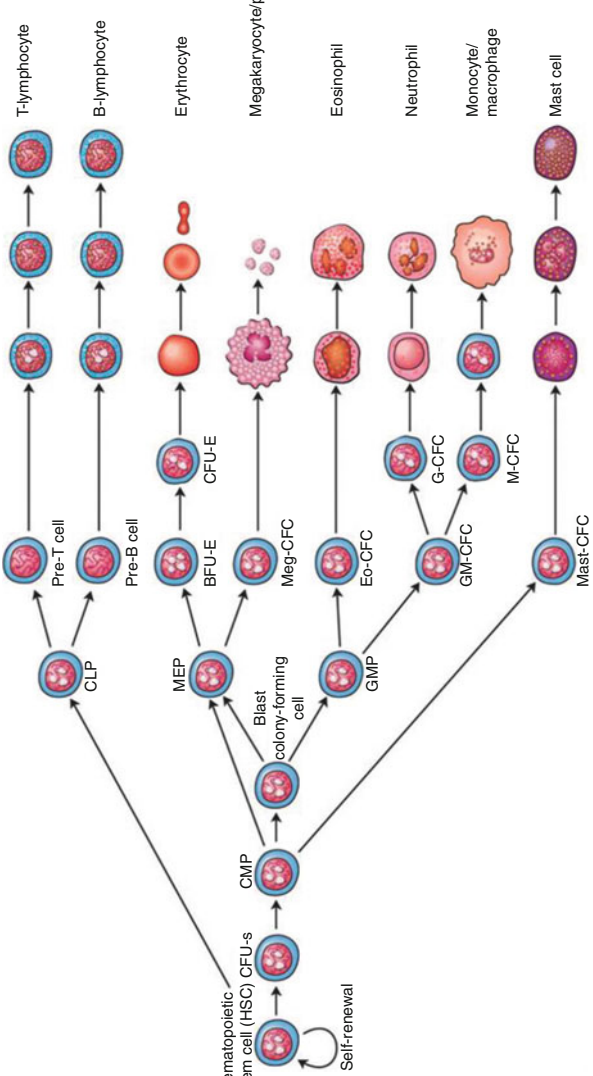


Fig. 5.3 Self-renewal and differentiation of haematopoietic stem cells and development of haematopoietic lineages into mature blood cells (Metcalf 2007)

Differentiation of stem/progenitor cells into the various mature blood cell types is regulated by HIMs (Hematopoietic Inductive Environments), with contributions made from stromal cells (fibroblasts, reticular cells, endothelial cells, adipocytes and osteoblasts), other hematopoietic cells, extracellular matrix proteins (including collagen, fibronectin and proteoglycans) cytokines (e.g. Interleukins, Thrombopoietin and Stem Cell Factor), and physiological factors such as O_2 gradients and pH.

5.3.1 Erythrocyte/RBC Development

From the MEP stage erythroid development passes through the progenitor stages of slowly dividing BFU-E (Blast Forming Unit-Erythroid) and rapidly dividing CFU-E (Colony Forming Unit-Erythroid); followed by maturation through various erythroid precursors – pro-erythroblasts, basophilic erythroblasts, polychromatic erythroblasts, and orthochromatic erythroblasts; orthochromatic erythroblasts then extrude their nuclei to form reticulocytes, which develop into mature erythrocytes (Fig. 5.4).

Many of the factors involved in supporting this developmental pathway were deciphered throughout the 1990s in studies using genetically modified mouse models and human ex-vivo erythroid cultures. Thus it was concluded that the early stages of culture are largely dependent on the cytokines Stem Cell Factor (SCF) (Dai et al. 1991) and Interleukin-3 (IL-3) (Papayannopoulou et al. 1993), and glucocorticoids, which maintain cells in an immature progenitor state to enable maximum proliferation (Muta et al. 1995; von Lindern et al. 1999). Erythropoietin (Epo), while not having a distinct effect on the progenitor stage on its own (Sui et al. 1996; Wu et al. 1997), can act in synergy with SCF in promoting proliferation (Muta et al. 1994; Panzenbock et al. 1998). Once cells reach the pro-erythroblast stage they are reliant on insulin and Epo (Muta et al. 1994), which enables erythroid differentiation through activation of the tyrosine kinase JAK2 and, in turn, the transcription factor STAT5, and prevents apoptosis through expression of the anti-apoptotic protein Bcl-X_L (Dolznig et al. 2002). As maturation proceeds cells become iron dependent, and adhesion to the extracellular matrix protein fibronectin has been shown to be important for terminal differentiation and enucleation (Hattangadi et al. 2011).

Throughout differentiation hemoglobin accumulates, chromatin condenses, cell size decreases and, finally, cellular organelles such as nuclei and mitochondria are packaged and extruded. This progression is regulated by growth factor-initiated signalling through various cell surface receptors leading to activation of specific transcription factors, chromatin modifying agents and micro-RNAs (Hattangadi et al. 2011).

During maturation in-vivo erythroblasts are located with macrophages in “erythroblastic islands”, where erythroblasts of varying levels of maturity are attached to a central macrophage through various adhesion molecules, with immature cells on

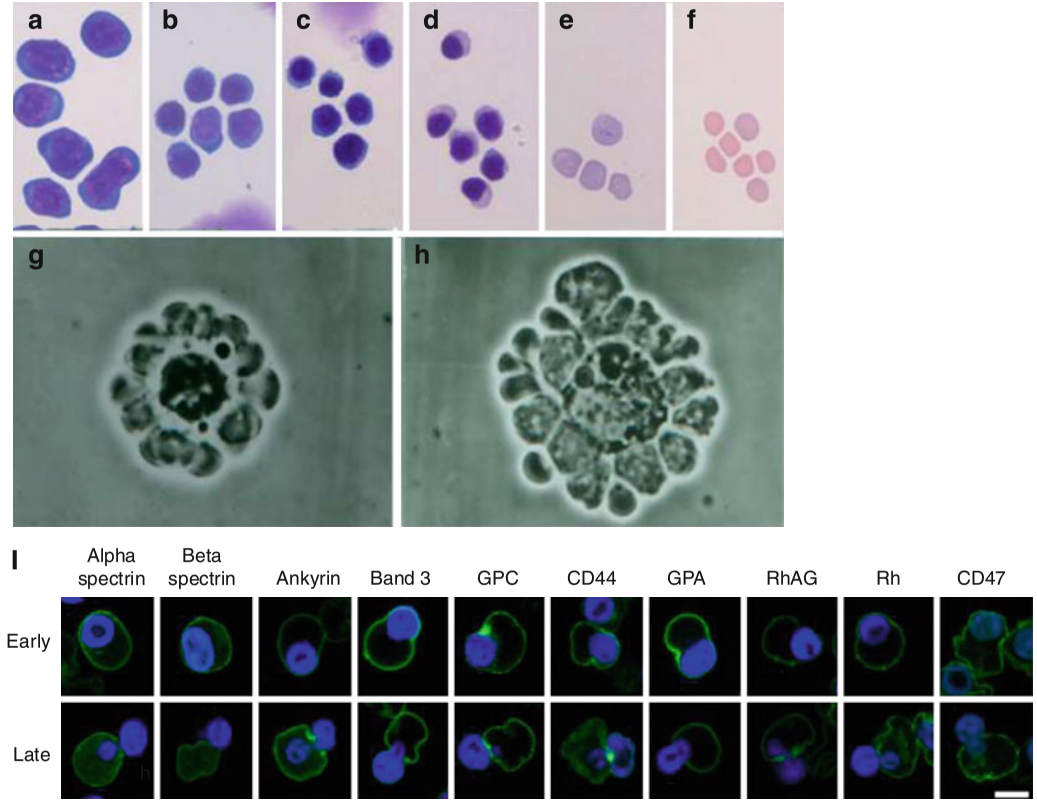


Fig. 5.4 (**a**–**f**) Wright-Giemsa stains of maturing erythroid cells, (**a**) pro-erythroblasts, (**b**) basophilic erythroblasts, (**c**) polychromatic erythroblasts, (**d**) orthochromatic erythroblasts, (**e**) reticulocytes, and (**f**) mature red blood cells. (**g**, **h**) Optical microscope pictures of erythroblastic islands (An and Mohandas 2011); (**i**) membrane sorting during enucleation – fluorescence microscopic images of cells undergoing early (*top row*) and late (*bottom row*) enucleation. Nuclei are stained with Hoechst and show in *blue* while surface proteins are stained with individual primary antibodies and detected with Alexa-488 conjugated secondary antibodies (Bell et al. 2013)

the periphery and cells of increasing maturity situated in advancing proximity to the central macrophage. After extrusion the nucleus is rapidly phagoctyosed and degraded by the macrophage. Although a direct involvement of macrophages in erythroid differentiation and maturation has not been conclusively proven, the close association of erythroblasts to macrophages during maturation, and the arrangement of the maturing cells with the least mature cells at the outermost edges and the most mature cells closest to the macrophage, has led many to suggest that macrophages somehow regulate the maturation process, possibly through the provision of iron (An and Mohandas 2011).

Although the main hypothesis surrounding the mechanism of erythroblast enucleation has been proposed as a form of asymmetric cytokinesis (Keerthivasan et al. 2012), the precise signalling processes involved are still unclear, although vesicle trafficking has been shown to be involved (Keerthivasan et al. 2010), as has cytoskeletal remodelling (Ubukawa et al. 2012), the event is likely the complex interaction of a number of factors (Ji et al. 2011). Enucleation involves the extrusion of plasma membrane enclosed nuclei, along with endoplasmic reticular proteins and several non-essential surface-proteins from the cell. All essential components required for the continued functioning of the nascent reticulocyte, and in turn the mature erythrocyte must be retained – these are mainly cytoskeletal and membrane proteins, along with some key cytoplasmic proteins and endocytic machinery (Bell et al. 2013). Excess surface proteins and protein complexes are sorted and discarded along with the nuclei (Fig. 5.4) (Bell et al. 2013). Extrusion of erythroblast nuclei (a process specific to mammals) and other cellular organelles leads to the formation of a pliable, deformable, non-replicating bi-concave erythrocyte – essentially a sac of haemoglobin the main purpose of which is to transport oxygen throughout the body.

Probably the most important aspect of erythroid differentiation and maturation is haemoglobin production. The haemoglobin molecule is a tetrameric structure consisting of four peptide chains each holding a heme molecule, containing an iron atom, at its centre (Fig. 5.5a). Globin-chain combinations vary with each stage of ontogeny, embryonic haemoglobin can have the following formats – $\xi_2\varepsilon_2$ (Gower I); $\alpha_2\varepsilon_2$ (Gower II), or $\xi_2\gamma_2$ (Portland); foetal chains are $\alpha_2\gamma_2$; while adult chains are $\alpha_2\beta_2$ (HbA1) or $\alpha_2\delta_2$ (HbA2 – a variant form) (Fig. 5.5b).

5.4 Sources of Hematopoietic Stem/Progenitor Cells

HSCs are characterised by their ability to reconstitute the hematopoietic system of deficient recipients following transplantation and are generally defined by the expression of the surface marker CD34 and the lack of CD38 and lineage specific markers, however, even within the $CD34^+$ population there is a large amount of heterogeneity, and different subsets with varying expansion and differentiation potential exist depending on the location from which they are isolated (Bender et al. 1994).

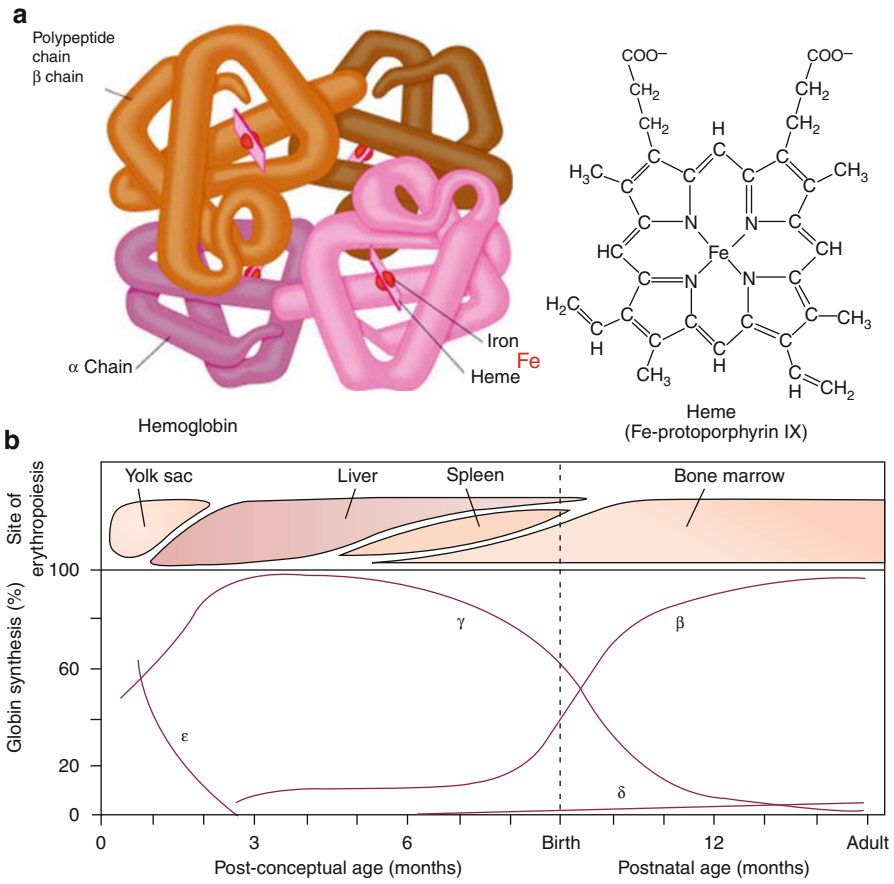


Fig. 5.5 Globin chain synthesis. (**a**) Schematic of the conformational structure of adult haemoglobin, showing two α-globin chains and two β-globin chains, each holding a central heme molecule, and also chemical structure. (**b**) Patterns of globin chain expression during human development (Higgs et al. 2012)

As well as bone marrow and foetal tissues (foetal blood, liver, bone marrow, pancreas, spleen and kidney, and also the extra-embryonic amniotic fluid and placenta), HSCs can be harvested from peripheral blood, mobilized peripheral blood (the administration of cytokines such as granulocyte colony-stimulating factor leads to increased mobilization of HSCs from the bone marrow to the peripheral blood), and umbilical cord blood (UCB) (Wang et al. 1996; Abdulrazzak et al. 2010).

The choice of HSC source for *in-vitro* production of blood cells is a trade-off between expansion potential in culture, functionality of the derived cells, and accessibility of the HSCs for differentiation (Table 5.2). Peripheral blood derived HSCs are highly accessible as they can be harvested from buffy coats (the white cell content of blood) which are separated from normal whole blood donations and, if not used for platelet retrieval, are discarded.

Table 5.2 Sources of hematopoietic stem cells

	Source	Accessibility	Yield/ unit	Advantages	Disadvantages	
Proliferative capacity	PB	High	-10^5–10^6 cells/ units PB	Readily available waste product Inexpensive Mature phenotype of derived cells	Relatively low proliferative capacity	Maturity of derived blood cells
	mPB	Moderate	-3×10^8 cells/ Donation (10–12 L apheresis vol.)	Higher stem cell yied than regular peripheral blood Mature phenotype of derived cells	Requires cytokine administration +/– chemotherapy Generally restricted to autologous stem cell retrieval for BM reconstitution	
	BM	Low	-10^{10} cells/1 L donation	Mature phenotype of derived cells	Difficult to obtain Generally restricted donation for BM reconstitution	
	UCB	High	-3×10^6 cells/100 ml unit	High number HSCs/TNC High proliferative capacity	Small unit size Immature phenotype of derived cells	
	Placenta	High	$-2 \times 1\ 0^7$ cells/unit	High number HSCs/TNC High proliferative capacity Significantly larger unit size than UCB	Inefficiency of current isolation protocols Immature phenotype of derived cells	
	Foetal tissues	Low	FBM -1×10^8/ foetus FL -1–1.5×10^8/ foetus	High number HSCs/TNC High proliferative capacity Lower immunogenicity	Ethical issues Small units size Immature phenotype of derived cells	
	ESC	Theoretically unlimited	N/A	Cell line – unlimited source	Ethical issues Immature phenotype of derived cells	
	iPSC	Theoretically unlimited	N/A	Cell line – unlimited source	Safety issues Immature phenotype of derived cells	

However, HSCs derived from peripheral blood have low proliferation potential compared to other sources and are present at a low frequency in peripheral blood. Although administration of cytokines to increase the frequency of HSCs in peripheral blood is possible, this is generally restricted to clinical transplantation for bone marrow reconstitution and has limited potential as a source for large scale *ex vivo* blood cell production. The same can be said for bone marrow, which has a high frequency of HSCs which have a high proliferation potential but, due to difficulties with accessibility and the low quantities available, this also represents an unlikely source material for medium to large-scale production.

UCB is relatively accessible, and although the unit size is small (approx. 100 ml), it has a high frequency of HSC per nucleated cell, and, like HSCs from foetal tissues

these have a high proliferation potential. In addition to the umbilical cord the placenta also contains HSCs, although, as with UCB the numbers of these decrease with gestational age; also retrieval is difficult with current protocols which typically yield about 1/10 the HSCs derived from UCB per unit (Robin et al. 2009). Foetal tissues, in spite of small tissue sizes, also have a high frequency of HSCs. However, access and acceptability are a sensitive issue as the harvesting of HSCs from aborted foetuses may be even more of an ethical challenge for many than the use of embryonic stem cells (see below).

Generally, more primitive cells have a higher proliferation potential. Foetal blood and liver HSCs proliferate to a greater extent than cord blood or bone marrow HSCs, which in turn proliferate to a greater extent than peripheral blood HSCs (Abdulrazzak et al. 2010). This is believed to be associated with their respective telomere lengths (Vaziri et al. 1994) – telomeres are nucleoprotein structures at the end of chromosomes which shorten with each cell division and maintain chromosome stability. In spite of this high proliferation potential though, these primitive cells also show a more primitive phenotype following differentiation e.g. erythrocytes generated ex-vivo from cord blood or foetal liver HSCs generate the embryonic (ζ and ε) and foetal (γ) globin chains as opposed to the adult β chains, with maturation, and show lower levels of enucleation (the final extrusion of nuclear content required to generate a functional RBC) (Neildez-Nguyen et al. 2002; Pourcher et al. 2011) (Fig. 5.5).

Due to the low number of cells/unit typically obtained for HSCs attempts have been made to increase the amount of starting material by maintaining self-renewal of primitive HSCs before differentiation into different blood cell types. Most of this work has been carried out in the context of increasing HSC numbers for bone marrow reconstitution in a clinical setting and includes recreation of the stem cell niche (Timmins and Nielsen 2011).

An alternative, and in theory unlimited, source material for differentiation into blood cells may be found in ESCs (embryonic stem cells) and iPSCs (induced pluripotent stem cells). ESCs are undifferentiated, pluripotent cell lines, derived from the inner cell mass of the blastocyst stage of the embryo (Fig. 5.6), which have a high telomerase activity, display normal karyotypes, and can be maintained

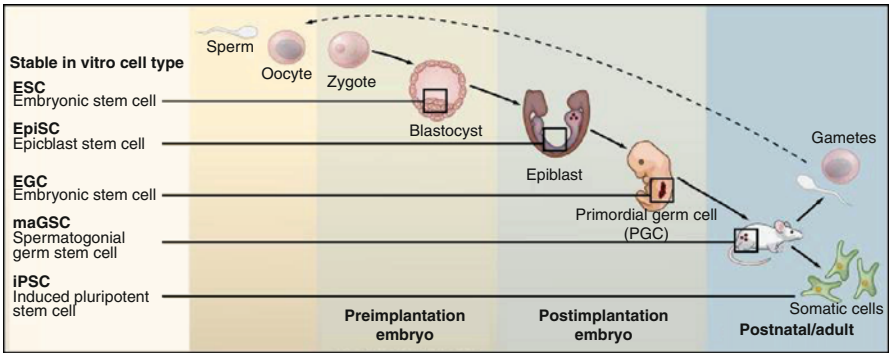


Fig. 5.6 Pluripotent stem cells (Hanna et al. 2010)

indefinitely in culture in an undifferentiated state when co-cultured with MEF (murine embryonic fibroblast) stromal lines (Thomson et al. 1998). Upon removal from these stromal layers they can differentiate into cells from all three germ layers – ectoderm, endoderm, and mesoderm – and theoretically into all cell types, with the mesoderm layer capable of differentiating into hematopoietic precursor cells; either through the formation of embryoid bodies, or by co-culture with tissue specific stromal lines e.g. when human ESCs are co-cultured on stromal lines derived from hematopoietic tissues (murine yolk sac and bone marrow) they give rise to $CD34^+$ haematopoietic precursors and differentiated cells expressing lineage-specific markers (GpA for Erythroid, CD15 on myeloid cells, and CD41 on mega-karyocytes) (Itskovitz-Eldor et al. 2000; Kaufman et al. 2001).

In somatic cells induction of pluripotency can be achieved through transfection with the transcription factors *oct4*, *klf4*, *sox2*, and *myc* (Takahashi et al. 2007) or *oct4*, *sox2*, *nanog*, and *lin28* (Yu et al. 2007). As with ESCs these iPSCs can also be maintained indefinitely in culture in an undifferentiated, pluripotent state followed by differentiation into hematopoietic cells (Fig. 5.6) (Lengerke et al. 2009). This allows the generation of autologous cells for transfusion/transplantation, removing the immune barriers that can be associated with ESC derived cells.

However, there are still many issues that need to be overcome with ESCs and iPSCs before they can become a credible starting material for blood cell production. First and foremost are the ethical issues associated with ESCs and the fact that ESC research is still not licenced in a number of countries; in this sense a more acceptable alternative to ESCs may be hiPSCs, although it has not yet been conclusively shown that ESCs and iPSCs are functionally identical and further research is needed in this area before iPSCs can be regarded as a definitive replacement for ESCs (Puri and Nagy 2012). Ex-vivo culture of ESCs and iPSCs is also much more expensive and complex than that of other HSC sources, in iPSCs reprogramming efficiency is low, and differentiation efficiencies are also low for both cell types.

Although they initially display normal karyotypes there is still the possibility of malignancy with ESCs with extended culture periods and there have been a number of reports of genetic instability (Puri and Nagy 2012). With iPSC residual expression of transgenes is an issue, and in particular the oncogenic potential of *c-MYC* needs to be taken into account. Further research has shown though that *c-MYC* expression is not essential for pluripotency which can be achieved with different combinations of transcription factors, and although induction time is longer in the absence of *c-MYC* and the efficiency of reprogramming is significantly decreased, it is more specific (Yu et al. 2007; Nakagawa et al. 2008; Miura et al. 2009). Further attempts to improve the safety of iPSC include the use of reduced numbers of transgenes, excisable vectors, non-integrating episomal vectors, RNA transfection, and direct protein transduction (Kim et al. 2008, 2009; Stadtfeld et al. 2008; Lacoste et al. 2009; Lin et al. 2009; Yusa et al. 2009; Yakubov et al. 2010; Hu et al. 2011).

In addition to the oncogenic potential and insertational mutagenesis associated with transgenes, iPSCs may also have an inherent susceptibility to genomic

infidelity. It has been proposed that somatic and germ cell lineages do not have the same level of protection of genome integrity. IPSCs undergo an initial reprogramming to a pluripotent state through enforced expression of synthetic transcription factors, as well as the transformation of an already semi-/fully-differentiated somatic cell type to a pluripotent state, and thus undergo two additional steps at which they can acquire genetic modifications (Puri and Nagy 2012); it has also been shown that some iPSCs retain an 'epigenetic memory' of the DNA methylation patterns of their original somatic cell type, or may retain specific micro-RNA patterns (Puri and Nagy 2012).

As with the "naturally occurring" sources of HSCs, with ESCs and iPSCs, the primitive state of the starting cells gives rise to a primitive hematopoietic program with generation of embryonic globin chains. Recently though, hematopoietic progenitors (progenitors of myeloid, erythroid and megakaryocytic, but not lymphoid lineages) have been generated directly from adult fibroblast cells, by-passing the primitive, pluripotent state and maintaining adult developmental patterns (Szabo et al. 2010).

5.5 Ex-Vivo Culture of Red Blood Cells

The complexity of hematopoietic cell development is a particular challenge when it comes to the production of blood cells *in-vitro*. Large scale cell culture has traditionally been confined to continuous cell lines which provide a relatively uniform cell population, thus the dynamic nature of blood cell maturation makes process optimization more difficult. Culture conditions, seeding densities and media formulations need to be adapted to each stage of maturation, efforts need to be made to maintain synchronicity of the cell populations as the culture progresses, and interactions with numerous soluble and cell/matrix-specific factors need to be taken into account. Although regulation of O_2 and pH in the bioreactor can remove some of this complexity in culture it is often difficult to maintain efficient gas exchange without excessive shear effects. Complications also arise from the reliance on serum supplementation of cell culture medium to support growth and protect cells from shear stress. Serum is undefined and contains unknown mitogens and growth factors, which can affect proliferation and differentiation of HSCs; there are also the safety implications to consider with the use of animal-derived serum, or components like albumin or transferrin, if the cells generated are destined for therapeutic use.

Due to their evolving nature, *in vitro* cultures of hematopoietic cell types typically follow a general format of multi-phase cultures with each phase enlisting different cytokine combinations and medium components with/without co-culture with stromal cells. Various physiological factors, such as dissolved oxygen, may also be regulated in a stage-specific manner. Below we will summarize research to date on the *in vitro* generation and expansion of RBCs.

5.5.1 *In-Vitro Erythrocyte Generation: Developing Culture Conditions*

The most obvious blood cell candidate for *in-vitro* production is the RBC, or erythrocyte. As discussed above, erythrocytes are by far the most transfused blood cell type. They are also the most populist cell type in the blood with at about 700-times the total number of leukocytes combined, and over 10-times the number of platelets (Table 5.1). A number of attempts have been made to create RBC substitutes including hemoglobin-based solutions and vesicles, and perfluorocarbon-based synthetic oxygen carriers (Sakai et al. 2008; Tsuchida et al. 2009; Castro and Briceno 2010); however, these products have yet to gain widespread regulatory approval and are unlikely to replace, or even supplement to any great extent, traditional blood donation. Thus research has become more focused on the *in-vitro* generation of blood.

There are three main challenges in producing RBCs *in vitro* – foremost is achieving the extremely high cell densities required through enhancement of cell proliferation in the stem/progenitor phase, followed by the maximization of differentiation and enucleation to generate a fully mature and physiologically functional erythrocyte population and finally, once cells have enucleated, removal of the extruded nuclei from culture.

Initial attempts at erythroid culture ex-vivo were based on colony-forming assays carried out in semi-solid medium. This format made it difficult to manipulate culture conditions and carry out prolonged culturing protocols, or achieve high cell densities to obtain sufficient cells for further analysis. However, improved availability of growth factors through the cloning of genes for SCF, Epo, and IL-3 in the late 1980s and their subsequent production in recombinant protein systems paved the way for the development of liquid culture systems that allowed expansion at a much larger scale and gave improved flexibility in sampling and analysis (Fibach et al. 1989, 1991). These liquid culture systems proved very effective for the study of pathways involved in both normal and pathological Erythropoiesis (Fibach and Rachmilewitz 1990; Dalyot et al. 1992; Fibach et al. 1993), and allowed further elucidation of the effects of different cytokine/growth factor combinations on proliferation and regulation of lineage commitment and differentiation of HSPCs (Hematopoietic Stem and Progenitor Cells) into each of the blood cell lineages (Brugger et al. 1993), and more specifically into exclusively erythroid cultures (Dai et al. 1991; Papayannopoulou et al. 1993; Muta et al. 1995; Wu et al. 1997) – see Sect. 5.3.1.

To date culture systems generating RBCs from the following stem cell sources have been described – PB, mPB, BM, FL, iPSC and ESC (Giarratana et al. 2005; Boehm et al. 2009; Anstee 2010; Pourcher et al. 2011). The processes employed typically follow a multi-phase format (two, three or four phases) in basal medium, supplemented with high levels of iron in the form of transferrin, bovine serum albumin (BSA), and insulin, with various cytokine cocktails (typically an expansion

phase with SCF, IL-3 and Epo, followed by a differentiation phase with Epo alone, and a maturation phase free from exogenous cytokines) and with/without feeder layers/stromal cells over the course of roughly 3 weeks of culture. Other optional additives include dexamethasone, a steroid hormone which can be added to the initial progenitor phase of culture to inhibit maturation and thus enhance proliferation; β-mercaptoethanol (to prevent excess generation of oxygen radicals), hydrocortisone (a glucocorticoid that promotes erythroid proliferation) and cyclosporine (to inhibit growth of contaminating lymhpocytes or monocytes).

The majority of erythroid cultures are carried out over the course of 21–30 days, yet it has been shown that by maintaining progenitor cells in an immature state, through sustained supplementation of the culture medium with SCF and steroids, much greater fold expansions can be achieved through extending proliferation phases of culture for up to 60 days (Leberbauer et al. 2005). However, it has also been shown that if the culture period is extended by too long differentiation is not as robust, and proper hemoglobinisation of cells is inhibited (Timmins et al. 2011).

By 2002 descriptions of mass production of mature erythroid cells was shown to be possible, however, at this point, no or low levels of enucleation were described (Neildez-Nguyen et al. 2002; Migliaccio et al. 2002). Luc Douays' group, at INSERM in Paris, suggested that enucleation *in-vitro* is not necessary and showed that erythroid precursors when injected into NOD/SCID mice, undergo terminal differentiation and enucleation in-vivo (Neildez-Nguyen et al. 2002). This first report looking specifically at the efficacy of producing RBCs *in-vitro* for transfusion managed a 200,000-fold mean expansion of $CD34^+$ cells isolated from UCB into a pure erythroid culture, however, as these cells were derived from UCB they produced predominantly foetal haemoglobin (26:74 HbA:HbF). Also using this serum-free system terminal differentiation was not possible *in-vitro* and the majority of cells died at the basophilic erythroblast stage in culture. In spite of this they were able to achieve terminal differentiation and enucleation by injecting Day 10 cells (predominantly BFU-E progenitors) into a mouse model. Injected cells remained viable, proliferated and completed maturation in-vivo with mature cells showing predominantly HbA production – the switch from HbF to HbA presumably being triggered by to the prevailing environmental conditions in the adult mouse (Neildez-Nguyen et al. 2002).

By 2005 the same research group had improved their expansion rates from UCB $CD34^+$s by tenfold to up to 2×10^6–fold expansion (with $1. \times 10^5$ and 1.2×10^5–fold expansions respectively from BM, and mPB derived $CD34^+$s) and saw full enucleation *in vitro*, producing cells that were functionally comparable to native, adult RBCs. This was achieved through co-culture with either the MS-5 murine stromal line or with mesenchymal stem cells throughout differentiation. Again, $CD34^+$s from adult sources produced HbA while those from UCB produced predominantly HbF (Giarratana et al. 2005).

The following year Yukio Nakamura's group in Japan showed that enucleation was possible *in-vitro* without the presence of stromal cells; however, these cultures

contained Plasmanate (a commercially available human plasma derivative). Between 40 and 80 % enucleation was shown with higher levels of enucleation in cultures containing a glucocorticoid inhibitor – highlighting the effect of serum/plasma glucocorticoids in blocking maturation (Miharada et al. 2006).

As well as functional studies of derived cells, molecular studies have shown the reproduction of in-vivo patterns of erythroid gene expression in these cultures, however, further understanding of the exact pathways involved, and the nutritional requirements of *in-vitro* erythroid development is still required as many of these cultures still show high levels of cell death in the later stages of differentiation – a significant problem if these cells themselves, rather than a secreted product, are the intended therapy (Ronzoni et al. 2008).

Once effective erythroid development was proven, optimisation studies began to look to further improving culture viability and increasing fold-expansions. By altering the feeding strategy to a daily passaging regimen, Boehm et al. (2009) managed to increase the fold-expansion of non-mobilized peripheral blood-derived $CD34^+$ cells to 1×10^6 – a level that had only previously been shown for UCB derived cells, a source with much higher proliferative capacity. This was likely achieved through the reduction in levels of inhibitory factors by daily dilution of culture medium.

Another strategy to improve proliferation is to maintain $CD34^+$s in an immature and self-renewing state for a period before allowing high-level proliferation and differentiation of cells. This can improve the proliferations achieved by almost 1,000-fold. Fujimi et al. (2008), achieved this by co-culturing CB derived $CD34^+$s with human telomerase (*h-tert*) transduced stromal cells for 2 weeks in serum-free medium containing SCF, thrombopoietin, and FLT-3/FLK-2 ligand before a second culture phase with Epo, a third with/without macrophage co-culture and a final, fourth, maturation phase, to yield the equivalent of 8 transfusable blood units from 1 CB unit (Fujimi et al. 2008). Removal of extruded nuclei and any cellular debris from cultures was achieved by using de-leukocyting filters.

As well as macrophages and murine or human stromal cells, mesenchymal stem cells have also been shown to be effective in co-culture studies (Giarratana et al. 2005; Baek et al. 2008). Co-culture with MSCs from day 8 of culture, either for 3 days (Giarratana et al. 2005; Baek et al. 2008), or for up to 2 weeks (Baek et al. 2008), has shown to be very effective in inducing terminal differentiation and enucleation; it would appear that the function of stroma is in its capacity as an adhesive substrate for developing erythroblasts, as cells co-cultured without direct cell contact were unable to enucleate. The source of cells for stromal co-culturing is also significant, as it has been shown that co-culturing with CB-derived mesenchymal stromal cells is more effective than using those derived from BM (Baek et al. 2008).

In spite of the high-fold expansions and maximized enucleation that have been shown in erythroid cultures these have generally been achieved through a reliance on serum/plasma-supplementation, conditioned medium (e.g. from bladder carcinoma cell line 5637-ref) and co-culture with stromal cells. Low viability at the later

stages of culture are contributed to by shear stress due to the fragility of erythroid membranes, and although one group has shown improvements on fold expansions and viability in serum- and stroma-free culture through the addition of the chemical surfactant Poloxamer 188 to protect from shear, the expansions achieved from UCB $CD34^+$s were relatively low compared to other reports (Baek et al. 2009). The most impressive reports of serum/stroma-free culture so far come from a group at the University of Queensland who have shown very high levels of expansion and full terminal maturation in defined culture conditions using $CD34^+$ cells from UCB owing to an extended proliferation phase (Timmins et al. 2011). Kim and Baek (2011) have shown a similar fold expansion of UCB $CD34^+$s up to day 13 of culture, although in this case the proliferation phase was not extended so the same overall expansion was not achieved. This group also showed an improvement in viability in the later stages of culture through the addition of vitamin C and hypothermic culture at 27 °C (Kim and Baek 2012).

In spite of this most groups still rely on a serum-free proliferation phase followed by a differentiation phase (at which stage erythroid precursor cells are more prone to apoptosis) using human plasma/serum (2–5 %) to prevent apoptosis of developing erythroblasts. Human plasma, although still not ideal, provides less worry in terms of safety and in certain situations autologous plasma could be used for patient-specific blood cell production.

5.5.2 Manipulation of Physical Conditions

A novel approach to enhancing ex-vivo erythroid cultures has been through the modification of cell culture surfaces. Although most of the research carried out on erythroid culture development is in cell culture plates or T-flasks maintained under static conditions, not much attention had been paid to the subject of culture surfaces as the cells themselves are not adherent. Yet the rationale in testing the effects of physical parameters in erythroid culture is well-founded given the impact of physical effects on in-vivo Erythropoiesis, such as the adhesive interactions with various stromal cell types and ECM proteins in the BM, and the intimate association in-vivo of erythroblasts with macrophages as they develop. Added to these adhesive interactions are various physiological gradients within BM such as pH, O_2, and even shear effects.

Bearing in mind the net negative charge that erythrocytes display Baek et al. (2010) tested the effects of altering the surface charges of culture plates, speculating that binding of negatively charged cells to positively charged culture surfaces could go some way to mimicking cell-stroma interactions. By altering culture surfaces with hydroxyl, carboxyl and amide groups, they showed that the use of positively-charged hydrophilic surfaces, as opposed to hydrophobic surfaces; negatively charged surfaces; and a combination of positive and negative charges, almost doubled the level of enucleation of erythroid cultures derived from CB $CD34^+$s (Baek et al. 2010).

The same group have also looked at temperature of culture in the later maturation stages that typically show a high proportion of cell death. Reduction of culture temperature to 27 °C when the cell population had reached the orthochromatic erythroblast stage led to a reduction in apoptosis, an increase in enucleation, and an improvement in cellular integrity (Kim and Baek 2012). The addition of vitamin C in the final stages of culture also showed a protective effect and a reduction in the levels of reactive oxygen species (Kim and Baek 2012). The effects of manipulating other physical parameters have also been explored, notably pH and oxygen tension.

Cell cultures are maintained at 20 % pO_2 (roughly atmospheric level) under typical laboratory conditions in a 5 % CO_2 incubator, even though physiological levels are generally in the range of 2–8 %. Thus an obvious parameter to analyse when optimising culture conditions for primary cells is oxygen tension. Results from various studies have, however, been contradictory in this regard.

The first demonstration of liquid erythroid cultures by Fibach et al. employed low oxygen tension (6 %), along with raised CO_2 (7 %), in phase 2 after an initial Epo independent phase but this report did not provide comparisons to normoxic conditions (Fibach et al. 1989).

Hypoxia (1 or 5 % O_2) has been shown to promote erythroid development and maintain early Erythropoiesis in colony-forming assays and liquid culture, and although further differentiation is inhibited at this level once cells return to normoxia differentiation proceeds as normal (Lu and Broxmeyer 1985; Cipolleschi et al. 1997). Analysis of growth at physiological levels (2–8 % O_2) throughout the course of proliferation and differentiation in a liquid culture system showed that decreasing O_2 levels had a cytotoxic effect compared to normoxic (20 %) conditions, and led to reduced growth, Epo-receptor expression and haemoglobinization, along with an increase in the ratio of HbF to HbA; although reduced O_2 did seem to promote differentiation of cells (Rogers et al. 2008). Analysis of altered O_2 concentrations in a phase-specific manner also showed an improvement in erythroid development following hypoxic-incubation of immature erythroid cells as opposed to more mature cells of later phases; this study also confirmed the increase in HbF production at lower O_2 tensions (Vlaski et al. 2009). A criticism of these studies however, is that O_2 concentration was regulated at the level of the incubator rather than taking measurements directly from culture. In-culture variations in oxygen tension in well-plates can be strikingly large, due to cellular uptake, altering cell densities as cultures proliferate, and diffusional effects of gas through culture media, thus the actual dO_2 levels in the cultures examined are likely to be quite different to incubator set-points.

Effects of shear stress and agitation of cultures, and the possibility of their knock-on effects on gas-diffusion and improved mixing of cultures, have also been examined. In 24-well plate format agitation at 15 rpm (rocks per minute) was shown to improve progenitor growth in the proliferation phase and hasten differentiation, possibly though the prevention of cell-surface adhesion (Boehm et al. 2010). However, under these same conditions, viability was decreased during the differentiation stage (Boehm et al. 2010).

A contrasting study described a negative effect of agitation on early proliferation in low density cultures, although the same effects on viability during differentiation were replicated in a 1 L wave-bioreactor set to the same agitation rate (Timmins et al. 2011). Initially in this larger-scale study the drop in viability was seen during maturation phase if cultures were maintained at normoxia, however, if the oxygen tension was decreased by 50 % the cultures remained viable and gave comparable results to the static control (Timmins et al. 2011). This lead the authors to speculate that the cell death effects of increased agitation during maturation were due to increased gas diffusion rather than a direct effect of increased agitation culminating in increased shear and/or decreased substrate adhesion.

As acknowledged previously the regulation of the culture environment during stem cell cultivation is very important for erythroid development *in-vitro*, and even seemingly small changes in culture parameters can affect proliferation and differentiation. Changes in pH as small as 0.2 points can affect lineage decisions in HSC culture, and a small shift from pH 7.2–7.6 has been shown to an increase in the proportion of erythroid cells from approximately 50–80 % along with increasing cell density (McAdams et al. 1998). Even cells grown in the same medium formulations purchased from different suppliers can show differences in proliferation and differentiation, presumably due to slight differences in buffering capacity (Susan Browne, personal observation).

Environmental regulation is difficult though in the static culture systems currently being used where cells are typically cultured in T-flasks with dilution feeding at fixed time-points days apart, rather than a constant perfusion of medium that is maintained at set points for pH and dissolved gases. These medium dilutions lead to sharp changes in the concentration of nutrients and cytokines, and dilution of autocrine and paracrine factors (Timmins and Nielsen 2011), and also cause a sudden change in dissolved O_2 profile of the culture (Susan Browne, unpublished data); thus it is difficult to maintain a culture environment that mimics in-vivo conditions.

5.5.3 Scale-Up of In-Vitro Erythropoiesis

In terms of scale up of RBC manufacturing, only one group has published data on culturing to terminal maturation at bioreactor scale at present, using a bag/wave-type bioreactor for the later stages of culture (from day 8). However, this process uses a large amount of media due to its feeding strategy, with cultures beginning at 1×10^4 cells/ml and diluted to 1×10^5 at regular intervals (Timmins et al. 2011). These low seeding densities are common in most culture protocols for *in vitro* RBC production with Lahoti et al. (2012) showing that, for optimum expansion, cultures need to be reseeded to a density of 3×10^5 cells/ml every 36 h during proliferation (Fig. 5.7). This requirement for low seeding densities has been suggested to be due to a cell-cell interaction associated inhibition, as cell density as a function of

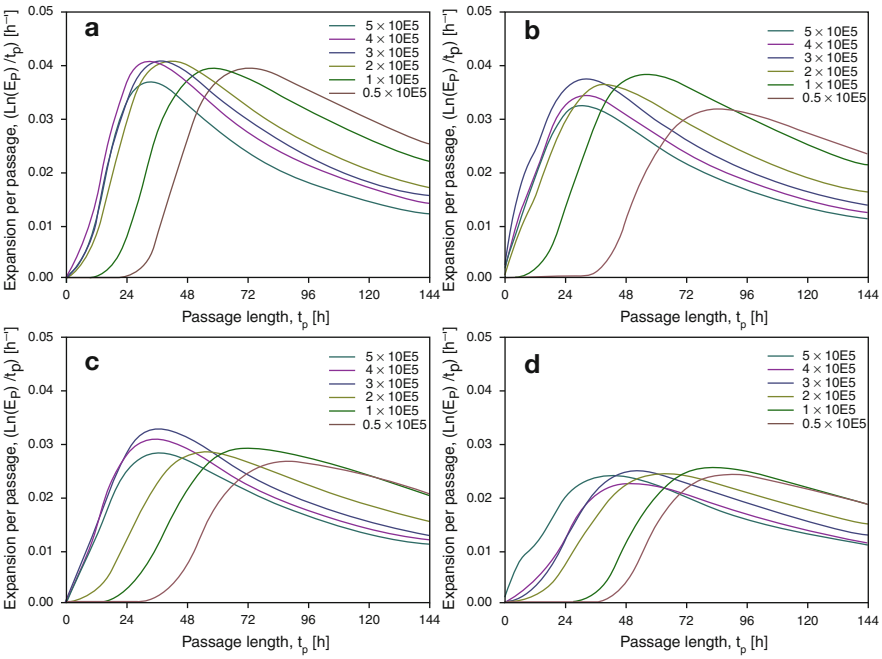


Fig. 5.7 Calculations of optimum seeding densities and medium renewal rates for erythroid differentiation of HSCs (Lahoti et al. 2012)

surface area, rather than media volume appears to affect proliferation, although the opposite is true during differentiation where increased cell density confers a protective effect (Boehm et al. 2009). Another likely reason is the induction of feedback-inhibition by paracrine factors secreted by more mature sub-populations in culture (Csaszar et al. 2012). This would also make sense in terms of surface area/cell volume findings, as although these are suspension cells, the majority of culture systems used to-date are maintained under static conditions and thus cells settle to the bottom of the well. Here there are likely locally high concentrations of these inhibitors, rather than homogenous dilution of cell-secreted factors throughout the culture medium.

Another recent report has shown generation of erythroid-lineage cells in a novel hollow-fiber reactor (Housler et al. 2012). These reactor types allow higher cell densities to be achieved through more efficient nutrient and gas-exchange, as they offer a structure that is closer in format to in-vivo conditions. Here cells were maintained in a "pseudo-continuous" culture with intermittent removal and re-inoculation of cells, although they did not show the same expansion and differentiation levels of other studies in static conditions, or in the wave-type reactor (Housler et al. 2012).

The type of bioreactor required for RBC production is likely to be of a complex formation rather than a simple stirred-tank due to the complexity of culture process and to enable isolation of fully mature cells in a homogenous population, and free of nuclei. It seems imperative however, that a move is made soon to bioreactor culture, whether small or exploratory scales, if significant improvements in this culture system are to be achieved. Stem cells are notoriously sensitive to their microenvironment, and thus that environment needs to be regulated as much as possible to enable repetition and homogenization of results.

5.5.4 Generation of Erythrocytes from Pluripotent Stem Cells

Inevitably research in this area has begun to focus on the generation of RBCs from ESCs and iPSCs. As cell lines these sources would, in theory, provide an infinite source of cells for differentiation into RBCs. Erythoid progenitor cells can be generated from pluripotent stem cells either by co-culture of cells with stromal layers such as foetal liver cells, yolk-sac endothelial cells, bone marrow or OP9 stromal cells; or via the generation of embryonic bodies and haemangioblasts (embryonic precursors of blood and endothelial cells) (Olivier et al. 2006; Lu et al. 2008). So far, however, ES and iPSC have shown limited expansion *in vitro* in culture systems designed to produce RBCs, are complex and expensive in terms of culturing conditions and cytokine cocktails required, do not show consistent growth in culture, show primitive patterns of differentiation (with expression of embryonic and foetal globin chains), and lower enucleation levels than more definitive sources . It is obvious also, that a greater understanding of the process of haemoglobin switching from primitive to definitive forms is required, although this is also applicable in the case of cells derived from UCB $CD34^{+}$s.

However, research is continuing at a fast pace in this area due to the potential that these cell lines offer once conditions for their growth and differentiation are optimised, with Peyrard et al., calculating that only 3 iPSC cell lines would be necessary to cover 90 % of alloimmunized Caucasians (Peyrard et al. 2011). Given that most of the research to date on these cell lines has been directed from the angle of molecular and cellular functionality, rather than taking a bioprocessing approach of cell line selection and culture optimisation, it is likely that there is a lot of scope in this area for further improvement of yields and proliferation efficiencies.

Pourcher et al. (2012) have proposed the *in-vitro* RBC generation from foetal liver derived HSCs as a study model to improve the amplification potential of these cell lines in the meantime. They suggest foetal liver HSCs to represent an intermediary state between ES/iPSCs and more mature UCB or adult derived HSCs as they show similar differentiation patterns to the more primitive cell types, with foetal globin chains and low enucleation, but show much higher proliferation than ES or

iPSC, at up to two-log folds higher than even bone marrow or CB derived HSCs (Pourcher et al. 2011).

A recent advancement by the Nakamura group in Japan has been the generation of cell lines following initial direction of ESCs towards the erythroid lineage, rather than using ESC cell lines and carrying out an extended erythroid differentiation step repeatedly with each culture. Initially showing this for murine cells (Hiroyama et al. 2008), they have since replicated the process for human cells to generate an immortalized erythroid progenitor cell line, that is capable of differentiation and maturation *in-vitro* (Hiroyama et al. 2013).

5.5.5 Current Status of In-Vitro Erythropoiesis

Proof of principle studies have already shown the efficacy of *in vitro* generated RBCs in vivo in both mice and humans, and functional studies have shown that *in-vitro* generated cells show comparable profiles to their ex-vivo counterparts (Neildez-Nguyen et al. 2002; Giarratana et al. 2005, 2013). It has also been shown that once generated, these cells can be stored (Migliaccio et al. 2009; Kim and Baek 2012) and in theory they could have an extended storage duration as the cells produced in culture are synchronous in terms of age and thus could be stored for up to 120 days, in comparison to the 42 day cut-off for donated blood.

Meeting the demands of the RBC market is still a long way off due to the huge volumes of cells required. A typical unit of RBCs (roughly 500 ml) contains 2×10^{12} cells. Timmins and Nielsen (2011) have calculated that, operating at current expansion levels, it would take 30,000 batch cultures in 20,000 L bioreactor systems operating at a maximum cell density of 5×10^7 cell/ml to meet the annual RBC requirements of the US alone, and at present even meeting the requirements of transfusion recipients who are alloimmunized or have rare blood types would be difficult – described in Table 5.3 (Timmins and Nielsen 2009, 2011).

However, the pace of research into optimising culture conditions is intensifying, including the move towards fully defined culture medium; the reduction in medium costs through the identification of suitable peptide mimetics and small molecules to replace expensive cytokines; and the development of improved and intensified bioreactor systems.

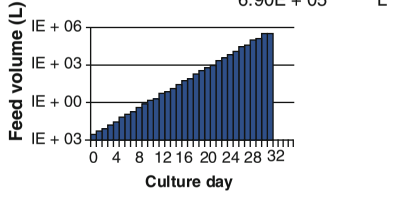
HSCs from peripheral blood and UCB/placenta probably represent the most likely sources for blood cell production for the short- to medium-term future for safety reasons while further optimisation and validation of pluripotent systems is carried out. The most favourable scenario is for research to proceed on all HSC sources for the foreseeable future, with PB and UCB HSCs used as source material in the interim until all safety concerns are met with iPSCs. It seems clear however, that the routine use of *in-vitro* generated blood products is no longer a case of if, but a case of when.

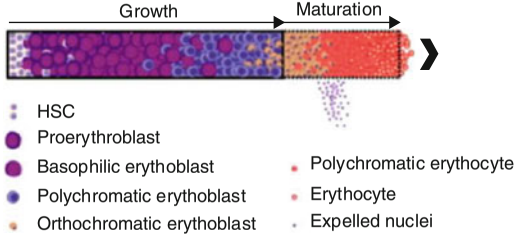
Table 5.3 Hypothetical costing for large scale RBC production (Timmins and Nielsen 2011)

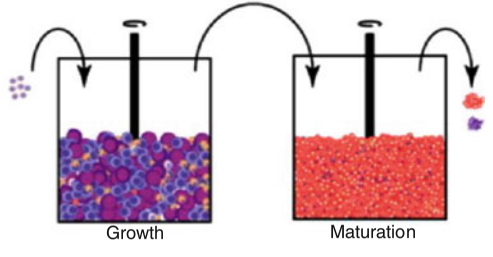
Production requirements		
Recipients	3.00E + 05	/year
Transfusion rate	3	units/recipient
Phenotypes	10	
Units/phenotypes	9.00E + 04	/year

Continuous process (per phenotype)		
Operating period	365	days/year
Production rate	247	units/day
Cell flux	2.05E + 13	cells/hour
Doubling time	12	hours
Number of growing cells	3.56E + 14	cells
Maturation time	48	hours
Number of maturing cells	9.86E + 14	cells
Total cell number	1.34E + 15	cells
Cell density	1.00E + 09	cells/mL
Operating volume	1342	L
Hollow fibre diameter	100	μm
Hollow fibre length	170873	km
Membrane area	53681	m^2
Media capacity	5.00E + 07	cells/mLday
Feed rate	2.68E + 04	L/day
Media consumption	9.80E + 06	L/year

Fed-batch process (per phenotype)		
Batch duration	35	days
Cycles	10.4	/year
Batch yield	8.63E + 03	units/batch
Maximum density	5.00E + 03	cells/ml
Maximum operating volume	3.45E + 03	L
Total volume	6.90E + 05	L
Media consumption	7.20E + 06	L/year







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Chapter 6
Bioprocessing Challenges Associated with the Purification of Cellular Therapies

Benjamin D. Weil and Farlan S. Veraitch

Abstract An ever increasing number of cellular therapies are in development for the treatment of a wide variety of diseases. One of the major challenges facing the translation of whole cell products into licensed therapies is the development of efficient, scalable purification processes. Current devices struggle to provide high resolution separations whilst ensuring sufficient cellular recovery. The removal of soluble and intracellular contaminants during processing is largely mitigated with precedence upon upfront validation of all materials and supplies. However, with an increasing number of cellular products reaching clinical trials, stricter regulation and adherence to biotech safety standards will require the design of novel, built-for-purpose purification systems. The most significant developments have been within the microfluidics field, where intrinsic physical and biochemical cell properties are utilised to fabricate high purity, high yield systems. With current research pushing the boundaries of throughput and looking to integrate microsystems into upstream processing, a new era of cell purification systems, based on regulatory requirements instead of retrofitting available technology from similar industries, may be fast approaching.

Keywords Cellular therapy • Purification • Cell isolation • Soluble contaminants • Intracellular contaminants • Impurities • Cell bioprocessing • Downstream processing • Cell sorting • Stem cells • Regulation • Novel separation devices • Microsystem • Microfluidic • Apheresis • FACS • MACS

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Abbreviations

ATPS	Aqueous Two-Phase Systems
CGMP	Current Good Manufacturing Practice
CGTP	Current Good Tissue Practice
CQAs	Critical Quality Attributes
CTCs	Circulating Tumour Cells
DEP	Dielectrophoresis
DEP-FFF	Dielectrophoretic Field-Flow Fractionation
DMSO	Dimethyl Sulfoxide
FACS	Fluorescent Activated Cell Sorting
FBS	Foetal Bovine Serum
FDA	Food and Drug Administration
GFP	Green Fluorescent Protein
GMP	Good Manufacturing Practice
GvHD	Graft versus Host Disease
HDE	Humanitarian Device Exception
hESCs	human Embryonic Stem Cells
HUD	Humanitarian Use Device
IND	Investigational New Drug application
LCM	Laser Capture Microdissection
mAb	monoclonal Antibody
MACS	Magnetic Activated Cell Sorting
mESCs	mouse Embryonic Stem Cells
MOFF	Multi-Orifice Flow Fractionation
MSCs	Mesenchymal Stem Cells
NCs	Nucleated Cells
PBPCs	Peripheral Blood Progenitor Cells
PDMS	Polydimethylsiloxane
PEG	Polyethylene Glycol
PMA	Pre-market Approval
QA	Quality Assurance
QC	Quality Control
RBC	Red Blood Cell
RPE	Retinal Pigment Epithelium
TRP	Tissue Reference Group
WBC	White Blood Cell

6.1 Introduction

Drug manufacture processes have been developed and refined over decades, hand-in-hand with regulatory bodies, to allow the creation of generic purification platforms. For example biopharmaceuticals based on recombinant protein production

frequently use a system comprising of three chromatography purification and polishing steps. For the production of clinical-grade material at commercially viable scales, techniques to remove viral and bacterial contaminants must be demonstrated and validated. Pharmaceuticals rely on harsh (usually pH dependent) viral inactivation steps, followed by nanofiltration to ensure viral removal (Yokoyama et al. 2004; Zhou et al. 2008). For cellular therapies, environmental conditions must operate within a much narrower bandwidth with cell viability, and consequently product efficacy, being paramount. Various instances of viral blood or respiratory infections following stem cell transplants have been reported (Yokoyama et al. 2004; Han et al. 2011; Liu et al. 2011a; Forest et al. 2011; Rynans et al. 2012; Busca et al. 2012). Similar risk factors exist for microbial contaminations. Large volume leukapheresis (purification) of peripheral blood stem cells was monitored over a 12 year period. Administration to patients demonstrated the transfer of microbial pathogens, with contamination through thawing and processing steps noted (Donmez et al. 2012).

The handling and manipulating of living material requires purification devices to maintain sterility, while operating with an environment which maintains biological viability. Intrinsic variation between the source of cellular material (from a cell bank, donor or autologous transplant) has led to unique biological, processing and engineering demands for cell therapies (see Fig. 6.1). Pluripotent cells require a directed differentiation towards a rare target cell type, from within a heterogeneous population (Serra et al. 2012). To identify and subsequently isolate the target cells, purity and process efficiency are vital due to low cell numbers. Therapies derived from blood constituents require a lower resolution separation, usually concentrating a cell type before intravenous administration. Maintaining sterility is key for these purification steps to ensure no foreign pathogens are introduced. Allogeneic donor material from a cell bank will be cryopreserved for storage and transportation. In this instance a further purification step to remove the cryopreservative is necessary. Due to the diverse purification concerns for different cell sources, few systems are designed for generic usage, with therapeutic purification strategies currently derived on an almost case-by-case basis.

Another challenge associated with cell purification is the cost, which varies greatly dependent on the process utilised (Seeger et al. 2007). Processing of autologous peripheral CD34-positive blood stem cells was assessed in three medical centres across America. The cost of pre-apheresis, the leukapheresis procedure followed by thawing, product assays and sterility testing was assessed (Meehan et al. 2000). Although the same procedure was used, the expenditure of each institution varied greatly, demonstrating the need for clarification and standardisation over cell therapy purification.

6.2 Purification Requirements

6.2.1 Removal of Cellular Contaminants

Due to their ability to differentiate into multiple cell types and proliferate extensively, stem cell based therapies have a range of explicit safety concerns specified

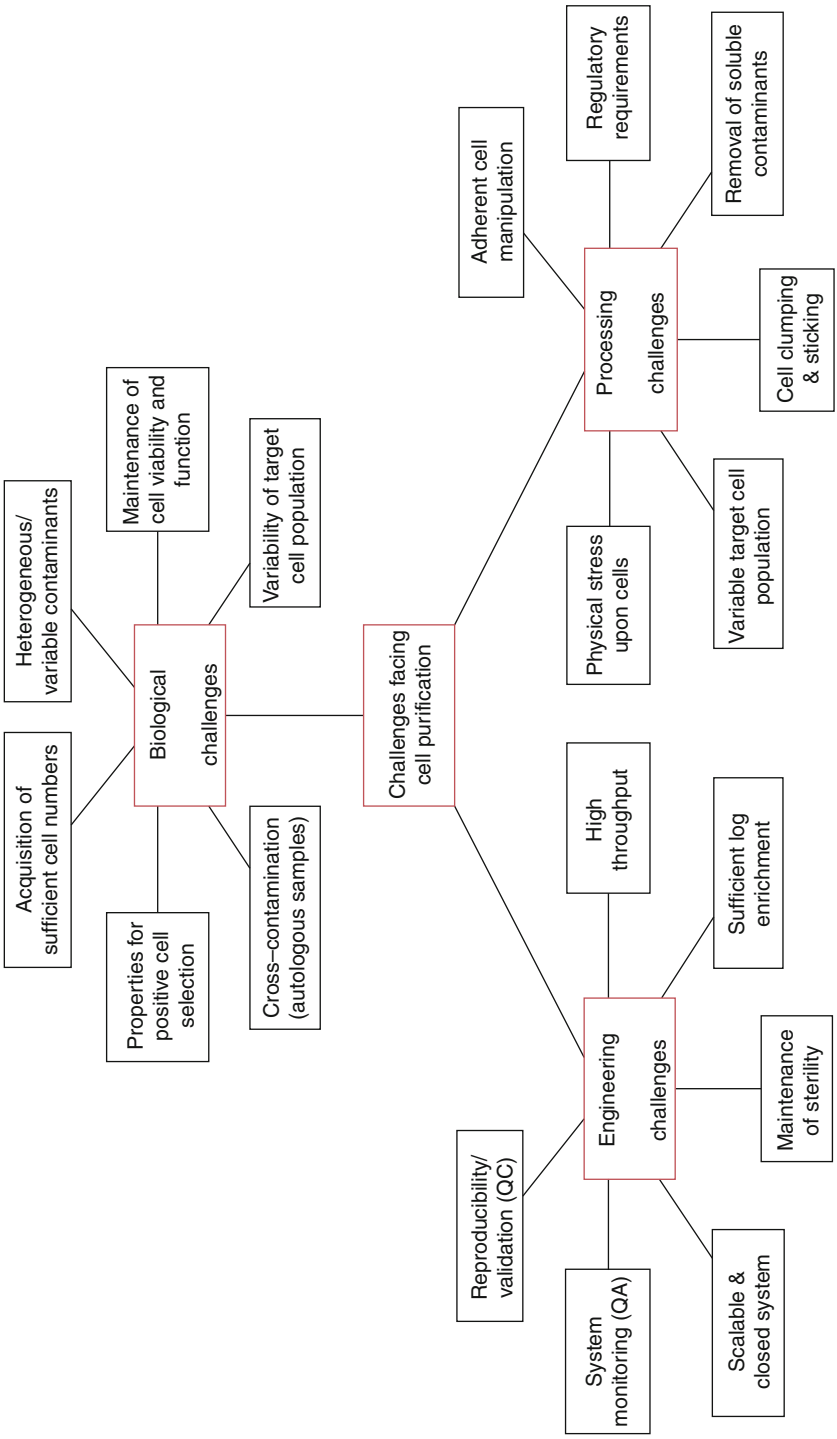

Fig. 6.1 Challenges associated with the purification of cellular therapies

Table 6.1 Specific burdens for cell-based products prior to human transplantation

Concern for cellular product	Assessment criteria for each concern
Product composition	• Individual cells types present
	Note: Product can contain more than one cell type and be classed as pure
	Relative cell proportions (analysis of marker expression)
	Cell viability
Product purity	• Key contaminants (e.g. undifferentiated stem cells)
	Note: The inability to detect a specific contaminant does not prove purity, safety studies in animal models are necessary
	Bacterial/viral release panel testing
Product potency	• In vitro testing of cellular functionality
	• Animal models

Adapted from Halme and Kessler (2006)

by the U.S. Food and Drug Administration (FDA). Key regulatory issues include the propensity for teratoma formation via undifferentiated or partially differentiated cells; the migration of cells from their intended transplantation site; elicited immunogenic responses and cellular impurities formed during differentiation protocols (Fink 2009). For GMP approval, quality control (QC) of cellular products must assess phenotype, functional activity, microbiological safety, and cell types present (Sensebé 2008). Quality assurance (QA) protocols are specific to each process, but general areas of interest for cellular products have been generated (see Table 6.1).

Autologous therapies have previously received greater lenience with regard to transplantation, as cells are sourced and returned to the same patient. However since 2006, the FDA has slowly trended towards tightening these guidelines (Freeman and Fuerst 2012). The Tissue Reference Group (TRG: an FDA-created panel to deal with the regulation of human cellular and tissue-based products) claimed that the autologous (bedside) separation of stem cells from adipose tissue, for either orthopaedic or breast reconstruction procedures, parallels the manufacture of a drug (Rodriguez 2012; Tissue Reference Group 2011). Tissue-based procedures which take place in the same operating session are currently exempt from FDA regulation, instead being controlled by medical professional societies. By classifying the procedure as a biologic, surgeons will have to submit an Investigational New Drug application (IND) to the FDA. This process is time consuming and expensive.

Core requirements for Current Good Tissue Practice (CGTP) centre on the prevention of communicable diseases being introduced or spread through treatment. This criteria is ensured through the screening and testing of critical quality attributes (CQAs). These CQAs include cellular identity – confirming presence of the cell population; potency – to ensure biological functionality is retained; purity – ensuring no unwanted cell types or residual impurities remain; and safety – to guarantee no pathogenic microorganisms or tumorigenic material is present (Carmen et al. 2012). More information regarding the regulation of cellular therapies has been published by the FDA and related sources (Fink 2009; Halme 2006; Burger 2003).

6.2.2 Removal of Soluble (or Intracellular) Contaminants

In addition to the removal of unwanted populations and cell debris, processing components and other external contaminant risks must be considered during purification. Supplements added during manufacture including growth factors, cryopreservatives, enzymatic solutions and serum must be removed before administration. Safety concerns over the introduction of endotoxin, bacterial, fungal, parasitic or viral contaminants must be addressed. Many process impurities such as host cell proteins are removed via chromatographic steps in biopharmaceuticals (Shukla et al. 2008). For cell therapies, instead of removing these contaminants during processing, upfront sourcing of GMP components is opted for. All materials used during manufacture must be purchased through regulatory approved suppliers, and validated for therapeutic usage. As a result, cell purification devices often only address extracellular contaminants (see Table 6.2). A complete buffer exchange is required to ensure soluble contaminant removal, with the buffer dilution volume dependent on purification demands. A compromise between wash steps (centrifugation) and the risk of cellular damage through shear or biological change must be made. Separating rare populations with low cell numbers exacerbates this requirement to mitigate excess washing. Cell losses of 20–30 % are expected with washing steps (Lemarie et al. 2005).

Viruses, Bacteria and Fungi

Preventing the transmission or introduction of communicable disease is the primary goal of regulator guidance. Viral contaminants present a significant challenge for cell therapies, with viral inactivation (via low pH treatment) impossible due to the need to maintain cell viability. Quality control combined with strict sourcing of material and process components is usually the required route. Detection systems now exist for bacterial and viral contaminants such as real-time PCR assays or immunomagnetic separation combined with functionalised quantum dots, which utilise fluorescent nanocrystals to detect specific contaminants (Liandris et al. 2011; Choi et al. 2009). These systems are cheap, robust and can be disposable (Cooney et al. 2012). It is also possible to utilise immunochromatographic strips (lateral flow tests) for the detection of viruses (Xiang and Li 2011). Multiple analytes can be tested simultaneously on the same strip, providing a fast response to any pathogen tested.

More recently novel cell isolation devices have been developed with virus removal in mind. Microfluidic platforms possess the ability to separate pathogens from human cell samples based on intrinsic cell and fluid properties, such as particle size-based diffusion (Zhao and Cheng 2011; Jung et al. 2011). Elsewhere, semi-automated high throughput devices have been designed to remove bacteria from blood samples (Wu et al. 2009). Over 80 % removal of fungal contaminants from whole blood has been demonstrated, with flow rates up to 20 ml/h (Yung et al. 2009). Similar techniques have been utilised for DNA isolation from whole blood,

Table 6.2 Breakdown of impurity removal from current cell separation techniques

	Contaminant to be removed											
	Extracellular		Soluble									
Cell separation procedure	Undesired cell populations + apoptotic cells	Cell debris	Extracellular protein (matrix/host cell proteins)	Trypsin/ collagenase	Serum	Viruses	Endotoxin	Bacteria	Cryopreservative (DMSO)	Xenogeneic proteins	Leachables	Growth factors
Density gradient centrifugation	✓[a]	✓	✓	✓	✓	×	×	×	✓	✓	✓	✓
Continuous flow fluidised bed (KSep)	×	✓	✓	✓	✓	×	×	×	✓	✓	✓	✓
Filtration	×	✓	✓	✓	✓	×	×	×	✓	✓	✓	✓
FACS	✓	✓	×	×	×	×	×	×	×	×	×	×
MACS	✓	✓	×	×	×	×	×	×	×	×	×	×
Chromatography (Cryogels)	✓	✓	✓	✓	✓	×	×	×	✓	✓	✓	✓
Field-assisted separations	✓	✓	×	×	×	×	×	×	×	×	×	×
Panning	✓[a]	×	×	×	×	×	×	×	×	×	×	×
Manual dissection	✓	×	×	×	×	×	×	×	×	×	×	×

[a]Addition higher resolution cell purification steps (such as MACS) may follow

suggesting that if sufficient throughputs can be achieved, soluble contaminants could be efficiently removed via microfluidic platforms (Wiesinger-Mayr et al. 2011). The combination of pathogen and cell purification has been achieved with a microfluidic device, operating to remove microbes as well as platelets and leukocytes from whole blood (Wei Hou et al. 2012). The device operates at 1 ml/h with over 80 % removal of pathogens and inflammatory cells.

Mycoplasma

The most notorious and prevalent external contaminant is often mycoplasma (Armstrong et al. 2010). Mycoplasma is a type of bacteria which does not contain a cell wall. The first noted contamination of human cell cultures was in 1956 (Robinson and Wichelhausen 1956). Infections present a significant challenge to the cell therapy industry today as Mycoplasma tends to proliferate at a slow pace in cell culture making infections difficult to detect. As a result, these properties can lead to facility-wide infections. Nucleic acid-based detection platforms exist to identify infected cells (allowing batch quality control testing), but the only real solution to prevent the spread of mycoplasma lies with well defined, characterised processes and adherence to CGTP (Lawrence et al. 2010). This is achieved by ensuring all process components are obtained through validated vendors and stored correctly (as detailed in the Code of Federal Regulations – FDA 2001).

Serum

Foetal Bovine Serum (FBS) is commonly used as a nutritional supplement for cell culture media (Kuznetsov et al. 2000). Although occurrences are rare and often only noted after long-term observation, immune reactions can occur in patients who have received transplanted cells which have been cultured in FBS-rich media (Phinney and Prockop 2007; Selvaggi et al. 1997). In addition, animal derived products present in the manufacturing process invite the risk of viral, fungal or bacterial contamination through the use of livestock (Focus on Alternatives 2009). Attempts to mitigate the serum demand from cell culture have demonstrated success, with various serum-free protocols now available (Amit et al. 2004; Ricci-Vitiani et al. 2007; Thomas et al. 2009). However, purification systems must be able to efficiently and reproducibly remove serum. An added complication is the retention of xenogeneic proteins from FBS in the cytoplasm of cells, removal of which would result in the loss of cell viability and functionality (Spees et al. 2004). Even with numerous drawbacks, FBS containing media remains a ubiquitous supplement for cell culture and is employed in research laboratories around the world, due to FDA approval for human clinical products. Further discussion with regard to the use of serum in clinical applications is necessary, with a predicted move towards serum-free media for cell therapies.

Cryopreservatives (DMSO)

Cryopreservation expands the commercial opportunity of cell therapies by permitting independent sites for collection, manufacture and administration. By extending the shelf life, product safety is improved during transportation and storage (Hubel 2011). Various side effects have been correlated with the presence of the cryopreservative dimethyl sulfoxide (DMSO) in clinical studies, leading to the conclusion that removal is necessary before infusion of cellular products. In transfusion studies of autologous peripheral blood progenitor cells (PBPC) without DMSO removal, 25.25 % of patients presented side effects (Donmez et al. 2007). Side effects ranged from hypertension and lung oedema to nausea, vomiting and dizziness. A correlation between DMSO concentration, infusion volume and instances of patient side effects was established. To confirm this assessment, studies into the prevalence of side effects following a (dilution) wash step have demonstrated a significant decrease in the number and severity of cases (Lemarie et al. 2005). Furthermore, wash steps have been shown to illicit negligible impact upon cell efficacy (Nagamura-Inoue et al. 2003). Various wash buffers are used to dilute the sample in stages to avoid cell osmotic shock, with the process lasting around an hour (Akkök et al. 2009). Various new microfluidic platforms are in development which aim to increase throughput while demonstrating efficient removal, without affecting cell recovery or viability (Mata et al. 2008). The FDA currently defines DMSO as a class 3 solvent in ICH Q3C, with an upper limit of 50 mg/day recommended dosage. In practice, regulators allow therapies to include up to a gram per kilogram per day (FDA 2011b). With mounting evidence against its injection, especially in solid tissue where diffusion is limited, regulation regarding safe dosage levels are expected to tighten.

6.3 Existing Cell Purification Systems

Operational characteristics of commercial cell separation devices vary greatly dependent on the method of separation and cell type isolated (see Table 6.3). The most commonly used cell purification method is apheresis – the gold standard for blood derived therapies (Szczepiorkowski et al. 2010). One alternative is filtration systems, which provide low shear separations for soluble and cellular contaminant removal (Aoki et al. 2004). For the isolation of rare cells within a heterogeneous population (such as progenitor cell purifications) fluorescence-activated cell sorting (FACS) is the most established method (Loken and Herzenberg 1975). For clinical separations of the same nature, magnetic-activated cell sorting (MACS) is the most common technique, with the most frequently used device being Miltenyi Biotec's CliniMACS (Grützkau and Radbruch 2010; Ringhoffer et al. 2004).

Table 6.3 Operational characteristics of commercial cell separations currently being applied in the cell therapy field

Clinical cell separation								
Device	Manufacturer	Method of separation	Cell type isolated	Processing time	Purity (%)	Viability (%)	Process yield (%)	Reference
Isolex 300(i)	Baxter	MACS	CD34+ cells	2–3 h	90	>92	25–51	FDA (1999), Gryn et al. (2002)
CliniMACS	Miltenyi Biotec	MACS	Haematopoietic progenitors/ Mesenchymal stromal cells/T-cells/Dendritic cells	2.5–3 h	78–90	78–99	15.7–43.4	Miltenyi et al. (1990), Miltenyi Biotec (2004)
CEPRATE® SC	CellPro	Continuous flow Immunoadsorption	CD34+ cells from autologous PBPC	<1 h	72	36.6	41.4	de Wynter et al. (1995)
Sepax®	Biosafe	Density gradient base separation	Mononuclear cells from bone marrow	35 min	–	80.5	83	Aktas et al. (2008), Henderson et al. (2010)
CELLector flask	Applied Immune Science	Antibody panning selection	Broad range of cell types	2–3 h	32.5	15.1	17	de Wynter et al. (1995)
SmartPReP 2 BMAC™	Harvest Technologies Corporation	Centrifugation	Autologous stem cells from bone marrow aspirate	<15 min	–	–	74.6+13.7	Harvest Technologies (2011)
COBE® 2991 Cell Processor	CaridianBCT	Centrifugation	Autologous stem cells from bone marrow aspirate	14–28 min	85–90	98	47	Olack et al. (1999)
Elutra® Cell Separation system	CaridianBCT	Counter-flow elutriation	Monocyte enrichment of apheresis products	1 h	13.6–79.5	93.8+2	79–100	Gulen et al. (2008)
Celution 800/ CRS	Cytori	Real-time cell processing	Adipose-derived regenerative cells	1–3 h	60	89.2+1.1	–	Lin et al. (2008)
FACS IV flow cytometer	Becton Dickinson Biosciences	FACS	Broad range of cell types	2–4 h	73.6	15.5	39.2	de Wynter et al. (1995)

Note: Purity, viability and yields are based on different cell and culture conditions as detailed in each reference. The percentages may vary based on the cell type isolated and experimental methods tested for each device

6.3.1 Antibody-Dependent Purification Techniques

Current strategies for the separation of progenitor or 'tissue-specific' stem cells are dominated by immunoseparation techniques (Wobus and Boheler 2005). A primary, target cell specific, antibody is incubated with the sample before a secondary antibody (possessing the ability to be isolated via a physical characteristic i.e. magnetism or fluorescence) is bound to the primary. Alternatively the primary can be directly conjugated onto magnetic beads or a fluorescent tag to eliminate the requirement of a secondary antibody.

A move towards affinity-based, positive separation has occurred through the discovery of specific surface antigens for target cell types. One example is the CD34 antigen which is expressed by haematopoietic cells. Selection of CD34-positive cells is commonly used for the purification of blood samples prior to transplantation (Berenson et al. 1988; Civin et al. 1984). This technique is now utilised in most bone marrow, cord blood or peripheral cell derived therapies, as well as in tests for diabetes, graft versus host disease and myocardial infarction (Phinney and Prockop 2007). Nine clinical trials in 2011 alone used CD34-positive selection of hematopoietic progenitor cells (Bersenev 2012). Affinity separations provide higher selectivity than centrifugation-based protocols (as discussed in 3.3) and have expedited the clinical translation of cellular therapies where rare cell types must be isolated (Wognum and Eaves 2003). Many other cellular products have been limited by the lack of a specific surface antigen with desired expression characteristics. Isolation of the target cell population is reliant upon the discovery of unique surface markers, to provide the purity required for clinical application.

Antibody panning is the simplest antibody-mediated cell separation technique. A flask is coated with a specific antibody which allows a corresponding antigen on the target cell population to bind (Morecki et al. 1990). Non adherent, unwanted cells are washed from the flask, before the target population is eluted via physical agitation. A low purity, around 50 %, is produced in comparison to current methods, but the fundamental strategy of cell antibody-mediated attachment was proven viable (de Wynter et al. 1995).

Immunoadsorption has also been used in scalable batch and continuous column-based devices (Baran et al. 1982; Mahara and Yamaoka 2010). Cells remain label-free through processing, and systems are cheaper than FACS and MACS. Column clogging and challenges with cell:bead interactions have limited the success of systems. Cryogels have also been used in column cell separations (Dainiak et al. 2007; Kumar and Bhardwaj 2008). The cryogel (a monolith structure, created from one solid piece of material) contains many channels running through the column which can capture cells via affinity, charge or hydrophobic interaction. Monolith columns are commercially available up to 8 L in size, with channel diameters greater than 30 μm diameter (Jungbauer and Hahn 2008). Low recoveries and concern about cell damage from shear force and pressure have restricted their clinical application.

The most common, established methods for separating rare cell types from a heterogeneous population are FACS and MACS (Amos et al. 2012). Purification via flow cytometry (or FACS) sorts cells based on their light scattering properties and

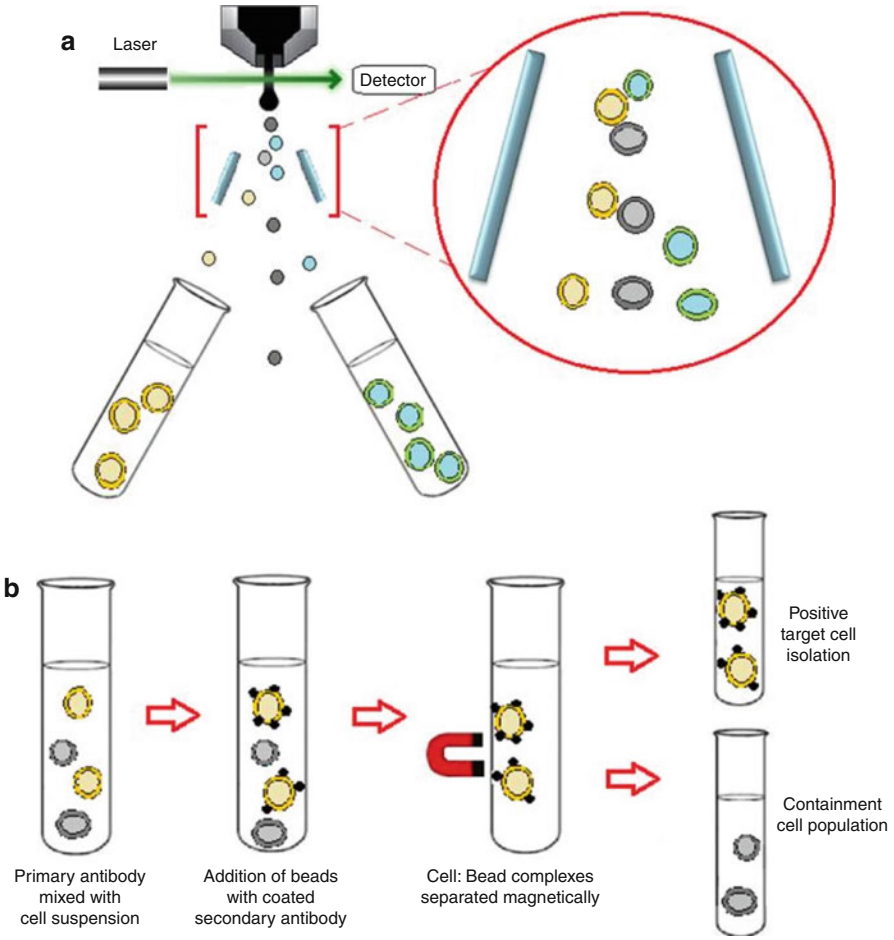

Fig. 6.2 (**a**) Fluorescence activated cell sorting: target cells of interest are tagged with an antibody which has been linked to a fluorescent marker. As the sample passes through a laser, the light will excite the fluorescent marker resulting in light emission. This interaction is analysed to determine the target cell population. An electrical charge is applied to cells types to allow sorting into different collection tubes. (**b**) Magnetic activated cell sorting: the sample is incubated with an antibody which binds specifically to the target cells of interest. Paramagnetic beads, coated in a secondary antibody capable of binding to the first, are then introduced to the sample. The cell:bead complexes are held in place with a magnetic field while unbound material is washed away. Alternatively, beads coated in an antibody which binds specifically to the target cells can be utilised to mitigate the requirement of a secondary antibody, and can be detached from cells after processing

fluorescence (Jensen 1989). The process sample is mechanically separated into a single cell suspension, before each cell is interrogated by a laser. A positive or negative charge is applied to each cell to facilitate isolation of phenotype-specific cells, labelled with a fluorescent marker (see Fig. 6.2a). A background and future look into the uses of FACS has been reviewed by Stanford University (Herzenberg et al. 2002).

Due to the ability to sort cells based on multiple parameters while achieving high purities, FACS has many applications in the biotechnology industry (Mattanovich and Borth 2006). Although single cell suspensions are sorted at rates of 10,000–20,000 cells per second, FACS is usually confined to research or analytical purposes. The capital costs to purchase the machine and (as with all immunoseparations) the high cost of the antibodies, sourced from a CGMP supplier, must be considered. However, at present no other non-antibody-mediated methods for the separation of rare populations which produce equally high target cell-fold purities, have been established.

Due to the lack of a unique marker for most cell types, some successful clinical purification strategies combine positive and negative selection (Grützkau and Radbruch 2010; Wognum and Eaves 2003). Negative selection defines procedures which remove unwanted cell types from the product. Specific contaminants which are known to cause therapeutic concern, such as undifferentiated cell types from stem cell derived transplants, can be purged via antigen specific selection (Thomas et al. 1999). The first positive clinical scale purification of CD34-positive T cells was the CEPRATE SC, created by Cellpro. Stem cells, labelled with biotinylated anti-CD34 antibodies, were bound to avidin coated beads. After a wash step to remove unbound cells, physical agitation was used to elute the target population. However, this method lacked the purity or recovery to operate as an efficient and scalable procedure. A T cell removal up to three-log was achieved, with a CD34-positive cell yield between 31 and 79 % (Handgretinger et al. 1997; Farag 2002). The product purity was insufficient to mitigate an immunological reaction with Graft versus Host Disease (GvHD) often noted post-transplantation (Hassan et al. 1996).

More recently MACS has combined antibody-mediated cell separations with paramagnetic beads. The primary antibody is incubated with the cell sample, before a secondary antibody immobilised onto paramagnetic beads is added. A magnetic field retains the cell:bead complexes while unbound material is eluted (see Fig. 6.2b). The Isolex 300i from Baxter was approved by the FDA in 1999 for the separation of CD34-positive cells from autologous peripheral blood progenitor cells for transplantation, following myeloablative therapy in cancer patients (FDA 1999). Currently the device can only be used off-protocol (at the discretion of physicians) for indications other than the FDA approved basis. The cells are mixed with a mouse anti-CD34 monoclonal antibody (mAb) in a batch system, before the addition of sheep anti-mouse IgG coated paramagnetic beads (Dynabeads M-450). Unbound cells are then washed away. The target CD34-positive cells captured on the beads are held within the device by a magnetic field. A peptide which competes with the primary antibody is used to elute bound cells. A range of 3.5–4.8 log depletion is achieved with a yield between 41 and 69 % (Martín-Henao et al. 2001; Farag 2002). The Isolex 300i software also allow for simultaneous negative contaminant removal with a positive selection step. Combining both positive and negative selection increased T cell log depletion by an average of 5.1 (Martín-Henao et al. 2001).

The addition of immuno-paramagnetic beads has greatly increased the purity of CD34-positive cell separations. A comparison between the Isolex 300i® and CEPRATE® devices resulted in final purities of 93 and 61.5 % respectively

(Björkstrand et al. 1999). Similar inefficient cell recovery was noted in both devices, with neither yielding over 50 % of CD34-positive cells.

Another competing immunomagnetic selection device is CliniMACS from Miltenyi Biotec. The CliniMACS device operates via incubation of anti-CD34 antibody-coated paramagnetic nanobeads (50–100 nm diameter) with the cell suspension. Bound cells are then retained via flow through a tube which contains a ferromagnetic core. This core is magnetised via a permanent magnet to capture cell:bead complexes, while permitting wash steps to remove unwanted material. Elution is achieved by removal of the magnetic field. A four to five log depletion of T cells can be expected, with a recovery of 71 % (McNiece et al. 1997; Schumm et al. 1999). Similarly to the Isolex 300i, significant cell loss during processing due to the limited selectivity of batch systems, cellular loss during washing steps and adherence to tubing and other materials presents a major problem for therapeutic function. Additionally in both systems, cell recoveries vary significantly batch-to-batch, with yields between 30 and 90 % noted (Björkstrand et al. 1999).

In September 2011, Miltenyi Biotec submitted a humanitarian device exemption (HDE) application for the isolation of CD34-positive cells from heterogeneous haematological cell populations (FDA 2011a). A humanitarian use device (HUD) must treat fewer than 4,000 individuals per year, with no other instrument available to perform the role. In this instance, clinical data which would demonstrate efficacy for a pre-market approval (PMA) application is not required. Currently 162 CliniMACS devices are distributed between 97 institutions in the US (Johansen 2011).

A common trend through MACS devices is the limitation of cellular recovery. Various factors affecting CD34-positive cell isolations from apheresis products have been investigated, including the variability of monocyte selection and constituent concentrations (red blood cells and platelets), as well as the initial percentage of CD34-positive target cells. The quantity of red blood cell and platelet contaminants were shown to have a negligible impact upon yield, suggesting that the wash steps in this instance adequately remove insoluble debris (Gryn et al. 2002). Most notable was the impact of the initial number of CD34-positive cells. Increasing the initial number significantly reduced the yield and was associated with an increase in purity (Gryn et al. 2002). This, in addition to the large variation of yields measured, suggests that the limiting factor for MACS devices is the binding efficiency of cells to beads. Currently all magnetic purification systems are performed in batch operating systems which does not allow for the high level of control and selectivity which can be achieved using column systems (Arnold et al. 1985; Roy and Pai 1999).

As well as concerns over cell:bead binding efficiency, other investigations into the therapeutic feasibility of MACS are on-going. A study evaluating the removal of pluripotent (teratoma forming) cells from an embryonic stem cell derived product, found that an estimated 31 steps would be required to achieve a purity of 10^{-1} undifferentiated cells per dosage (Schriebl et al. 2010). The highest selectivity available at present is affinity-based magnetic cell sorting, however, new engineering approaches are required to increase the target cell yield. By combining positive and negative selection techniques product purities could be increased, but running multiple runs is impractical at present due to cell losses.

6.3.2 Fluorescent Tagging

If no distinct physical feature or unique surface protein can be found, fluorescent tagging has been shown to provide an effective purification platform for selection via intracellular gene expression. Green fluorescent protein (GFP) tagging of target populations has been used to provide 'proof of principle' studies for progenitor cell transplantations. One common use is to identify a target cell type from within a heterogeneous population, derived through the directed differentiation of stem cells (such as CK19-positive insulin-producing cells derived from mouse embryonic stem cells (mESC)) (Naujok et al. 2008). Fluorescence provides a method to easily identify the required population for extraction via FACS, when no specific surface marker is present for alternative affinity purification techniques.

Lentiviral vectors are often used for stable gene delivery in both in vitro and in vivo application. However, for clinically relevant processes, genetically modifying cells presents an additional regulatory burden, such as with induced pluripotent cells (Schambach et al. 2010). A risk of viral vector shuffling, resulting in the transduction and consequent transplantation of undesired cell types, presents a significant challenge (Blömer et al. 2005). Additionally viral vectors used for GFP tagging have been shown to remain within cell suspensions, even after extensive washing (Blömer et al. 2005). As a result, tag-less cell sorting methodologies have been developed such as fractionation or immunological separation techniques (Roda et al. 2009a, b; Braakman et al. 2008).

6.3.3 Physical Separation of Target Cell Population

Gradient density separation, exploiting physical variations in size and density between cell populations, is frequently used as a low resolution isolation step for processing whole blood, cord blood or bone marrow derived material. Various indications from haematological to neurological or autoimmune treatments use an apheresis purification step. Density gradient media, such as Ficoll or iodixanol-based media, is often added to cell suspensions for processing, which can achieve two to five fold concentration (Wognum and Eaves 2003). Fractionation of whole blood by apheresis is exploited to isolate buffy coat (the fraction of blood containing white blood cells and platelets), with various saline wash steps integrated to remove the gradient density solution (Fadel et al. 2011).

Tissue derived cells, such as mesenchymal stem cells isolated from adipose tissue, are enzymatically dissociated before filtration, washing and centrifugation steps (Fadel et al. 2011). Devices, such as Cytori's Celution 800/CRS, now automate the clinical extraction and processing of adipose-derived stem cells in a sterile, closed system. This includes a wash step and subsequent centrifugation, allowing supernatant removal after pellet formation.

The success of apheresis devices is indicative to the simplicity of the process. The procedure is fast, relatively cheap and can operate in a closed, point-of-care environment. Separation is reliant upon variation between cell size and density to

create clear bands of material post-centrifugation. The separations are fairly crude, only removing select bands of cellular waste, and often performed in combination with higher resolution purification steps such as MACS.

Filtration is the main competitor to gradient separation at present. With the ability for continuous operation, a wider range of materials and scales can be processed (Choi et al. 2007). Furthermore, filtration devices are commonly closed, disposable systems which do not generate aerosols (Hibino et al. 2011). Various novel microfluidic devices using filtration have been developed recently (see Table 6.4 and Chap. 4).

Table 6.4 Novel microsystems utilising different intrinsic cell or affinity properties for research-grade cell separations

Method of separation	Cell type isolated	Throughput	Purity (%)	Reference
Microbeads coating CTCs to allow Continuous size-based isolation	CTCs from peripheral blood samples	–	–	Hughes et al. (2012b), Lin et al. (2012)
E-selectin and antibody coated microfluidic device	CTCs from buffy coat samples of patients diagnosed with metastatic cancer	–	–	Hughes et al. (2012a)
Alcohol-dispersed elecrospun magnetic nanofibers	Primary CD3+ T lymphocytes	–	–	Kim et al. (2012)
Continuous microfluidic margination	Microbes, platelets and leukocytes from whole blood	~1 ml/h per channel	80/90 % removal of bacteria & inflammation cellular components	Hou et al. (2012)
Particle deformation in a continuous microfluidic channel	Platelet isolation from diluted whole blood	–	99.9 %	Nam et al. (2012)
Microscale vortices isolating cells based on diameter	CTCs spiked in blood	7.5×10(6) cells/s [ml/min scale]	–	Hur et al. (2011)
Biomimetic postcapillary expansions	NCs from whole blood	5 nl/s	94±4.5 %	Jain and Munn (2011)
Porous membrane in a microfluidic device	Separating whole blood samples into constituents	–	99.9 %	Wei et al. (2011)

Table 6.4 (continued)

Method of separation	Cell type isolated	Throughput	Purity (%)	Reference
Pinched flow dynamics (size-based separation)	CTCs spiked in blood	~10(8) cells/min	>80 % cell recovery	Bhagat et al. (2011)
Combination of MOFF and DEP	CTCs spiked in blood	126 µl/min flow rate	RBC removal – 99.24 % WBC removal – 94.23 %	Moon et al. (2011)
Non-contact acoustic cell trapping in disposable glass capillaries	Aspirating, trapping and dispensing red blood cells	–	–	Hammarström et al. (2010)
Two-phase laminar flow in a microfluidic chip	Leukocyte and erythrocyte cells from whole blood	2 µl/min	–	Tsukamoto et al. (2009)
Membrane antigen aptamers	CTC cells from peripheral blood	2.5 mm/s (29 min with 1 ml volume)	90 % of LNCaP cells (prostate cancer cell line)	(Dharmasiri et al. 2009)
Continuous flow ribbon-like capillary device	MSCs from epithelial cells	30 min	Near 100 %	Roda et al. (2009a, b)
DEP-FFF	Stem cell enrichment from adipose tissue suspension	–	–	Vykoukal et al. (2008)
Microfluidic device with mAbs covalently attached	CTC isolation from whole blood	–	–	Adams et al. (2008)
Cell-capture microtubes coated with adhesion molecules	Haematopoietic stem and progenitor cells	–	16–20 % CD34+ cell purity	Narasipura et al. (2008), Wojciechowski et al. (2008)
LCM	Isolation of rare cell types from tissue sections	–	–	Li et al. (2008)
3D mammalian cell separator biochip based on cell deformability	Non-invasive separation of heterogeneous populations	2,800 cells/min	–	Choudhury et al. (2012)

Aqueous two-phase systems (ATPS) have been utilised for low specificity recovery and primary purification operations. When two incompatible aqueous solutions are mixed together, they form two clear phases. The biphasic system can be composed of either polymer-polymer or polymer-salt formations. The most commonly used polymer is dextran and polyethylene glycol (PEG) (Diamond and Hsu 1990). Cells or macromolecules can then be partitioned into either phase (or between the liquid:liquid interface) dependent on size, net charge and hydrophobicity (Albertsson 1970). Affinity ATPS has demonstrated recovery of CD34-positive cells with a 75 % yield and 80 % purity (Kumar et al. 2001). Two-phase flow has also been shown in microfluidic chips, separating leukocytes and erythrocytes from whole blood at 2 μl/min (Tsukamoto et al. 2009).

For certain applications, manual dissection may be acceptable with no requirement for larger, more complicated purification systems. If a distinct morphology can be noted for target cell populations, phase contrast images can be used to identify and subsequently isolate cells. If this criterion can be met, a cheap, highly selective method is available for separations. Manual dissociation has been used for a number of controlled differentiations from pluripotent or multipotent cells, when a distinct morphology is observed. Retinal pigmented epithelium (RPE) cell transplantation to treat dry Age-Related Macular Degeneration (AMD), such as the on-going clinical trials with Advanced Cell Technology (ACT), uses a manual isolation. Pigmented cells are selected via a stereomicroscope and a monolayer, established through enzymatic dissociation with trypsin or collagenase (Klimanskaya 2006). Another example of manual dissociation is the separation of cardiomyocytes derived from pluripotent stem cell, where beating clusters of cells can be observed and isolated (Shinozawa et al. 2012). Laser microdissection can be utilised to dissociate smaller or fluorescently tagged populations (Bonner et al. 1997). Accurate, relatively gentle separations can be achieved, but high levels of technical training and expertise are required to operate the instrument. Furthermore, issues with scalability and equipment costs still remain. Recently more commercial laser dissection instruments have been created which reduce the once significant capital cost (Hanson et al. 2011).

If no notable visual distinction can be identified within a population, other physical dissociation techniques are available. Raman spectroscopy uses the principle of cell polarisation to discriminate between populations, based on visible or near infrared light resonance (Long 1977). Instead of MACS or FACS, where indirect interrogations of externally labelled cells are carried out, Raman spectroscopy measures a direct, intrinsic cell signal. The interaction between cells and monochromatic light from a laser results in a shift of energy states, or excitation, which is referred to as inelastic scattering or Raman scattering (Kneipp et al. 1997) (see Fig. 6.3). To date, Raman activated cell sorting (RACS) has been integrated into fluidic platforms with proof of concept established (Lau et al. 2008). Disadvantages with this technique include low throughput, labour intensive processing and extensive operator training requirements. A more detailed report on the uses of Raman spectroscopy for cell sorting and imaging has been created by the Kroto Research Institute in Sheffield (Li et al. 2012).

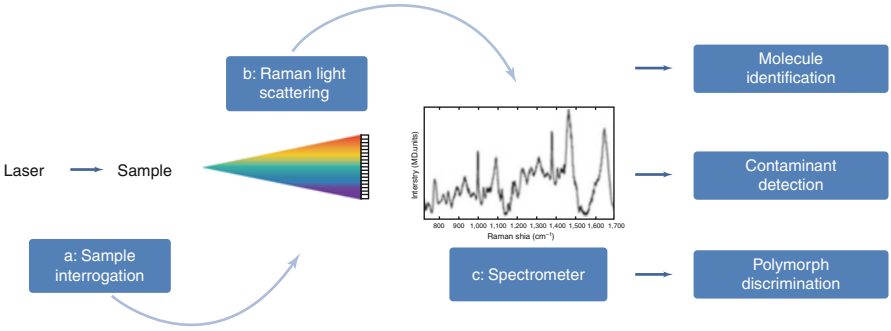

Fig. 6.3 Raman spectroscopy: monochromatic light from a laser interacts with a sample (**a**). Photons are absorbed and re-emitted at an altered frequency as the molecules are excited (**b**). This shift in frequency is called the Raman Effect. Scattered light is focused with a lens, before a spectrometer is used to create a Raman spectrum for the sample (**c**). The spectrum can be used to determine cell types in situ, detect contaminants present, or identify a chemical compound's polymorph (crystalline structure)

Another physical separation method is adhesion, such as the isolation of human mesenchymal stem cells by their adherence to plastic (Kassem 2004). A purification combining Percoll gradients with cell culture has shown rat foetal liver cells could be separated with a purity of 95 % (Liu et al. 2011b). Limitations to adherent processes include time and labour dependence, as well as frequently requiring further processing steps (Tondreau et al. 2004).

6.4 Novel Systems

6.4.1 Future Trends in Cell Purification

The broadest area of new development for cell purification is within the microfluidic field. Many novel devices for the isolation of rare cell populations and removal of pathogenic materials have been developed in microscale systems. Microsystems significantly reduce cell losses, while allowing for automation and minimising contamination risks (Dharmasiri et al. 2010). By assessing recently accepted patent applications (accumulated through Google Patents) for cell separation devices, microfluidic operations such as filtration and fractionation were shown to comprise 40 % of patents. Patents relating to current clinical cell separations devices, such as MACS, FACS and apheresis, totalled to only 31 %. This data agrees with the hypothesis that fewer clinical devices exist for common purification techniques and predominantly, new development is within the microfluidic field.

6.4.2 *Novel Cell Separation Devices*

Devices which produce high purities in addition to high cellular recovery tend to suffer from issues with low throughput and scale. As an analytical technique, microfluidic platforms now exist with the capacity to isolate rare cell populations for diagnostic or therapeutic monitoring applications (Plouffe et al. 2012). Although microfluidic devices sort cells at a slower rate than FACS and other methods, the flexibility bestowed through laminar flow allows cells to be accurately isolated by intrinsic physical or biochemical properties. Lab-on-chip devices are fabricated out of polydimethylsiloxane (PDMS), creating closed system, disposable devices to separate small volumes of cell material. Integration with existing devices, such as in situ imaging instruments, has greatly increased the clinical feasibility (Li et al. 2012). With research focusing on increasing the throughput of microsystems, feasible alternatives to centrifugation and other clinically utilised devices are being developed (see Table 6.4).

One limitation to cell isolation is the method to identify the desired cell population for removal. A novel approach to compete with antibody-dependent separations now exists through the use of aptamers (Guo et al. 2008; Nery et al. 2009). Aptamers are nucleic acids which can target specific biomarkers which are often inaccessible to antibodies. The ability for greater molecular recognition, due to their small, flexible structure, complements microsystem designs with distinct flow channels for cell sorting (Dharmasiri et al. 2009).

Label-free cell sorts have also been developed, such as field flow fractionation (FFF) or dielectrophoresis (DEP). Both take advantage of morphological or biological differences to separate cells based on variable cell density, size or shape. FFF operates by an applied field, perpendicular to the direction of flow which results in differential flow dynamics for cell populations. Elution time can be correlated to the complexity (density) of cellular material. Instead of separation via flow, DEP captures or directs cells by applying a non-uniform electric field across microfluidic channels (Vykoukal et al. 2008). The force experienced by cells is dependent on cell polarisation. Slight variations in intracellular constituents of cells can be utilised to generate a polarisation gradient. Field-dependent cell sorting has often been viewed as an analytical scale technology, however, microscale separations can now operate up to 1 ml/min (Hou et al. 2012). Applications for DEP also include non-viable and bacterial contaminants (Karumanchi and Doddamane 2002). For the small volumes required for most cell therapies, especially autologous treatments, these novel label-free physiology-based separations provide a cheap, efficient alternative to FACS processing. Other label-free separation techniques include: cell margination (Wei Hou et al. 2012), deformation (Choudhury et al. 2012), and size (Bhagat et al. 2011).

6.5 Concluding Remarks

At present, purification devices for cellular therapies are still in their infancy, encompassing a wide breadth of different challenges dependent on source material, physical cell characteristics and specific formulation requirements. The selection of

purification system is vital to ensure success of therapies. Although a diverse range of both antibody-dependent and physical separations are available at commercial scales, challenges still exist with each technique that limit a gold standard system which fulfils all purification requirements. General limitations include: low selectivity; low cellular recovery; the risk of cell damage and biological instability; limited throughput and difficulties maintaining sterility. At present, cell purification systems are often retrofitted around existing technology, most utilising centrifugation-based separation. Apheresis systems are one of the most widely used devices to separate constituents from whole blood. Procedures are fast and relatively cheap but separation is crude and further processing is often required. Affinity-based technology such as FACS or MACS provides higher resolutions, however, the lack of unique surface markers restrict clinical application. Furthermore, few systems address soluble or intracellular contaminants, with additional wash procedures necessary (such as for the removal of DMSO). A shift towards purification systems designed around regulatory specification is a foreseeable outcome of these challenges. New engineering approaches are being pursued to create closed system, high throughput processes. Microsystems combine fluid flow properties with intrinsic biophysical cell characteristics, which enable high resolution, automated devices. With a dense field of development, the possibility to integrate cell culture, processing (such as a differentiation step), purification and formulation into one system is looking more feasible. Miltenyi Biotec's CliniMACS Prodigy looks to fully integrate an automated closed system, which can standardise cell manufacture. The device combines a cell culture chamber (also capable of running other cell protocols such as differentiation), a centrifuge for washing and fractionation steps, and their own MACS system for target cell isolation. With recent improvements in cell culturing techniques, such as suspension culture of adherent cell lines (Steiner et al. 2010; Larijani et al. 2011) and large scale production using micro-carriers (Alfred et al. 2011), the demand on purification is increasing. Consequently a consideration of the whole bioprocess is required, with integrated microfluidic systems currently presenting the most feasible solution.

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Chapter 7
Separation Technologies for Stem Cell Bioprocessing

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Abstract Stem cells present an enormous potential in a number of fields with a great impact on human health including Regenerative Medicine, drug discovery, toxicology studies and fundamental stem cell biology. Crucial to the accomplishment of this potential is the development of stem cell-based bioprocessing strategies based on the rational integration of cell culture procedures with separation methods towards the isolation of specific stem cell types from tissues and/or purification of stem cells and derivatives after *in vitro* culture. Separation methods/strategies have been applied to stem cells since many years ago, namely the isolation of hematopoietic stem/progenitor cells (HSPC) from bone marrow for the treatment of hemato-oncological diseases using density gradient centrifugation followed by immunoaffinity-based techniques. More recently, novel approaches have been proposed including affinity-based methods that take advantage of the use of more cost-effective ligands (*e.g*. aptamers, lectins), as well as novel biophysical-based methods requiring no cell labelling and integrated with microscale technologies. This chapter presents a critical assessment of these traditional and novel separation methodologies and their present or potential applications to the stem cell field. The techniques are grouped according to their fundamental principles, which are defined by the main physicochemical, biophysical and affinity properties of cells. Nevertheless, enormous challenges still need to be overcome in order to make available a wide range of strategies combining scalability potential with high-resolution abilities, allowing the cost-effective large-scale production of highly purified stem cell populations and/or derivatives. Further developments in this field are thus expected to greatly impact and potentiate the medical translation of stem cell-based therapies.

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7.1 Separation Technologies for Stem Cell-Based Therapies: Relevance and Challenges

The isolation and/or purification of stem cells and their derivatives to be used for cell therapy applications, including fully established stem cell-based therapies, as well as for fundamental biomedical research have been performed over the last decades (Diogo et al. 2012; Gonzalez-Gonzalez et al. 2012). Overall, separation techniques have been used for isolation of stem cell populations from tissues, for separating different stem cell populations from a heterogeneous cell mixture and for purification of stem cell derivatives obtained upon differentiation of stem cells *in vitro*. The most classical example of a stem cell-based separation is the isolation of human hematopoietic stem/progenitor cells (HSPC) from different sources such as bone marrow (BM), umbilical cord blood (UCB) and mobilized peripheral blood (mPB) for the treatment of hemato-oncological diseases. For this purpose, a separation strategy was conceived including a density gradient centrifugation followed by immunoaffinity-based techniques, including magnetic-activated cell sorting (MACS), and fluorescence activated cell sorting (FACS), for targeting of $CD34^+$ cells. The main objective of these procedures is the enrichment of rare HSPC present in these sources for further transplantation to restore the blood and the immune system of cancer patients following high-dose chemotherapy or to treat autoimmune, metabolic and genetic diseases (Weissman and Shizuru 2008).

In addition to this widely established procedure, it is nowadays believed that the successful establishment of stem cell based-therapies at different stages of preclinical and clinical tests and other stem cell applications in the biomedical field is highly dependent on the development of more sophisticated and efficient separation technologies and strategies. According to the final stem cell-based therapy application envisaged, different challenges must be faced in this field. One of these challenges is the need for novel techniques with a higher resolution, either for depletion of contaminating cells, or for the separation of stem cell populations sharing similar physicochemical and affinity characteristics but presenting different clinical features. On the other hand, there is also the necessity of scaling-up the separation processes when the cellular product is intended to be used for clinical applications or as a tool for drug screening and pharmacological testing.

These challenges present a different relevance according to the stem cell-based therapy envisaged. Of note, several pre-clinical studies and clinical trials have pointed to the therapeutic potential of mesenchymal stem/stromal cells (MSC), based on their multilineage differentiation potential, but especially on their intrinsic immunomodulatory and pro-regenerative features (Caplan 2007, 2009; Santos et al. 2011; Uchida et al. 2000). These cells have been isolated from different sources such as BM, adipose tissue (AT) and umbilical cord matrix (UCM) typically based

on their adhesion to plastic surfaces, which yields a very heterogeneous cell population. A more rational clinical use of MSC would thus strongly benefit from the development of novel high-resolution separation strategies to capture specific MSC sub-populations with defined properties from a variety of different sources. Other stem cell applications requiring the development of separation techniques with high-resolution abilities are the ones relying on the depletion of rare contaminating cancer stem cells (Geens et al. 2007) and the removal of tumorigenic stem cells from *in vitro* differentiating cultures of pluripotent stem cells (PSC), both embryonic (ESC) and induced pluripotent stem cells (iPSC) (Levenberg et al. 2010). Without these developments, the potential application of human PSC-derived tissue specific cells in clinical settings will remain hampered, among other reasons, by the presence of pluripotent cells or naïve proliferative progenitors that can form teratomas upon *in vivo* transplantation. Importantly, for all the applications that may be considered, separation technologies should be rationally integrated with cell production methods in wider bioprocessing strategies towards the large-scale manufacturing of stem cells and/or their progeny. Overall, the examples aforementioned illustrate the challenge and the relevance of separation technologies to potentiate the medical translation of stem cell-based therapies.

7.2 Cell Separation Technologies: An Overview

Cell separation technologies are selected according to general criteria including the final application of the cellular product, the cellular properties, the resolution capabilities required and the scalability of the process. When considering the cellular properties, different techniques have been explored that can take advantage over the differential physicochemical and biochemical characteristics of cells, including size, density or electrostatic and hydrophobic character, as well as the differential expression of cell-specific surface markers or adhesive properties (Fig. 7.1 and Table 7.1). The techniques that explore the physicochemical properties of cells are generally traditional methods such as centrifugation or membrane filtration. These techniques are characterized by a low resolution capacity and they are typically used at the first stages of cell processing for the separation of very distinct cell types and/or for cell concentration. Moreover, the differential adhesion of distinct cells to tissue culture plastic can also be explored as a low resolution separation/concentration method that is generally used during the first stages of the bioprocess. However, for similar cell phenotypes, high resolution techniques are required and in these cases cell separation has been generally performed by taking advantage of the differential number and type of molecules present on the cell surface that can be targeted by specific monoclonal antibodies, lectins and, more recently, by aptamers. This group of techniques is named immunoaffinity methods. Immunoaffinity cell separation strategies can be conceived by using a single specific ligand only (*e.g.* monoclonal antibody), but by targeting cells with several immunoaffinity ligands at the same time, or in sequential steps, more complex strategies can be conceived. Separation

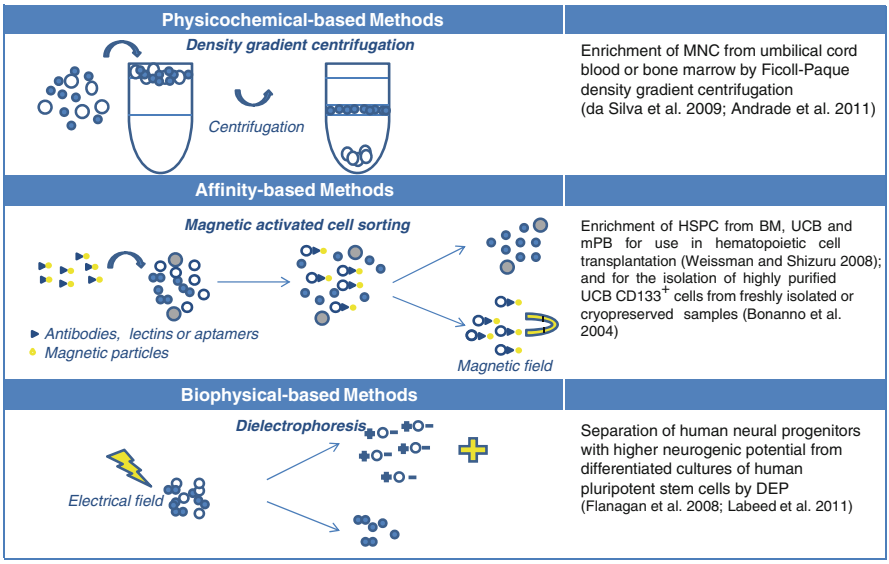

Fig. 7.1 Separation methods in the stem cell field based on cell's physicochemical, affinity and biophysical properties. Physicochemical-based methods such as density gradient centrifugation, take advantage of cell size, cell density, or the capacity to adhere to tissue culture plastic. Affinity-based methods, like MACS, employ specific monoclonal antibodies, lectins or aptamers targeting different types of molecules present on the surface of the cells; Biophysical-based methods, such as DEP, do not require cell labeling and promote selective separation based on inherent differences in cell's biophysical properties

techniques taking advantage of the use of antibodies that bind to surface markers to specifically pick out cells of interest include the very widely used fluorescence-activated cell sorting (FACS), immunomagnetic cell sorting (MACS), affinity chromatography and aqueous two-phase systems (ATPS) using antibody-modified polymers. Nevertheless, although immunoaffinity separation methodologies typically provide a high resolution in cell separation, for many stem cell or stem-cell derived populations, surface markers for separation and analysis are limited. In addition, these immunoaffinity strategies rely on the formation of a complex cell-antibody or cell-antibody-magnetic particle, which could affect cell function (*e.g.* differentiation) or activation state (Chou et al. 2010). Alternative selection strategies avoiding the use of antibodies and magnetic particles have been more recently provided by novel "tag-less" methods, such as dielectrophoresis (DEP) (Pethig et al. 2010), integrated with microfluidics and other microfabricated structures, and also fluid flow fractionation (FFF) (Reschiglian et al. 2005). DEP and FFF do not require labelling of cells but they allow a selective cell separation based on the inherent biophysical properties of cells.

According to the final purpose and to the characteristics of the starting material, stem cells can be separated by negative or positive selection. A positive selection operation is more adequate for the isolation of specific and low proportion

Table 7.1 Advantages and limitations of cell separation technologies

Cell separation technique	Cell properties	Advantages	Problems
Physicochemical-based			
Centrifugation Membrane filtration Cell adhesion	Size, density, adhesion properties	Cell concentration Avoids cell labelling	Low selectivity
Immunoaffinity-based			
FACS	Expression of surface markers	Highly selective, automated, multiparametric	Low Scalability, requires cell labelling with fluorescently labelled antibodies which might affect cell function; low yield; low throughput; requires skilled technicians; expensive reagents; conveys shear stress to the cells
MACS		Lower cost than FACS, automated, closed-system technology	Requires cell labelling with monoclonal antibodies conjugated with magnetic particles which might affect cell function; lower selectivity when compared to FACS; conveys shear stress to the cells
Affinity chromatography		Highly scalable, highly selective	Requires cell labelling with antibodies which might affect cell function; conveys shear stress to the cells
Aqueous two-phase systems with antibody-modified polymers		Highly scalable	Requires cell labelling with antibodies which might affect cell function; low selectivity
Tag-less methods			
DEP FFF	Biophysical properties	Avoids cell labelling	Low throughput and scalability
Microfluidic devices	Several (according to the technique that is being explored)	Laminar flow, can be automated and integrated in lab-on-a-chip platforms	Low throughput and scalability

populations from a complex cell mixture. In the stem cell field, this strategy has been successfully used for the specific capture of $CD34^+$ HSPC (either by FACS or MACS) from different sources. On the other hand, negative selection techniques are advantageous and required if the target cells have to be untouched

(without magnetic particles or antibodies) for subsequent analysis or application in clinical settings. Moreover, negative selection techniques may also be required if no surface marker/monoclonal antibody specific for the cell of interest is known or available or if the main objective is the high-resolution depletion of an undesired cell type (*e.g.* tumorigenic cells). According to the desired cell phenotype, different separation techniques in different modes may be selected and integrated in order to take advantage of different cellular properties and achieve more efficient separation strategies. Importantly, these downstream processing techniques should also be strategically combined with cell culture operations in order to design a cost-effective and efficient bioprocess for production of stem cells and derivatives.

The following sections of this chapter describe the basic principles of traditional and novel cell separation techniques and their applications in the stem cell field. A critical assessment is provided here concerning their advantages and limitations considering the final usage of stem cells and/or their derivatives in particular for applications in stem cell-based therapies.

7.3 Stem Cell-Based Separation Technologies

7.3.1 Physico-chemical Methods

Centrifugation

One of the most traditional and widely used techniques for primary cell separation is discontinuous density gradient centrifugation (Fig. 7.1). In this separation method, two distinct solutions with different densities are put together forming a system with two immiscible layers. The two-layered system is generally composed of sucrose and a polymer, such as Percoll or Ficoll-Paque. After obtaining this system, cells are added to the less dense solution on the top and a centrifugation is performed causing the cells to cross the system and to be separated according to their densities. Thus, cells with higher density than the more dense solution beneath will cross the interface between the two immiscible layers and settle at the bottom whereas the cells that have a lower density will settle at the interface. This technique is characterized by a low resolution capacity and for that reason it is generally used for enrichment, concentration or as a preparative step before using other separation techniques with higher resolution capabilities, namely immunoaffinity-based methods, such as FACS or MACS (see following sections).

One of the most popular applications of density gradient centrifugation in the stem cell field is the enrichment of mononuclear cells from human UCB or BM by a Ficoll-Paque density gradient (1.077 g/mL) (Andrade et al. 2011; da Silva et al. 2005, 2009). In this case, erythrocytes and granulocytes sediment to the bottom layer, whereas lower density lymphocytes and other slowly sedimenting cells, including stem/progenitor cells, as well as platelets and monocytes, are retained at

the interface between the plasma and the Ficoll-Paque. Cells can then be collected from the interface and subjected to subsequent isolation of HSPC or MSC populations using higher resolution immunoaffinity techniques. More recently, enrichment of mononuclear cells through density gradient-based separation has also been performed using commercially available equipment, the Sepax (Biosafe SA, Switzerland). This equipment is a fully-automated, closed, single-use and mobile system that can be used in GMP compliant environments or directly at bedside in the operating room for Regenerative Medicine applications. In addition to this application, gradient centrifugation was also already used with success for enrichment of human PSC derivatives. In particular, Percoll centrifugation was applied after differentiation of human ESC into cardiomyocytes using a monolayer adherent protocol (Laflamme et al. 2007). This purification methodology increased the purity of cardiomyocytes in cell suspension from 30 to 80 %.

An alternative type of centrifugation that has been recently applied for stem cell isolation is counter-flow centrifugal elutriation (CCE). In this case, cells are separated inside a centrifugal chamber where a continuous pumping of a fluid occurs. This technique was already used with success for the fractionation of umbilical cord (UC)-derived cells. In fact, through CCE it was possible to isolate a sub-population of small-sized UC-derived primary cells with MSC-like characteristics (Majore et al. 2009). This subpopulation exhibited a higher proliferative capacity as compared to the total UC-derived primary cultures and demonstrated a reduced amount of aging cells. The separation of this self-renewing MSC-like subpopulation by CCE provides a valuable tool to be used in Regenerative Medicine and may be an alternative to BM derived MSC.

Membrane Filtration

An alternative physico-chemical method for cell separation that has been recently used in the stem cell field is membrane filtration. In this case, cell separation is achieved based on cell size, according to the membrane pore size, but may also be based on the differential intensity of cell adhesion to the membrane. This technique is characterized by a high processing speed, simplicity, relatively low cost and, importantly, a high potential for scaling-up. In fact, the equipment necessary to perform this operation is already available at an industrial scale.

The isolation of $CD34^+$ cells from mPB was already performed using unmodified polyurethane (PU) foaming membranes, as well as PU membranes modified with -COOH groups and coated with Pluronic F127 or hyaluronic acid at different blood permeation rates (Higuchi et al. 2006, 2008). The permeation ratio of $CD34^+$ HSPC through the membranes was the lowest among blood cells regardless the type of PU membrane used while erythrocytes, platelets, T cells and B cells permeated more freely through the PU membranes. This behaviour was potentially due to the high expression of cell-adhesion molecules on the surfaces of the more primitive HSPC. More recently, the successful isolation of human adipose-derived stem cells (ADSC) with a superior capacity for osteogenic differentiation from a suspension

of human adipose tissue was achieved by this technique also using PU membranes (Wu et al. 2012). Importantly, these cells were isolated in less than 30 minutes whereas the conventional method of adhesion to plastic surfaces (see following section) requires 5–12 days. Although these results are encouraging, many improvements are still needed to increase the potential of this technique for stem cell-based separation settings.

7.3.2 Cell Culture-Based Methods

The differential behaviour of different cell types in culture settings can also be used as a means to obtain their separation. The most classical example is the isolation of human MSC from different sources, namely BM, based on their ability to adhere to tissue culture plastic, which allows the separation of MSC from the majority of hematopoietic cells. In fact, these cells are mainly non-adherent, being eliminated during culture medium exchange (Lennon and Caplan 2006). Differential enzymatic treatment can also be used to eliminate the major cell contaminants in primary cultures of human MSC, namely monocytes, as these need longer incubation times with the enzymatic agent in order to be harvested from culture plastic. Another alternative and relatively straightforward method for the isolation of human MSC in culture settings is based on osmotic selection due to their uncommon resistance to osmotic lysis (Parekkadan et al. 2007).

7.3.3 Immunoaffinity Methods

As previously mentioned, high resolution cell separation can be performed by a group of techniques entitled immunoaffinity methods, in which the cells are targeted by specific immunoaffinity ligands, such as antibodies. This group of methods has been widely used for the high-resolution targeting of stem cells and their derivatives using specific cell surface markers (Table 7.2). One of the first immunoaffinity stem/progenitor cell selection strategies was developed in the hematological field and consists on the isolation and enrichment of human HSPC based on the expression of the surface marker CD34. CD34 antigen is indeed the most utilized in hematopoietic studies, identifying cells from the stem through progenitor states. However, since the expression of CD34 by truly self-renewing HSPC populations is not exclusive, a combination of CD34 and other antigens is often used for isolation and characterization of HSPC (Table 7.2). For example, the more immature hematopoietic stem cells (HSC) should possess a $CD34^{+}CD38^{-}$ phenotype (da Silva et al. 2005). However, this primitive phenotype has been shown to inherently modulate in culture (da Silva et al. 2009), leading to erroneous quantification of engraftment competent cells upon *ex-vivo* cultures. Other phenotypes for human HSP have been proposed (Weissman and Shizuru 2008) including the expression of Thy-1 (or CD90). Another approach

Table 7.2 Surface molecular markers of stem cells and stem cell-derived cells for affinity-based separation

Cell type	Surface molecular markers
ESC and iPSC	SSEA-3, SSEA-4, TRA-1-60 and TRA-1-81, SSEA-5 (Tang et al. 2011) in human and SSEA-1 in mouse; lectin UEA-I (Wang et al. 2011c)
human PSC-derived Neurons	NCAM (Pruszak et al. 2007)
human PSC-derived Cardiomyocytes	VCAM1 (Uosaki et al. 2011)
Human HSC	$CD34^+$, $CD34^+CD38^-$, $CD34^+CD90^+Lin^-$ (reviewed in (Weissman and Shizuru 2008))
Human MSC	STRO-1 (Goncalves et al. 2006; Gronthos and Zannettino 2008), CD73 (Barry et al. 2001) and CD105 (Tondreau et al. 2004) or negative selection for CD45 (Jones et al. 2002)
NSC	CD133 in human (Uchida et al. 2000) and CD15 (Capela and Temple 2002), CD184 (Corti et al. 2005) and CD24 (Rietze et al. 2001) in mouse

Lin^- CD2, CD3, CD11b, CD14, CD15, CD16, CD19, CD56, CD123, CD235a (Glycophorin A)

consists of the use of negative selection for lineage markers associated with terminal maturation of specific blood cell types (Lineage negative (Lin-) cells) combined with CD34 expression (Table 7.2).

When concerning to other multipotent stem cell types, such as human MSC, the scenario is different since few surface markers have been identified for the analysis and isolation of these cells. Moreover, the existence of universal markers for these stem cell types and their derivatives still remains elusive. For that reason, on a routine basis, most laboratories perform the isolation of MSC from BM samples or adipose tissue/UC based on its adherence to culture plastic (Lennon and Caplan 2006). Nevertheless, alternative immunoaffinity methods have been proposed in order to isolate MSC based on surface marker expression, either by positive or negative selection (Table 7.2) to obtain more homogeneous populations. One illustrative example is the purification of human MSC through positive selection using the STRO-1 (Goncalves et al. 2006; Gronthos and Zannettino 2008), CD73 (Barry et al. 2001) and CD105 (Tondreau et al. 2004) antibodies. Negative selection of human MSC has also been performed through the use of the CD45 surface marker (Jones et al. 2002). When considering other multipotent stem cell types, surface markers have also been identified for the analysis and isolation of neural stem cells (NSC) and neural progenitors from brain tissue such as Lex1 in mouse (Capela and Temple 2002) and CD133 in human (Uchida et al. 2000).

In the field of human PSC, expression of the surface markers SSEA-3, SSEA-4, TRA-1-60 and TRA-1-81 has been typically used for identification and separation of these cells, both ESC and iPSC. More recently, efforts have been devoted towards the identification of novel and more specific surface markers to ensure the complete depletion of these teratoma-forming cells from differentiating cultures, such as the SSEA-5 antigen (Tang et al. 2011), as well as novel lectin biomarkers (Wang et al. 2011c). In parallel, the design of complete immunophenotyping screens for

identification and isolation of NSC, neurons and glia obtained during *in vitro* neural differentiation of human PSC has also been focused (Pruszak et al. 2007; Sundberg et al. 2011; Yuan et al. 2011). In what concerns to mouse ESC, SSEA-1 is generally used as universal surface marker for identification and separation of these cells.

The following sections will describe different immunoaffinity-based separation techniques that take advantage of these surface markers for targeting stem cells and their derivatives.

Fluorescence-Activated Cell Sorting (FACS)

Fluorescence-activated Cell Sorting (FACS) is one of the most widely used high-resolution techniques for isolation and purification of cells including stem cells and their derivatives. Cell separation in FACS relies on by Flow Cytometry exploiting cell's size and light-scattering properties. However, the full potential of flow cytometry as a preparative separation technique is only achieved when fluorescently-labelled monoclonal antibodies are used to bind to specific antigen markers responsible for a particular surface phenotype. As previously mentioned, one of the major limitations associated to immunoaffinity techniques is the absence of surface markers to identify and isolate specific stem cell types or their derivatives. In FACS this limitation can be surpassed through the insertion of reporter constructs inside the cells to make fluorescent labels (*e.g.* a fluorescent protein expressed under the control of a specific promoter) (Aubert et al. 2003; Wang et al. 2011a). In addition, the use of reporter constructs also avoids the time-consuming step of immunostaining. Nevertheless, the genetic modification of stem cells to be used in clinical applications can raise important regulatory concerns and others that should be carefully analysed.

Flow cytometry is a powerful analytical and preparative technique with three major components: fluidics, optics and electronics (Brown and Wittwer 2000). After analysis and identification of the target population to be sorted, the cells in the liquid stream will be separated into small droplets through the use of mechanical vibrations. These droplets can be positively or negatively charged and when flowing parallel to electrodes are deflected into sampling tubes depending on the charge of the droplet (*i.e.* a cell generates a negative charge if fluoresces, and a positive charge if not). Since FACS allows the fractionation of one cell at a time this technique presents unique resolution capacities.

When concerning the stem cell field, FACS is widely used for sorting of human HSPC based on CD34 expression after removing the more mature cells and reducing the sample volume by density gradient centrifugation (Weissman and Shizuru 2008). This application is widely used in clinical settings. In fact, FACS would be very time consuming and expensive to process whole blood directly. Importantly, FACS was effective to obtain highly purified mPB $CD34^{+}CD90^{+}$ cells allowing the preparation of cancer-free transplants in breast cancer patients (Negrin et al. 2000). More recently, FACS has also been used for the depletion of PSC from heterogeneous cell populations obtained after cell differentiation, based on the expression of

SSEA-4 in human and primate ESC (Fong et al. 2009; Shibata et al. 2006), TRA-1-60 in human ESC (Fong et al. 2009) and the expression of SSEA-1 in mouse ESC (Fukuda et al. 2006). It was also used for purification of ESC-derived neurons expressing the cell surface marker neural cell adhesion molecule (NCAM or CD56) (Pruszak et al. 2007) and for the isolation of ventricular-like cardiomyocytes differentiated from mouse ESC using a reporter cell line (Muller et al. 2000).

Although FACS presents an impaired high-resolution capacity, the equipment is large, very expensive and requires skilled technicians to operate it. In addition, it imposes significant contamination risks and a high shear stress to the cells. Importantly, the throughput of the technique is limited, with processing times of 3–6 hours including the pre-processing steps for immunostaining. Due to these characteristics, FACS is particularly adequate for purification of cells for biomedical research mainly, but very limited at a process scale for the manufacturing of cells for clinical applications. Therefore, other more scalable immunoaffinity techniques have been proposed.

Immunomagnetic Cell Separation

Magnetic-activated cell sorting (MACS) is a trademark name (Miltenyi Biotec) for a magnetic-based cell separation technique using small, magnetically susceptible beads bound to a monoclonal antibody. To achieve cell separation, cells are mixed with the beads and this mixture is then loaded into a column that is placed under the influence of a magnetic field. Due to this, the bead-carrying cells will be retained in the column whereas the unbound cells will be washed away (Fig. 7.1). The bead-carrying cells can then be recovered by elution after turning off the magnetic field. This technique can be used both for the enrichment of a target cell type (positive selection) or for the depletion of unwanted cells (negative selection).

Since the implementation of the original concept of MACS, this technology has been the focus of important developments (Grutzkau and Radbruch 2010). The most recent advances were the development of column-free systems (*e.g.* EasySep, Stem Cell Technologies) that rely on the use of very small submicron magnetic particles. Due to their biological and optical inertness, colloidal super-paramagnetic particles ranging from 20 to 100 nm have become the gold standard for magnetic cell separation (Grutzkau and Radbruch 2010). These microbeads are always in suspension allowing fast binding kinetics and short labelling procedures. Moreover, due to their small size, these particles do not saturate cell epitopes and thus they do not have to be removed for downstream applications. For example, these particle-antibody complexes do not interfere with subsequent flow cytometric analysis in opposition to cells labelled with microparticles, where the optical properties are changed. Until the introduction of this separation technology, cells labelled with submicron magnetic particles had to be magnetically separated on a column containing a magnetic matrix (*e.g.* StemSep™, Stem Cell Technologies) requiring an extra step to remove the purified cells from the column. Other developments of MACS technology consisted on the development of multimagnetic devices that

allow parallel processing of samples (autoMACS™ Pro, Miltenyi Biotec). In addition, for clinical applications requiring automated cell separation on a large scale, closed and sterile system, the target cells can be enriched from up to 1.2×10^{11} cells using the CliniMACS® system (Miltenyi Biotec). These automated systems have been widely used to enrich stem/progenitor cells from BM, UCB and mPB for use in hematopoietic cell transplantation (Weissman and Shizuru 2008) and for the isolation of highly purified UCB CD133$^+$ cells from freshly isolated or cryopreserved samples (Bonanno et al. 2004).

Traditional MACS technology was already used for the separation of cardiomyocytes derived from human PSC after having identified VCAM1 as a cell surface marker (Uosaki et al. 2011). With this method it was possible to obtain more than 95 % of cells expressing TNNT2 (cardiac troponin – T). It was also used for the separation of undifferentiated mouse ESC from a pool of differentiated and undifferentiated cells in a batch system. By using a mathematical model it was predicted that MACS technology alone would be insufficient to achieve the necessary clearance of teratoma-forming undifferentiated cells for a therapeutic application (Schriebl et al. 2010). However, in a more recent work it was shown that by using MACS followed by selective killing of residual human ESC with a specific cytotoxic antibody, the required purity of human ESC-differentiated cells can be achieved (Schriebl et al. 2012).

When compared to other separation technologies, MACS may present undesirable biological effects from the use of magnetic particles, which can interfere with cell features and further cell analysis, for example using flow cytometry, since the cell's optical properties can change. However, MACS is very easy to use, it is faster than FACS and provides comparable purity (especially if 2 consecutive cycles of magnetic separation are combined) and efficacy, and potentially conveys lower shear stress to the cells (Grutzkau and Radbruch 2010). For instances, concerning the enrichment of UCB CD34$^+$ cells we recently reported average values of 69 % purity (%CD34$^+$) after one round of purification (first column, cell recovery of 93 %). The purity of the cell population obtained could be further increased to 93 % (similar to the purities reached by FACS) after a second round of purification (second column, 52 % cell recovery) (Andrade et al. 2011). Both techniques can be strategically combined, by using MACS for pre-enrichment of rare cells and a subsequent FACS purification. However, both methods may be expensive and unsuitable for large-scale processing. Importantly, MACS has been considered the gold standard for stem cell isolation since it has been approved by FDA for clinical purposes, in particular for the enrichment of CD34$^+$ cells in neuroblastoma ex-vivo therapy (Handgretinger et al. 2002).

Affinity Chromatography

One of the most important requirements when selecting a separation technique for stem cell-based isolation and purification for cell therapy applications is their potential for large scale bioprocessing. Chromatography is one of the most powerful and

widely used separation and purification techniques in downstream processing of biomolecules and the adoption of this method for the separation of different cell types potentially offers many advantages with respect to scalability when compared to FACS and MACS. Since many years ago, several examples have been described in the literature regarding the use of packed bed chromatography for stem cell purification using for example a chromatographic column of avidin-coated Sephadex beads for $CD34^+$ cell enrichment (Johnsen et al. 1999). However, this technique presents several important limitations such as the high shear stress conveyed to the cells, long processing times and the slow rate of diffusion within the pores of the matrix. Indeed, the large size of the cells, their low diffusivity as well as their complex surface structure and chemistry pose severe challenges. Therefore, novel alternatives have been proposed for efficient and gentle cell separation under the principles of chromatography. An alternative to packed bed chromatography can be potentially provided by immunoaffinity expanded bed chromatography (EBC) since this technique is characterized by a high interparticular porosity, high adsorbent surface area and a lower shear hydrodynamic environment. This makes EBC a potentially adequate technique for stem cell-based purification but until now it was only used for the recovery of other human cells, such as human monocytes, from a heterogeneous mixture of blood cells (Ujam et al. 2003). Another alternative can be provided by the use of monolithic chromatographic columns. In this case, columns are made of a continuous matrix rather than beads with porous channels. One possible type of these monolithic chromatographic columns are supermacroporous cryogels (Lozinsky et al. 2003). These columns are prepared by gelation or polymerization at sub-zero temperature under frozen conditions and they have large (10–100 μm) and interconnected pores, allowing micrometer size particles between 1 and 15 μm (like cells) to pass through the columns non-retained (Kumar and Bhardwaj 2008; Kumar and Srivastava 2010). In addition, the hydrophilic nature of pore walls results in a gentle separation system very well suited for large and fragile cells. Envisaging a precise fractionation, these chromatographic columns can be derivatized with a specific antibody ligand introduced at the surface of the pores, allowing the affinity capture of a specific cell type. The application of cryogel-based affinity chromatography was reported for the capture of $CD34^+$ cells from UCB (Kumar and Srivastava 2010). Protein A is a protein obtained from *Staphylococus aureus*, which binds to the Fc portion of IgG from a wide range of species. When it is covalently coupled to Cryogel surfaces it can be used as an efficient adsorbent for cells that have been coated with a specific antibody (IgG type) that can thus be separated from cells that lack the surface antigen against which the antibody is directed. In this specific case, Protein A-captured $CD34^+$ cells were recovered from the Cryogel by mechanical squeezing. Indeed, since these cryogels are elastic and soft they can withstand the pressure and can be compressed four-to-six fold without getting damaged and they re-swell to their original shape upon addition of more liquid. More than 95 % of bound cells were recovered through this method and these cells maintained their proliferative capacity and the expression of CD34 cell surface marker. Nevertheless, the application of this methodology for other stem cell types remains to be explored.

Aqueous Two-Phase Systems (ATPS)

An alternative separation method with a high scalable potential is aqueous two-phase systems (ATPS). ATPS a liquid-liquid fractionation technique used for recovery and primary purification of biological products, including for separation and/or purification of cells (Cabral 2007). This biphasic system is composed of two aqueous solutions at critical concentrations inducing the formation of two immiscible phases. The technique explores the differential partitioning of the biomolecules or cells between the two phases since they will preferentially partition to one of the phases and avoid the other based on their affinity for the compounds that constitute the two phases or the interface. The separation can be performed in one or more steps in negative and/or positive mode according to the required purity degree and cell yield. ATPS can be classified as polymer-polymer and also as polymer-salt systems. Most commonly used polymers include polyethylene glycol (PEG) and dextran whereas the most widely used salts are phosphates, sulphates and citrates (Cabral 2007).

In the case of the more traditional ATPS systems, the affinity of the molecules or cells for one of the two phases or interface is solely defined by their physicochemical properties such as hydrophobicity, size and net surface charge. Nevertheless, novel ATPS strategies for cell separation have been developed that include the use of antibody-conjugated polymers, namely utilizing temperature-sensitive polymers (Kumar et al. 2001). This combined strategy was first employed for type-specific separation of acute myeloid leukemia (KG-1) cells expressing the CD34 antigen in a PEG/dextran system using an antibody conjugated with a temperature-sensitive polymer, the poly-N-isopropylacrylamide (PNIPAM) (Kumar et al. 2001). Under these conditions, the target cells were purified with a high viability, a yield of 75 % and a purity of 80 %. Moreover, the use of PNIPAM allows the potential recovery and re-utilization of the antibody, which turns this method very cost-effective. This separation system was more recently adapted to stem/progenitor cell isolation in our laboratory, more precisely for the isolation of human $CD34^+$ cells directly from whole UCB samples. In this case, the initial population of $CD34^+$ cells (0.2 % of the initial sample) was enriched to values up to 42 % with a yield above 90 % in a single partitioning step. When compared with MACS technology, ATPS provides similar recovery yields (Sousa et al. 2011) and is a more simple method since avoids the use of magnetic particles. Moreover, when compared to FACS, ATPS is more scalable and can be used at an industrial scale. Nevertheless, ATPS still have not addressed the purity standards required for a clinical application (Ruiz-Ruiz et al. 2012) and these systems require the separation of the cells from the phase polymer, which consumes a significant amount of time. Indeed, a repetitive extraction may be required for a sufficient selectivity to be achieved. Considering these characteristics, ATPS can be an adequate solution for purification of stem cells and derivatives for applications in Regenerative Medicine when a precise fractionation is not required but a fast processing is needed. In particular, the ATPS separation method developed in our laboratory is expected to pave a new way to purify HSPC for use in a variety of clinical settings (Sousa et al. 2011).

Other Immunoaffinity Techniques

Immunoadsorption techniques may also be applied for cell separation. One of the most traditionally used is the so-called Panning that consists on the covalent immobilization of antibodies to the surface of polystyrene flasks. The cells with surface receptors that bind to the immobilized ligand will tend to adhere to the plastic, while the loose cell fraction can be removed by gentle washing. One classical example of the use of this technique in the stem cell field is the isolation of $CD34^+$ cells from human BM that was achieved with a purity of about 93 % and with a 74 % yield of the multipotent colony-forming units (CFU-GEMM) (Cardoso et al. 1995). This technique, however, is characterized by a low resolution and scalability.

The distinct transient interactions of different cell types with antibodies or lectins immobilized in a surface under fluid flow can also be used as a strategy for cell separation. It has been described that this characteristic, entitled rolling velocity, can be applied for cell separation when the velocity of a specific cell type is significantly lower than the velocity of a non-interacting cell near the surface (Hammer and Apte 1992). These differential interactions were already used for the separation of primitive populations of HSPC from adult BM and fetal liver ($CD34^+$ and $CD34^+CD38^-$) from more differentiated cells ($CD34^-$ and $CD34^+CD38^+$) since the $CD34^+$ and the $CD34^+CD38^-$ cells were found to roll slowly especially on P-selectin and L-selectin immobilized in a parallel plate flow chamber when compared to more differentiated $CD34^-$ and $CD34^+CD38^+$ cells (Greenberg et al. 2000). The same basic principle was applied for developing an anti-CD34 antibody-immobilized cell-rolling column that can separate cells according to CD34 density on their surface (Mahara and Yamaoka 2010a). This strategy was already applied with success for the separation of different stem cell populations from BM namely MSC with distinct osteoblastic differentiation potential (Mahara and Yamaoka 2010b).

Novel filtration methods that take advantage of affinity interactions have also been applied to the stem cell field. As one example, a separation device that was developed for the isolation of MSC harvests cells via a nonwoven fabric filter composed of rayon and polyethylene, in a semi-closed system reducing contamination risks, without centrifugation (Ito et al. 2010). The filter selectively traps MSC among mononuclear cells based on affinity and not cell size.

7.3.4 Novel Stem Cell-Based Separation Methods

Aptamer-Based Separation

As previously mentioned, immunoaffinity methods are generally used for stem cell-based separation when a high resolution is required. However, this group of methodologies is very expensive mainly due to the necessity of using monoclonal antibodies which may turn unfeasible the application of these technologies on a large-scale for production of stem cells and their derivatives for Regenerative

Medicine. In order to overcome this limitation, novel immunoaffinity alternatives have been recently proposed to the use of monoclonal antibodies, such as the use of synthetic peptides or highly-specific nucleic acids generated by combinatorial chemistry for cell capture, the so-called aptamers (Nery et al. 2009). The use of aptamers may be advantageous since their inherent flexibility enables the molecule to bind to target sites that are not normally accessible for typical antibodies. Due to this characteristic, aptamers can be potentially used to distinguish stem cells of the same lineage and with very similar molecular features but with different degrees of commitment. These novel ligands can potentially be adapted to all types of immunaffinity-based techniques. For example, aptamers can be bound to polymeric matrices such as cryogels, magnetic beads or polymers in order to directly replace them in affinity chromatography, immunomagnetic sorting or ATPS, respectively. Aptamers were already used for stem cell separation, in particular for the isolation of BM MSC (Guo et al. 2006) as an alternative to the traditional adherence to plastic surfaces (Lennon and Caplan 2006) or antibody-based separation. Biotinylated aptamers were developed for the recognition of the molecular signature of MSC that were then used for capturing MSC from BM using anti-biotin microbeads and using a cell sorter after being labelled with fluorescein isothiocyanate (FITC). A phenotypic characterization revealed that the purified cells were positive for CD29, CD44 and CD90 expression and most of the cells did not express CD45 being consistent with previous phenotypic characterisation of these cells (Lennon and Caplan 2006). Moreover, following re-plating, the purified cells revealed an increased proliferative capacity and also osteogenic and adipogenic differentiation ability when compared to MSC isolated trough the traditional plastic-adherence method.

Tag-Less Methodologies

In addition to the use of synthetic peptides and aptamers, other alternatives have been proposed to overcome the high-expensive nature of the immunoaffinity-based methods. One recent trend in this field is the development of novel tag-less separation methodologies in which no affinity ligands are required. In this case, cell separation is governed by the biophysical properties of cells. One possible tag-less methodology is Field Flow Fractionation (FFF). FFF encompasses a group of label-free and gentle separation techniques whose principles are based on cellular morphological and biophysical differences such as mass, charge, size, density, shape and rigidity. FFF is achieved within an empty capillary channel by the combined action of a transporting laminar flow of mobile phase and a field that is applied perpendicularly to the flow (Reschiglian et al. 2005). Different types of FFF have been used for stem cell separation (Comte et al. 2006; Guglielmi et al. 2004; Roda et al. 2009a, b) but the simplest variant is gravitational FFF (GrFFF) that makes use of the gravity field. GrFFF was already used for isolating human HSPC from mPB (Roda et al. 2009b) and human MSC from a variety of different sources (Roda et al. 2009a). Undifferentiated human HSPC have “simpler” biophysical properties when compared to more differentiated/committed cells with a spherical/ovoidal shape and

with a low cytoplasm-to-nucleus ratio while committed cells acquire features related to their function that generally correspond to a more irregular shape and to more complex cytoplasm contents and a lower nucleus-to-cytoplasm ratio (Roda et al. 2009b). For this reason, in GrFFF, spherical particles elute later than non-spherical ones of similar size. In the case of human MSC, this technique was used for characterizing MSC populations from different sources, sorting different MSC subpopulations with a high differentiation potential and purifying MSC from epithelial contaminants (Roda et al. 2009a). As a major disadvantage, GrFFF is generally considered an analytical-scale methodology since a low number of cells can be isolated in each run.

Another label-free alternative for identification and separation of stem cells and stem cell-derived cells is dielectrophoresis (DEP). DEP devices consist of microchannels filled with an adequate buffer solution into which the sample is injected. A non-uniform electric field is generated and the cells can be separated, moved or trapped (Fig. 7.1). The response of a cell to DEP-mediated forces depends on the polarization between the suspending medium and the intrinsic dielectrical properties of the cell such as cytoplasm, membrane and cell wall conductivities which are dependent on cell density, size, physiology and differentiation state. In the stem cell field, DEP has been used for enrichment of $CD34^+$ HSPC from BM or mPB (Stephens et al. 1996; Talary et al. 1995). More recently, DEP was also applied to NSC populations derived from PSC (hPSNSC), which allowed to correlate the biophysical properties of cells with their differentiation potential. These studies indicated that the ultimate fate of these cells after differentiation can be predicted by distinct changes in their dielectrophoretic properties before the presence of cell-surface proteins can be detected (Flanagan et al. 2008; Labeed et al. 2011). In particular, recent data demonstrates that membrane capacitance, an electrophysiological property of cells, is inversely correlated with the neurogenic potential of human PSC-derived neural stem/progenitor cells (Labeed et al. 2011). This information indicates a potential mechanism to separate stem cells with different neuronal differentiation potential. DEP does not require a large number of cells nor expensive equipment, which are important advantages when compared to FACS.

Microfluidic Devices

One of the most recent trends in the field of cell separation is the adaptation of the different methodologies to microscale devices. Distinct flow channel designs have been developed for cell sorting based both on the physicochemical (Kim et al. 2008), affinity (Didar and Tabrizian 2010) and biophysical properties of cells. One illustrative example of a classical cell separation method that was recently incorporated into microfluidics is ATPS (Hardt and Hahn 2012). In this case, in opposition to the classic standard batch ATPS, a number of co-flowing streams of immiscible phases are guided through a microchannel while the biological samples partition between the phases. This type of continuous-flow process presents many advantages such as a more rapid mass transfer, an easy separation of the two phases, since

they are recovered from different exit branches, and there is virtually no lower limit for the sample amount to be processed (Hardt and Hahn 2012). Microfluidics present important features for cell separation such as laminar flow, easy integration with mechanical, electrical and optical systems and a low cost fabrication (Wang et al. 2011b). In addition, the use of microfluidics allows handling of very small sample volumes and cell processing on closed systems, which avoids contamination. Although these microscale technologies are very recent, they can potentially be applied to stem cell separation. One example of application of microfluidic devices for stem cell-based separation reported in the literature combines the use of the microfluidic chip technology with optical tweezers, photonic devices that exploit a tightly focused laser beam to manipulate the dielectric properties in three dimensions in a non-invasive manner, for the isolation of human ESC from a mixture of different cells with similar sizes (Wang et al. 2011b). Digital image processing was used for recognition of cell size and fluorescence for separation of human ESC modified with green fluorescent protein (GFP). This microfluidic device thus presents a great potential for depletion of human ESC from cell suspensions obtained after differentiation to eliminate residual tumorigenic, undifferentiated cells.

As another potential application to the stem cell field, microfluidic systems can be used for the development of modern cytometers with enhanced portability for on-site measurements. As one example, micro-fabricated magnetoresistive sensors can be integrated within microfluidic channels for detection of magnetically labelled cells. It was recently described the real-time detection of single magnetically labelled cells with a magnetoresistive based cell cytometer (Loureiro et al. 2011). For KG1-a cells ($CD34^+$) magnetically labelled to anti-CD34-conjugated beads (Miltenyi) and flowing through a 150 μm wide, 14 μm high microchannel, with speeds around 1 cm/s, bipolar signals with an average amplitude of 10–20 μV were observed. This system demonstrated to be effective for cell counting and has potential to be further exploited for stem cell-based separation.

Overall, despite the huge potential of these microscale devices to provide a high resolution separation it should be emphasized that the scaling up of these technologies is limited to the integration of several units in parallel configurations and for this reason the amounts of cells processed is not sufficient for a clinical application requiring $1–2 \times 10^8$ cells per patient. Thus, the application of these novel technologies is still for now restricted to the diagnosis field.

7.4 Large-Scale Manufacturing of Stem Cells and Derivatives for Cell Therapies: Bioprocess Integration

When considering the application of separation technologies for stem cell-based therapies, one of the most important issues to consider is their rational integration with culture technologies, especially bioreactor culture systems. A particularly relevant example in which the rational bioprocess integration will be required is the large-scale production of human PSC-derived cells for Regenerative Medicine applications. In fact, among other issues, the application of human PSC derivatives

in clinical settings is critically hampered by the absence of highly-efficient separation techniques for purification of the desired cell phenotype after human PSC differentiation. An important aspect to consider when designing such a bioprocess is the integration of at least one high-resolution separation operation in a negative mode for the depletion of human PSC. This is a critical issue mainly because these cells can cause the formation of tumours upon transplantation but also because the presence of human PSC in culture, especially at high densities, can negatively influence the outcome of the differentiation process. Another critical issue associated to the use of PSC derivatives in cell therapies is the low efficiency of human PSC differentiation protocols, with the desired phenotype being obtained with relatively low yields. Due to this, high-resolution separation techniques must also be integrated in a positive mode after the differentiation process for capturing the desired cell phenotype with a relatively high yield and purity. A successful bioprocess for the *in vitro* production of PSC-derived cells should thus perform a rational integration of these purification operations with the different steps of PSC expansion, commitment and differentiation in bioreactors.

Another example of an integrated bioprocess based on stem cell technology includes a scalable bioreactor system towards the efficient production of human HSPC featuring *in situ* cell selection to maximize cell productivity. The major objective of this operation is the depletion of mature blood cells arising in culture and their overall effects on culture microenvironment, which is expected to result in a more efficient expansion of HSPC. In fact, these cells are known to secrete negative regulators inhibiting stem cell proliferation and/or inducing differentiation, which may be a major limitation on the expansion of the more primitive HSC. This separation can be performed by using our previously established immunoaffinity ATPS system for UCB HSPC isolation (Sousa et al. 2011). Therefore, the integration of this separation method in a bioreactor system will allow the *in situ* elimination of the more mature cells. The ability to successfully isolate, purify and expand the numbers of human HSPC *ex-vivo*, especially those from the UCB, will be an enormous boost to all current and future medical uses of these cells.

Overall, the ability to purify the cultured stem cell populations or their derived progeny along with cell production in a bioreactor, as well as at downstream processing, will represent a major breakthrough in terms of stem cell processing. The integration of these operations should thus be rationally performed in order to develop robust, scalable and cost-effective Bioprocesses towards the large-scale production of stem cells and their derivatives, which ultimately will have a major impact on the potential clinical use of these cells.

7.5 Conclusions and Future Trends

Considering the present and future applications of stem cells in Regenerative Medicine, drug screening, pharmacological testing as well as in fundamental studies on developmental biology and human disease mechanisms it has been recently identified the urgent necessity of developing novel separation techniques and

strategies that can be successfully used for the isolation and purification of stem cells and their derivatives. According to the challenges to be faced in this field, separation techniques to be adopted should fulfill major requirements including a high-resolution capacity as well as a high potential for scalability. However, as described in previous sections, presently available techniques have a number of limitations such as their low selectivity, low scalability and contamination risks. Stem cell isolation from a variety of tissues has been performed over the years through the use of physicochemical-based methods, such as density gradient centrifugation, membrane filtration and adhesion-based separation, but these techniques are characterized by a low resolution capacity and they are only efficient for the separation of very different cell types. Affinity-based traditional techniques for cell purification, such as FACS and MACS, have also been systematically used in the stem cell field but these methods are not easily scalable and they are very expensive, due to the necessity of labeling the cells with specific targets for surface markers (*e.g.* monoclonal antibodies). In addition, cell labeling procedures (*e.g.* with magnetic particles) can potentially compromise the therapeutic application of cells. Furthermore, with the exception of the hematopoietic family, several stem cell (*e.g.* human MSC) and stem cell-derived populations (*e.g.* human PSC-derived cells) still lack a panel of surface markers that can be used as targets during affinity-based separation methods. A great effort should thus be performed towards the identification of novel more specific antigens that can distinguish similar cell populations of the same lineage. For that purpose, future efforts in this field should be focused on performing a thorough characterization of cell populations towards obtaining panels of novel surface markers similar to the ones already available in the haematological field. In addition, different techniques have been proposed to overcome some of the obstacles associated with affinity-based separation methods. Affinity chromatography and ATPS have been considered attractive alternatives since they present a higher potential for scalability but they still require cell labeling. An alternative to overcome this problem is the use of novel cell ligands, such as lectins and aptamers, that can potentially be less expensive for large-scale applications (*i.e.* aptamers are obtained by chemical synthesis). Microscale technologies based on microfluidic devices have also recently emerged as another powerful tool to overcome these limitations. As a novel trend in this field, microfluidic devices can be designed to perform a high-resolution separation based on the distinct biophysical properties of different cell populations after submitting cells to an electrical stimulation or a gravitational field. These novel "tag-less" biophysical techniques can be combined with other methodologies based on complementary cell properties, such as affinity characteristics, providing an integrated separation strategy. Moreover, the use of laminar flow that characterizes microfluidic systems potentiates a high-resolution separation and parallelization of microfluidic channels in compact arrays increasing the scalability and throughput of the method. Importantly, these devices can be operated as closed systems avoiding cell contamination. Future developments in this field should focus the integration of several microfabricated devices in *lab-on-a-chip* platforms to perform cell separation, culture, monitoring and concentration in a fully controlled, automated and closed system. Finally, when considering the

different applications in stem cell-based therapies, future technological improvements should envisage the development of separation systems with unprecedented selectivity in order to deplete residual pluripotent and immature phenotypes that can cause tumours upon transplantation. Moreover, as an important future trend, integration of separation techniques with stem cell bioreactor culture and cryopreservation is an essential requisite for the translation of stem cells and stem cell-derived products to the clinics and drug discovery. Overall, these progresses are expected to boost the application of stem cell-based therapies in the near future.

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