CHAPTER 32

Embryonic Stem Cells as a Cell Source for Tissue Engineering

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INTRODUCTION

It has been estimated that approximately 3,000 people die every day in the US from diseases that could have been treated with stem cell-derived tissues [1]. Given the therapeutic potential and growing public awareness of stem cells to treat disease, it is not surprising that embryonic stem cell (ESC) research has been rapidly expanding since mouse ESCs (mESCs) were first isolated in 1981 [2,3] followed by the isolation of human embryonic stem cells (hESCs) in 1998 [4,5] from the inner cell mass (ICM) of human blastocysts (Fig. 32.1).

Adult stem cells have been used clinically since the 1960s for therapies such as bone marrow transplantation, and these cells hold great therapeutic promise. ESCs also offer major benefits, including their ease of isolation, ability to propagate rapidly without differentiation, and — most significantly — their potential to form all cell types in the body. Additionally, ESCs are an attractive cell source for the study of developmental biology, drug/toxin screening studies, and the development of therapeutic agents to aid in tissue or organ replacement therapies. Regarding the latter application, which is the focus of this chapter, ESCs have the potential to exhibit a considerable impact on the field of tissue engineering, where current treatments for large tissue defects involve graft procedures which have severe limitations. Specifically, many patients with end-stage organ disease are unable to yield sufficient cells for expansion and transplantation. In addition, there exists an inadequate supply of harvestable tissues for grafting, and that which is available has associated risks, such as donor site morbidity, infection, disease transmission and immune rejection.

Tissue-engineering-based therapies may provide a possible solution to alleviate the current shortage of organs. Expectations for the potential of stem cells have increased even more after

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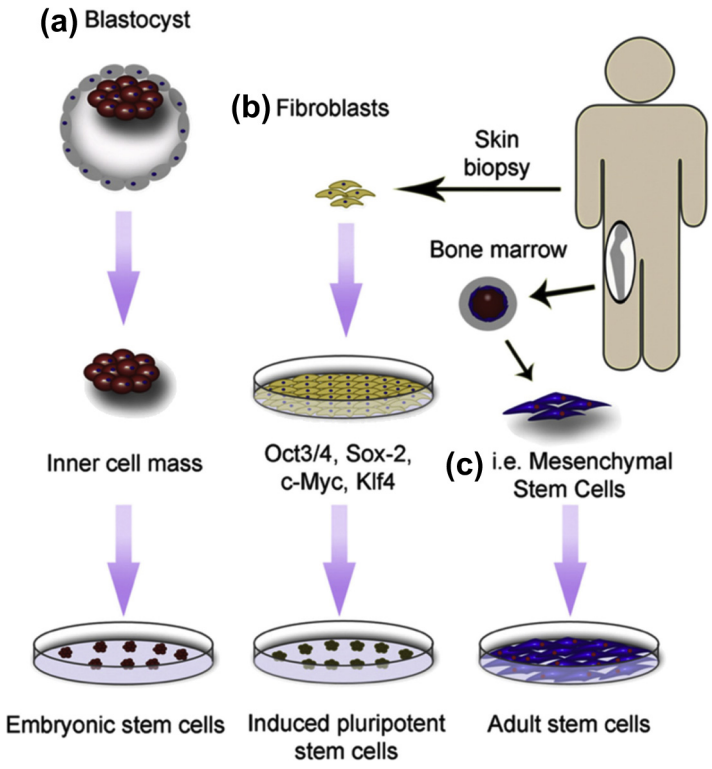

FIGURE 32.1
Schematic diagram of the derivation of stem cells. (a) Derivation of embryonic stem cells from the inner mass of the blastocysts and differentiation to different cell types; (b) Generation of induced pluripotent stem cells from somatic cells overexpressing Oct3/4, Sox2, c-Myc and Klf4; (c) Formation of adult stem cells (ASCs) during ontogeny (e.g., bone marrow mesenchymal stem cells). *(Adapted from [131]).*

the revolutionary finding of the generation of induced pluripotent stem cells (iPSCs) that profoundly modified the principles of cell fate and plasticity, and at the same time may represent a novel remarkably important cell therapy tool. iPSCs were originally generated by the introduction of four transcription factors (Oct3/4, Sox2, Klf4 and cMyc) in a somatic committed cell, the fibroblast, converting it to a pluripotent ESC-like state [6]. This work gave rise to a completely new field that is not covered in this chapter, and the reader is directed to a number of excellent papers and reviews on this topic.

Tissue engineering has been defined as an interdisciplinary field that applies the principles of engineering, materials science and life sciences toward the development of biologic substitutes that restore, maintain, or improve tissue function [7]. Thus, tissue engineering may provide therapeutic alternatives for organ or tissue defects that are acquired congenitally or produced by cancer, trauma, infection, or inflammation. Tissue-engineered products would provide a life-long therapy and may greatly reduce the hospitalization and health care costs associated with drug therapy, while simultaneously enhancing the patients' quality of life.

A central part of such strategy is the cell source to be used and the methods whereby sufficient numbers of viable differentiated cells can be obtained. ESCs represent a powerful source of cells capable of multi-lineage differentiation because they can potentially provide a renewable source of cells for transplantation. ESC-derived cells can be used directly as cellular replacement parts, or in combination with other materials (typically in the form of scaffolds, Fig. 32.2). Despite this promise, the application of ESC to tissue engineering faces numerous challenges, including appropriately differentiating the cells to the desired lineage in a controlled and homogenous fashion, and avoiding implantation of undifferentiated ESCs which can potentially form teratomas. Currently, ESC-based tissue-engineering research is focused on elucidating soluble and immobilized cues and respective signaling mechanisms that direct cell fate, on characterization and isolation of differentiated progeny, and on establishing protocols to improve the expansion and homogeneity of differentiated cells.

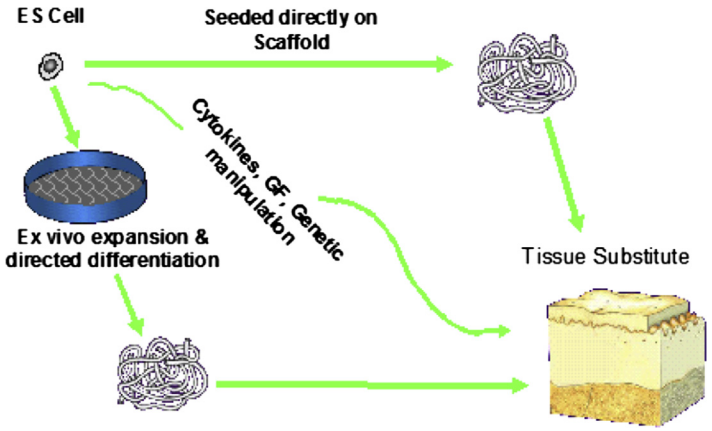

FIGURE 32.2
Approaches for using ES cells for scaffold-based tissue engineering. ES cells can be used in tissue-engineering constructs in a variety of methods. ES cells can be expanded in culture and then seeded directly onto scaffold where they are allowed to differentiate. Alternatively, stem cells can be directed to differentiate into various tissues and enriched for desired cells prior to seeding the cells onto scaffolds.

This chapter discusses key concepts and approaches for:

1) The propagation of undifferentiated ESC,
2) The directed differentiation into tissue specific cells,
3) The isolation of progenitor and differentiated phenotypes,
4) The transplantation of progenitor and differentiated cells, and
5) The remaining challenges for translating ESC-based tissue-engineering research into the clinical therapies.

Whenever possible, approaches using hESCs are reported.

MAINTENANCE OF ESCs

The self-renewal of ESCs is a prerequisite for generating a therapeutically viable amount of cells. Over the past few years much insight has been gained into the self-renewal of ESCs. Both murine and human ESCs (mESC and hESCs) were first derived and maintained in culture using mouse embryonic fibroblast feeder (MEF) layers and media containing serum. Considerable behavioral, morphological, and biochemical differences have been observed between mESCs and hESCs, and the research of animal ESCs is not easily translated to human ESCs [5,8,9]. For example, mESCs form tight, rounded clumps whereas hESCs form flatter, looser colonies, grow more slowly and demand strict culture conditions to maintain their normal morphology and genetic integrity. Unlike mESCs, which can be maintained in an undifferentiated state in the presence of leukemia inhibitory factor (LIF), hESC cultures require, in addition to LIF, supplementation of the basic fibroblast growth factor (bFGF) or the use of a feeder layer. Although both mESCs and hESCs express common transcription factors of 'stemness', such as Nanog, Oct4, and alkaline phosphatase, in the human system undifferentiated ESCs express Stage Specific Embryonic Antigen-3 (SSEA-3) and SSEA-4 and SSEA-1 is only expressed upon differentiation, whereas the opposite expression is observed in the mouse system. Due to these differences, efforts in hESC research focus on understanding the mechanisms of hESC self-renewal.

Mouse and human iPSCs have marker expression profile and biological properties very similar to the mouse and human ESCs, respectively, and this is a general proof of principle of their real pluripotent state. However, after initial studies, data are emerging that iPSCs are actually not identical to ESCs [10], and a growing body of evidence indicates that the epigenetic memory of the original cell type reprogrammed is at least partially maintained (reviewed in reference [11]). This may result in a more limited level of pluripotency in terms of spectrum of differentiation if compared to ESCs, but not necessarily a more limited spectrum of possibility of tissue differentiation for transplantation purposes if the original cell type is chosen accordingly [12].

Therapeutic applications of stem cells require moderate to large numbers of cells, hence requiring methods amenable to scale-up. Therefore, xenograft cell sources have also been considered. Using cultures of hESCs on human feeders it was found that human fetal muscle fibroblasts, human fetal skin fibroblasts and adult fallopian tubal epithelial cells supported the pluripotency of hESC culture *in vitro* [13]. The same group derived and established a hESC line on human fetal muscle fibroblasts in entirely animal-free conditions [13]. Since then, different fetal and adult cells have been examined and shown to support the continuous growth of hESCs [14–17]. However, the use of hESCs for therapeutic application requires defined culture medium and controlled cell derivation, maintenance and scale-up. To overcome these obstacles, combinations of self-renewal signals for hESCs have been investigated including soluble factor, extracellular matrix (ECM), cell-cell interactions and mechanical forces.

Significant attempts have been made to identify culture conditions and media components which can regulate hESCs self-renewal. Growth factors in culture media can bind the cell surface receptors to promote self-renewal. These soluble factors include bFGF [18,19], tumor growth factor β1 (TGFβ1)/ActivinA/Nodal ligands [20,21], insulin-like growth factor (IGFs) [18,22], Wnt ligands [23,24] and glycogen synthase kinase-3 GSK-3 inhibitors [25]. In one study, it was shown that hESCs can be expanded on human fibronectin using a medium supplemented with bFGF and TGFβ1 [26]. Noggin, an antagonist of bone morphogenetic protein (BMP), was found to be critical in preventing the differentiation of hESCs in culture. The combination of Noggin and bFGF was sufficient to maintain the proliferation of undifferentiated hESCs [27].

It has been also demonstrated that Wnt ligands affect ESCs self-renewal and differentiation. For example, spontaneous differentiation of mouse embryonic fibroblast inhibits by addition of Wnt1 to culture media [23]. In addition, hESC differentiation induces by using Wnt3 [28]; however, hESC self-renewal perturbs by the activation of canonical Wnt/β-catenin pathway through the expression of stabilized β-catenin [29]. Furthermore, hESCs maintained in media containing high concentrations of bFGF (24–36 ng/ml), alone or in combination with other factors, show characteristics similar to cultures maintained with feeder cell-conditioned medium [30,31].

The derivation of hESCs has also been achieved with minimal exposure to animal-derived material, using serum replacement (SR) and human foreskin fibroblasts as feeder cells [32], instead of the feeder layer [33], providing well-defined culture conditions [34]. Research is currently under way to determine how these conditions maintain cell integrity over long-term culture. For example, mTeSR, which contains TGFβ1, LiCl, bFGF, pipecolic acid, and GABA, supports long-term self-renewal of feeder-independent hESC cell culture [34]. In addition to growth factors, lipid molecules such as sphingosine-1-phosphate (S1P) [35–37], albumin [38], and synthetic lipid carriers [39] have been shown to regulate the self-renewal and differentiation of hESCs. Although growth factor and media compositions can control hESC self-renewal, challenges including maintenance of pluripotency, and production of biologically and functionally identical cells still remain.

In addition to soluble factors, a defined ECM or biomaterial may be required for maintaining the hESC self-renewal ability. Various biomaterials, such as Matrigel [40], human fibronectin [41], human vitronectin [42], collagen I [43], complex humanized matrices [44], hyaluronic acid hydrogels [45] or calcium alginate hydrogel [46], have been used as a structural support for hESC self-renewal. As an example, Xu et al. showed that hESCs can be maintained on Matrigel or laminin and MEF-conditioned media [47]. Cells grown in these conditions meet all the criteria for pluripotent cells: they maintain normal karyotypes, exhibit stable proliferation rate and high telomerase activity and they differentiate into derivatives of all three germ layers, both *in vitro* and *in vivo*. In an attempt to find ideal ECM components or biomaterials for *in vitro* feeder-cell-free culture of hESCs, Hakala et al. compared various biomaterials including ECM proteins (i.e., collagen IV, vitronectin, fibronectin, and laminin), human and

animal sera matrices, and Matrigel in combination with a variety of unmodified or modified culture media. Matrigel in combination with defined mTeSR1 culture medium was found to be superior matrix for hESC culture compared to other biomaterials used in this study [40]. Similarly, in a combinatorial study, Brafman et al. developed a high throughput technology, an arrayed cellular microenvironment, to assess the self-renewal of hESC cultured on different ECMs in media composed of different growth factors. Long-term self-renewal of hESCs was obtained on a biomaterial consisting of collagen IV, fibronectin, collagen I, and laminin in defined StemPro media and MEF-conditioned media [48].

Self-renewal and differentiation of ESCs can be also regulated through intercellular interactions [49–52] and mechanical forces [53–55]. Cell-cell interactions and formation of ESC colonies affect the self-renewal and spontaneous differentiation of ESCs. It has been shown that the size and shape of colonies play an important role in controlling ESC expansion [49,52]. Various microfabrication technologies have been employed to control ESC shape and size, such as micropatterning of substrate with ECMs [49] to confine colony formation to patterns, or formation of hESCs colonies in 3D microwells [50,51]. Another important factor in hESC self-renewal is the application of mechanical forces (e.g., cyclic biaxial strain [53,54] or shear stress [55]) to the cells. Although the physiological effects of mechanical forces on self-renewal and proliferation of ESCs remain unknown, it has been shown that these forces can regulate cellular differentiation. For example, fluid flow-induced shear stress has been demonstrated to enhance the elongation and spreading of undifferentiated hESCs and induce vascular differentiation of hESC at higher shear stress [55].

Large-scale production of hESCs is critical for tissue-engineering applications, which require large numbers of cells. It is generally accepted that 'classical' laboratory culturing methods are not suitable for the large-scale production of ESCs for therapeutic applications, and new culture systems are needed. Although two-dimensional (2D) methods such as the high density cultures of ESCs have been developed by combining automated feeding and culture methods [56], three-dimensional (3D) culture may be a more suitable technology for large-scale expansion of ESC production.

At the present time, the aggregation of multiple ESCs is necessary to initiate EB formation. The formation of large cellular aggregation may prevent nutrient and growth factor diffusion as well as metabolic waste removal from the aggregates in suspension cultures in large-scale systems. A small number of methods have been developed for the differentiation of mESCs in controlled cultures. Hanging drops and methylcellulose cultures have been shown to be somewhat efficient in preventing the agglomeration of EBs, but their complex nature makes their upscaling a rather difficult task.

A much simpler process in spinner flasks resulted in the formation of large cell clumps within a few days, indicative of significant cell aggregation in the cultures [57]. Compared to static culture system, spinner flasks enhance homogenous expansion of hEBs and can be easily scaled up to 10,000L bioreactor tanks [58]. In one study, it was demonstrated that the growth rate of hEB is higher when cultured in stirred vessels than in other culture systems (e.g., static culture and rotary cell culture system) [59]. However, an increase of the culture medium stirring rate to avoid agglomeration within the stirred vessels resulted in massive hydrodynamic damage to the cells due to the extensive mixing in the vessels. Therefore, in order to establish a scaleable process for the development of EBs, there is a need for dynamic cultivation under controlled mixing conditions. One approach used a static system for an initial aggregation period of four days, followed by a period in dynamic culture in spinner flasks, to successfully achieve the bulk production of cardiomyocytes from differentiating mES cells [60].

In addition to suspension cultures using hEBs, stirred vessels can be also used for the scale-up expansion of undifferentiated hESCs through the combination of a microcarrier with the stirred culture systems. Various microcarriers such as polystyrene [61], collagen-coated dextran

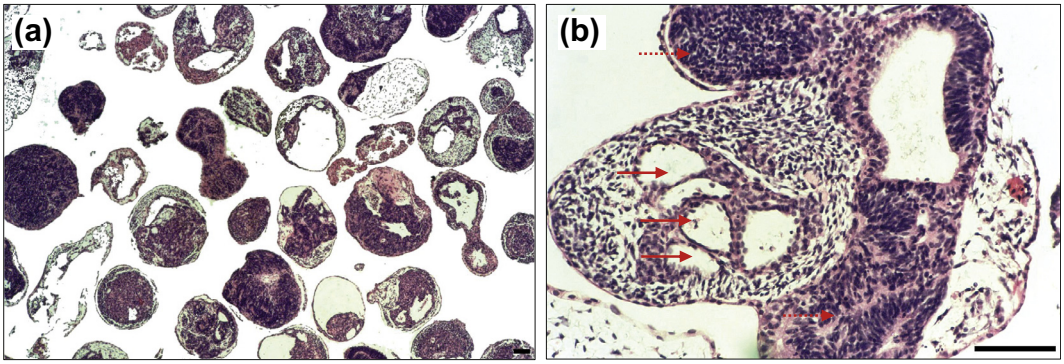

FIGURE 32.3

Formation of human EBs in a rotating cell culture system. (a—b) Haematoxilin- and eosin-stained sections of EBs after one month in culture, showing the formation of (a) a small and relatively homogenous population of human EBs, and (b) a variety of cell types such as: epithelial neuronal tubes (dashed arrows), and blood vessels (solid arrows) (Scale bars: 100 μm). *(Adapted from reference [66].)*

[62] and Matrigel-coated cellulose [63] have been used to promote hESC expansion in spinner flasks. In addition to hESC expansion, this combination of microcarriers and stirred culture systems has been used for directing hESC differentiation to definitive endoderm [64] and cryopreservation and recovery of undifferentiated hESCs adhered to microcarriers [65].

Another dynamic approach which was highly effective for hESCs is to generate and culture EBs within rotating cell culture systems [66]. These bioreactors provide exceptionally supportive flow environments for the cultivation of hESCs, with minimal hydrodynamic damage to incipient EBs, reduced EB fusion and agglomeration, and they allow the uniform growth and differentiation of EBs in three dimensions, as they oscillate and rotate evenly (Fig. 32.3a). hESCs cultured within these systems formed aggregates after 12 hrs that were smaller and more uniform in size and evenly rounded due to minimal agglomeration; the yield of EBs was three times higher than that measured for static cultures. Also, dynamically formed EBs exhibited steady and progressive differentiation, with cyst formation and elaboration of complex structures such as neuro-epithelial tubes, blood vessels and glands (Fig. 32.3b) [66].

Different rotary cell culture systems, including slow turning lateral vessel (STLV) and high aspect rotating vessel (HARV), have been used to promote the efficiency of EB formation and differentiation of stem cells [66—69]. Generally, STLV systems are preferable to HARV for the EB aggregate formation and differentiation. It has been shown that the HARV system can lead to significant aggregation with large necrotic areas at the center and differentiations at the peripheries of aggregates. The aggregation rate of hESCs can be controlled by using the STLV system, which results in the formation of small-size hEBs [58,66].

To further enhance the large-scale differentiation of hEBs, a perfused STLV system was combined with a dialysis chamber to allow the diffusion of media as well as removal of waste products from the bioreactor [70]. Compared to static cultures, uniform growth and differentiation of hEBs to neural lineage was promoted when the combined rotary cell culture system/dialysis chamber was used [70]. Although rotary cell culture systems provide low-shear environments for hESCs cultivation and differentiation, they can only be scaled-up to volumes of 5—500 mL, which is much lower than the scalability of stirred culture systems. Although still an area of active research, these technologies have demonstrated the potential of engineering for the development of scalable technologies to expand ESC provision for research and therapies.

DIRECTED DIFFERENTIATION

Perhaps the biggest challenge in the clinical use of ESCs is the lack of knowledge of how to predictably direct their differentiation. For example, although ESCs can generate cells of

hematopoietic, endothelial, cardiac, neural, osteogenic, hepatic and pancreatic tissues, it has been very difficult to achieve directed differentiation into these tissues. The lack of homogeneous differentiation may be attributed to the intrinsic property of ESCs of differentiating stochastically in the absence of proper temporal and spatial signals from the surrounding microenvironment. Various techniques have been employed to control the differentiation of hESCs and to isolate a specific germ layer for tissue regeneration applications. The limitation of current techniques used for controlled differentiation is the low transformation efficiency, which results in a cell population containing ectoderm and mesoderm germ layers. The segregation of these germ layers can be achieved by using appropriate differentiation protocols. In this section we describe some of the current approaches used to direct the differentiation of ESCs and give examples of their use.

Genetic reprogramming

This approach includes the introduction of specific gene(s) into hESCs, which enable the production (by enhancement or selection) and propagation of specific cell type populations. Different techniques for knocking-in and knocking-out genes into hESCs have already been established. Transfection of undifferentiated hESCs with specific plasmid was established using either chemical reagents or electroporation. The latter was further shown to be useful for the generation of homologous recombination events [71]. Another technique is the introduction of transgenes into hESCs by self-inactivated lentiviruses. This transduction technique was shown to be efficient, with sustained expression in undifferentiated hESCs as well as in hESCs, which undergo differentiation [72,73]. However, both undifferentiated and differentiated hESCs were successfully infected by using adenoviral and adeno-associated viral vectors [74]. Another approach, which uses genetic manipulation, is the introduction of suicidal genes, which permit the ablation of the cells if necessary [75]. Using this approach, hESCs were transfected to express the herpes simplex virus thymidine kinase gene [76].

Genetic techniques involve both positive and negative regulators. The positive regulators include the constitutive or controlled expression of transcription factors that have been shown to derive the differentiation into particular tissues. For example, the over-expression of the Nurr transcription factor has been shown to increase the frequency of ESCs that differentiate into functional neural cells [77]. Alternatively, the negative regulators can be incorporated to induce the apoptosis of cells that differentiate to varying pathways. For example, neomycin selection and suicide genes that are activated by certain transcription factors can be used [78]. In a recent study, Zoldan et al. developed a 3D siRNA delivery system using lipid-like materials, lipidoids, for the efficient transfection of hESCs. This system was used to direct differentiation of hESCs to a specific lineage by knocking down Kinase Insert Domain Receptor (KDR) to prevent the differentiation of endoderm layer, leading the separation of this germ layer from mesoderm and ectoderm [79]. The developed 3D RNA delivery technique have shown to be preferable over a 2D environment for directing hESC differentiation, in which the transfection reagents are added to the media used for *in vitro* culture of hESCs-seeded 2D substrates. Clearly, all these techniques would benefit from a deeper understanding of inner workings of transient cells and knowledge of the differentiation pathways and lineages. Further analysis of the stem cell and progenitor hierarchy through high-throughput analysis of gene and protein profiles should accelerate this process. Despite the power of these approaches, one potential concern is that the genetic modifications may make the cells unsuitable for transplantation.

Microenvironmental cues

Another approach to directing ESC differentiation is through the use of microenvironmental cues that are important in regulating adult and ESC fate decisions. During development, cells of the inner cell mass are exposed to a series of tightly regulated microenvironmental signals. However, in tissue culture the complex expression patterns and spatial orientation of these signals can be lost. Currently, ESCs are grown in their primitive state as aggregated colonies of

cells. To stimulate differentiation, two main methods have been examined. In one method, differentiated cells are derived from EBs. EBs can be formed from either single cell suspensions of ESC or from aggregates of cells. EBs mimic the structure of the developing embryo and recapitulate many of the stages involved during its differentiation, and clonally derived EBs can be used to locate and isolate tissue specific progenitors. EBs initiate many developmental processes and create suitable conditions for differentiation of cells into all three germ layers and are generally formed through suspension or hanging drop methods.

In general, the differentiation of ESCs in EBs produces a wider spectrum of cell types, due to the EBs' ability to better mimic the temporal pattern of cell differentiation seen in the embryo. In some applications, the combined use of EBs and adherent cultures has resulted in better cell yields. For example, to induce ESC differentiation to cardiomyocytes, EB formation in suspension cultures followed by differentiation in adhesion cultures has been shown to optimize the percentage of cells that give rise to cardiomyocytes [80,81]. Similarly the production of hepatocytes has been shown to be induced by first culturing the cell in EBs and then in 2D cultures [82].

Neural progenitor cells were isolated from hESCs that showed positive immunoreactivity to neuron-specific antigens, responded to neurotransmitter application, and presented voltage dependent channels in the cell membrane [83–86]. Various differentiation approaches, including adherent culture or EB suspension culture, have been used to direct the hESC differentiation to the neural lineage [87] (Fig. 32.4a).

To promote neural differentiation, different soluble factors such as BMP-inhibitors, retinoic acid (RA), and other supplements (e.g., N2, B27, ITS) are added to the media in adherent culture methods. In EB suspension culture systems, neural induction factors should be added during differentiation to induce neural differentiation of hESCs. The differentiated cells are then cultivated on adherent culture to allow for the neural cell growth. In both approaches, the morphological characteristics of the neural progenitors can be maintained and expression of NP-markers in the medium supplemented with FGF2 and B27 [87] (Fig. 32.4b). Highly enriched cultures of neural progenitor cells were isolated from hESCs and grafted into the stratum of rats with the Parkinson's disease [88]. The grafted cells differentiated *in vivo* into dopaminergic neurons and corrected partially behavioral deficits in the transplanted animals. A subsequent study showed that hESCs implanted in the brain ventricles of embryonic mice can differentiate into functional neural lineages and generate mature, active human neurons that successfully integrate into the adult mouse forebrain [89].

Oligodendrocytes and their progenitors were also isolated in high yield from hESCs [90]. Transplantation of these cells into animal models of dysmyelination resulted in integration, differentiation into oligodendrocytes and compact myelin formation, demonstrating that these cells displayed a functional phenotype. In addition to *in vivo* differentiation of hESCs to neural lineages, ESCs can be combined with a biomaterial to induce the *in vitro* differentiation of ESCs to specific neural lineages in the presence of differentiation-inducing agents. For examples, electrospun fibrous scaffolds not only enhanced the differentiation of mouse ESCs into specific neural lineages such as neurons, oligodendrocytes and astrocytes, but also supported the neurite outgrowth [91]. In recent years, the use of carbon nanotubes (CNTs) for the neuron differentiation from hESCs and neural growth has been also explored [92,93]. It has been shown that 2D scaffolds composed of poly(acrylic acid) grafted CNT thin films promoted hESCs' neuron differentiation efficiency as well as protein adsorption and cell attachment compared to poly(acrylic acid) scaffolds without CNTs [92].

The differentiation of hESCs to neural lineages is induced by supplementation of the culture medium with biochemical agents. Recently, it has been demonstrated that nanopatterning of the substrate can effectively control hESC differentiation to neural lineages in the absence of

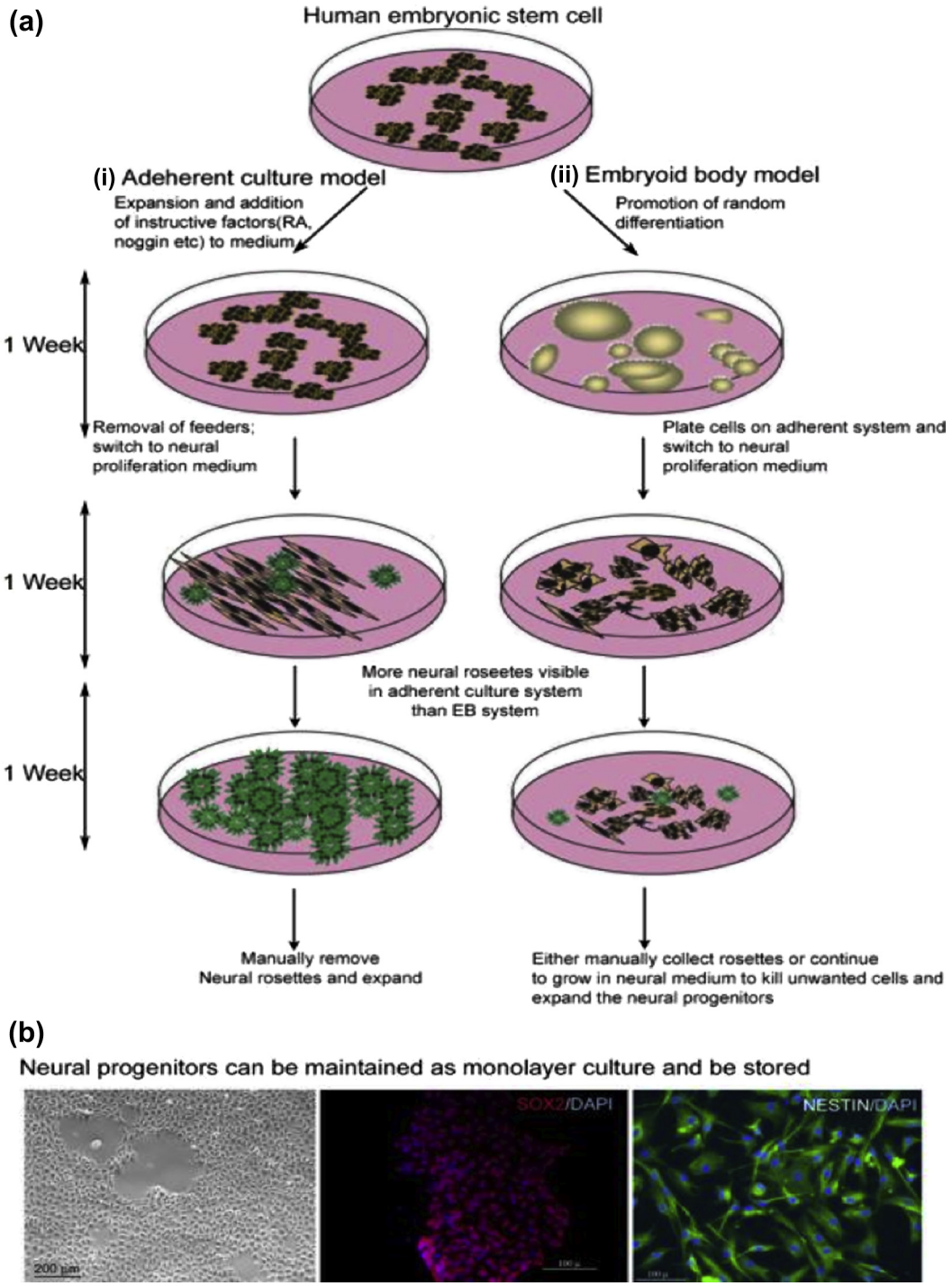

FIGURE 32.4

Differentiation of hESCs into neural progenitor cells. (a) Directed differentiation of hESC to neural lineages by adherent or EB suspension culture, (b) bright field image (left panel), and expression of neural progenitor markers including SOX2 (red, middle panel) and Nestin (green, right panel), indicating that the neural progenitor generated by these methods preserved their characteristic morphology and exhibited the expression of neural progenitor by culturing in medium containing FGF2 and B27. *(Adapted from reference [87]).*

any biological and biochemical agents. In one study, a UV-assisted capillary force lithography technique was developed to generate 350 nm pattern arrays using polyurethane acrylate [94]. The hESCs seeded on these patterns differentiated to neuronal lineage after five days of culture without the addition of differentiation-inducing agents [94].

ESCs have been shown to give rise to functional vascular tissue. Three different strategies have been employed to induce vascular differentiation of ESCs:

1) EB formation;
2) Uco-culture with fibroblast feeder layers or target cells; and
3) 2D monolayer culture of ESCs in defined chemical conditions combined with differentiation stimuli [95] (Fig. 32.5).

Spontaneous differentiation of ESCs to EB aggregates in a medium supplemented with cytokines has been shown to promote their differentiation to smooth muscle cell, pericytes, and endothelial cells [96,97]. One limitation of this strategy is that the ESCs differentiate to a heterogeneous cell population composed of vascular cells and other cell types from different germ layers. Various approaches have been employed to improve the efficiency of EB protocols to promote vascular cell differentiation, such as addition of VEGF-A [98] or BMP4 [99] to culture media and using magnetic-activated cell sorting (MACS) or fluorescence-activated cell sorting (FACS) [100].

Another approach to directing the vascular differentiation of hESCs is the use of co-culture systems, where undifferentiated ESCs are seeded onto mouse fibroblast feeder layers such as stromal cells [101], MEF [102], or mouse ECs [103] to enhance vascular differentiation. Alternatively, 2D monolayer culture on Matrigel [104], collagen IV [105], and fibronectin [106] combined with differentiation stimuli (e.g., addition of GFs/Cytokines [104] or RA [107], mechanical stimulation [108], and hypoxia [109]) have been used for vascular hESC differentiation. Although these strategies increase the differentiation efficiency, isolation of progenitor cells expressing markers (e.g., CD34, Stem Cell Antigen [Sca]-] or Flk1) during ESC differentiation is a crucial requirement for deriving homogenous vascular cell populations [104].

Early vascular progenitor cells isolated from differentiating mESCs were shown to give rise to all three blood vessel cell types: hematopoietic, endothelial and smooth muscle cells [110]. Once injected into chick embryos, these vascular progenitors differentiated into endothelial

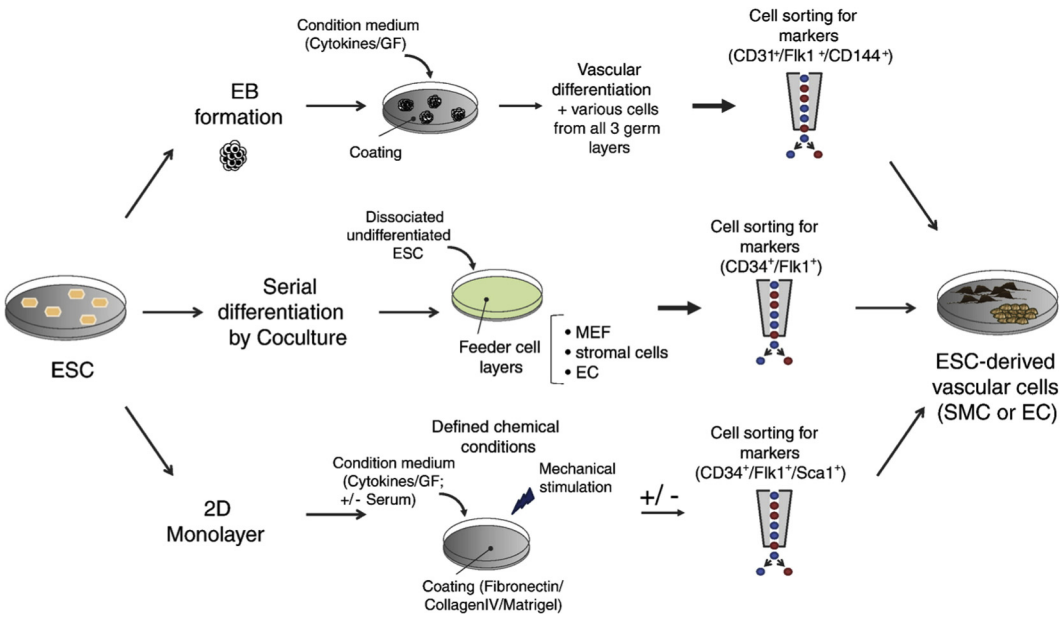

FIGURE 32.5

Approaches used for vascular differentiation of ESCs. Vascular cell differentiation is mainly induced by three culture methodologies: differentiation through EBs, co-culture with fibroblast feeder layers or target cells, and monolayer culture of ESCs in defined chemical conditions. *(Adapted from reference [95].)*

and mural cells and contributed to the vascular development. hESCs can also be differentiated into endothelial cells by using platelet endothelial cell adhesion molecule-1 antibodies [111]. *In vivo*, when transplanted into immunodeficient mice, these cells appeared to form microvessels.

Furthermore, it has been shown that monkey ESCs can give rise to endothelial cells when the embryonic cells were exposed to a medium containing combinations of growth factors. The isolated cells were able to form vascular-like networks when implanted *in vivo* [112]. Endothelial progenitor cells have been isolated from hES cells which presented hematopoietic [113] or smooth muscle cells competency. hESCs have been reported to differentiate into hematopoietic precursor cells when co-cultured with bone marrow and endothelial cell lines [114]. When these precursor cells are cultured on semisolid media with hematopoietic growth factors, they form characteristic myeloid, erythroid and megakaryocyte colonies.

Cardiomyocytes have been isolated from hES cells for the treatment of cardiac diseases. The most common approach to induce *in vitro* differentiation of hESC to cardiomyocytes is the formation of EB aggregates followed by few days post-plating on a 2D substrate to obtain cells with cardiomyocyte characteristics [115]. Cardiomyocyte differentiation of hESCs can be also induced by co-culturing undifferentiated hESCs with a mouse visceral endoderm-like cell line (END-2) [116]. Cardiomyocytes isolated from hES cells expressed sarcomeric marker proteins, chronotropic responses, and ion channel expression [116]. Upon differentiation, beating cells were observed after one week of culture under differentiation conditions. These increased in number over time, and could retain contractility for over 70 days [117]. The beating cells expressed markers characteristic of cardiomyocytes, such as cardiac α-myosin heavy chain, cardiac troponin I and T, atrial natriuretic factor, and cardiac transcription factors GATA-4, Nkx2.5, and MEF-2. Electrophysiology demonstrated that most cells resembled human fetal ventricular cells. Despite the progress made over the last decade, knowledge about the mechanism of the formation of functional cardiomyocytes from hESCs remains limited.

Insulin-producing β cells were also generated from hESCs [118], by spontaneous differentiation of hESCs in adherent or suspension culture conditions [119] and using media which contained growth factors [120,121]. Reverse transcription-polymerase chain reaction detected an enhanced expression of pancreatic genes in the different cells [120]. Immunofluorescence and *in situ* hybridization revealed high percentages of insulin-expressing cells [120].

Recently there has been great interest in examining the osteogenic potential of ESCs derived from both mice and humans. hESCs can differentiate into osteogenic cells with the same media supplements that are used to differentiate adult mesenchymal stem cells (MSCs). Due to their high self-renewal capability, ESCs are preferable to MSCs for bone regeneration, as the latter have limited abilities for self-renewal, proliferation, and differentiation into the osteogenic lineage [122]. Current issues associated with the osteogenic differentiation of hESCs include the formation of non-homogeneous cell populations and limited numbers of differentiated cells. To overcome these limitations, various growth factors and reagents such as β-glycerophosphate, ascorbic acid, dexamethasone, and osteogenic factors have been used to create osteoprogenitor cells from hESCs [123–126]. In addition, the differentiation efficiency of hESCs into a homogeneous osteogenic cell population was improved through co-culturing of hESCs with human primary bone-derived cells (hPBDs) in the absence of exogenous factors [127].

The osteogenic cells derived from hESCs (OC-hESCs) were seeded on a apatite-coated poly(D,L-lactic-co-glycolic acid)/nano-hydroxyapatite (PLGA/HA) composite scaffold and subcutaneously implanted in immunodeficient mice to examine *in vivo* bone formation [128]. The results of *in vivo* studies demonstrated that the implanted OC-hESCs and apatite-coated PLGA/HA scaffold induced the formation of large amounts of new bone tissue within the defect site, demonstrating the suitability of hESCs for bone regeneration [128]. Our group

showed that culturing hESCs without EBs leads to an over seven-fold increase in the number of osteogenic cells and to spontaneous bone nodule formation after 10–12 days [129]. In contrast, when hESCs were differentiated as EBs for five days followed by plating of single cells, bone nodules formed after four weeks only in the presence of dexamethasone.

We show that the cultivation of hESC-derived mesenchymal progenitors on three-dimensional osteoconductive scaffolds (derived from fully decellularized trabecular bone) in bioreactors with medium perfusion was shown to lead to the formation of large and compact bone constructs. Notably, the implantation of engineered bone in immunodeficient mice for eight weeks resulted in the maintenance and maturation of bone matrix, without a single incidence of the formation of the teratomas that were consistently observed when undifferentiated hESCs are implanted, alone or in bone scaffolds. This showed that tissue-engineering protocols can be successfully applied to hESC-progenitors to grow bone grafts for use in basic and translational studies [209].

In another study, native heart extracellular matrix (ECM) was successfully used to direct the cardiac differentiation of human embryonic stem cells (hESCs) *in vitro* [210]. A series of hydrogels was prepared from decellularized heart ECM blended with collagen type I at varying ratios. Maturation of cardiac function in EBs formed from hESCs was documented in terms of spontaneous contractile behavior and the mRNA and protein expression of cardiac markers. Hydrogel with high ECM content (75% ECM, 25% collagen, no supplemental soluble factors), increased the fraction of cells expressing cardiac marker troponin T (*c*TnT), when compared to either hydrogel with low ECM content (25% ECM, 75% collagen, no supplemental soluble factors), or collagen hydrogel (100% collagen, with supplemental soluble factors). The ability of native ECM to induce cardiac differentiation of hESCs without the addition of soluble factors makes it an attractive biomaterial system for basic studies of cardiac development and potentially for the delivery of therapeutic cells into the heart.

3D versus 2D cell culture systems

In an appropriate environment, ESCs can differentiate into complex 3D tissue structures. These environments are designed to resemble the key features of the hESC's niche and are favored over the 2D systems, which limit the cellular interactions and signaling, and hamper the subsequent differentiation of hESC into functional tissues [130,131]. The scaffold may act as a temporary ECM; providing physical cues for cell orientation, spreading, differentiation and the remodeling of tissue structures.

It has been demonstrated that the biochemistry, topography, and physical properties of the scaffold can regulate stem cell differentiation and function [131,132]. Culture of hESCs in PLGA scaffolds in specific media containing transforming growth factor β, activin A, or insulin-like growth factor induced the differentiation of the cells into 3D structures with characteristics of developing neural tissues, cartilage, or liver, respectively [133].

It was also demonstrated that the 3D environment created by cell encapsulation in Matrigel failed to support hESC growth and 3D organization, and this was likely due to the fact that this gel was unable to resist the force of cell contraction. Furthermore, when these cells were cultured in PLGA and poly(lactic acid) (PLA) scaffolds in the presence of media containing nerve growth factor and neurotrophin 3, enhanced numbers of neural structures were observed [134]. In one study, hESC-derived EBs cultured with a 3D collagen scaffold exhibited liver-specific genes expression and albumin production in the presence of exogenous growth factors and hormones [135]. The addition of signaling factors such as activin A and Wnt3a to this system may improve the efficiency of hESCs differentiation and production of functional hepatic endoderm [136].

Similarly, the culture of ESCs in a 3D collagen scaffold, stimulated with exogenous growth factors and hormones, led to the differentiation of the cells into hepatocyte-like cells. These

cells were characterized by the expression of liver-specific genes and synthesis of albumin, and the differentiation pattern observed compared favorably to cells differentiated in a 2D system. It was also reported that the differentiation of rhesus monkey ESCs in 3D collagen matrices was different from that which took place in monolayers [137]. Alginate scaffolds were also used for the differentiation of hESCs [138]. These scaffolds induced vasculogenesis in encapsulated cells to a larger extent than cells grown in bioreactors. Tantalum scaffolds also increased the differentiation of mESCs into hematopoietic cells as compared to traditional 2D cultures [139]. Therefore, the 3D culture systems can promote hESC differentiation and assembly into functional tissues, through better mimicking of the 3D structural organization of native tissue compared to 2D systems.

High-throughput assays for directing stem cell differentiation

Today, chemists and engineers are equipped with tools which give them the ability to synthesize molecules and test their effects on cells in a high-throughput manner. For example, libraries of small molecules, polymers and genes have been generated and used to screen candidate molecules to induce osteogenesis [140] and cardiomyogenesis [141] in ESCs as well as the dedifferentiation of committed cells [142]. The use of chemical compound libraries may provide a method of addressing the complexities associated with native microenvironments by directing cell behavior through interacting with transcription factors and cell fate regulators.

Microscale technologies can facilitate high-throughput experimentation and provide a powerful tool for screening whole libraries of molecules and biomaterials. Robotic spotters capable of dispensing and immobilizing nanoliters of material have been used to fabricate microarrays, where cell-matrix interactions can be tested and optimized in a high-throughput manner. For example, synthetic biomaterial arrays have been fabricated to test the interaction of stem cells with various extracellular signals [143]. Using this approach, thousands of polymeric materials were synthesized and their effects on differentiation of hESCs [144] and human mesenchymal stem cells (hMSC) [145] have been evaluated. These interactions have led to unexpected and novel cell-material interactions. Although the molecular mechanisms associated with the biological responses have yet to be clarified, such technology may be widely applicable in cell-microenvironment studies and in the identification of cues that induce desired cell responses.

Also, these materials could be used as templates for tissue-engineering scaffolds. Such an approach is a radical departure from traditional methods of developing new biomaterials, where polymers have been individually developed and tested for their effect on cells. In addition to analyzing synthetic material libraries, the effect of natural ECM molecules on cell fate can be evaluated in a high-throughput manner [143]. In one example, combinatorial matrices of various natural extracellular matrix proteins were tested for their ability to maintain the function of differentiated hepatocytes and to induce hepatic differentiation from murine ESCs [146]. Recently, Huang et al. used a micro-scale direct writing (MDW) technique to print ECM components (e.g., collagen IV, gelatin, and fibronectin) into diverse geometries and compositions on 2D surfaces for assessing the effect of ECM geometry and composition on ectodermal differentiation of murine ESCs [147]. It was shown that ECM compositions, soluble factors, and surface topography could regulate ESC attachment and differentiation [147].

Microfabrication techniques have been also used to control cell-cell interaction and to form hESC-derived EB aggregates with defined sizes and geometries. For example, in one study, soft lithography was used to fabricate cell-repellant poly(ethylene glycol) (PEG) microwells for the formation of EB aggregates with controlled sizes and shapes, determined by the geometry of the microwells (Fig. 32.6a). The EB cell aggregates formed within the microwells remained viable and maintained their geometries over at least 10 days of culture. Using this system, the EB aggregates could pattern into various shapes and sizes (Fig. 32.6b–d) [148]. To control the

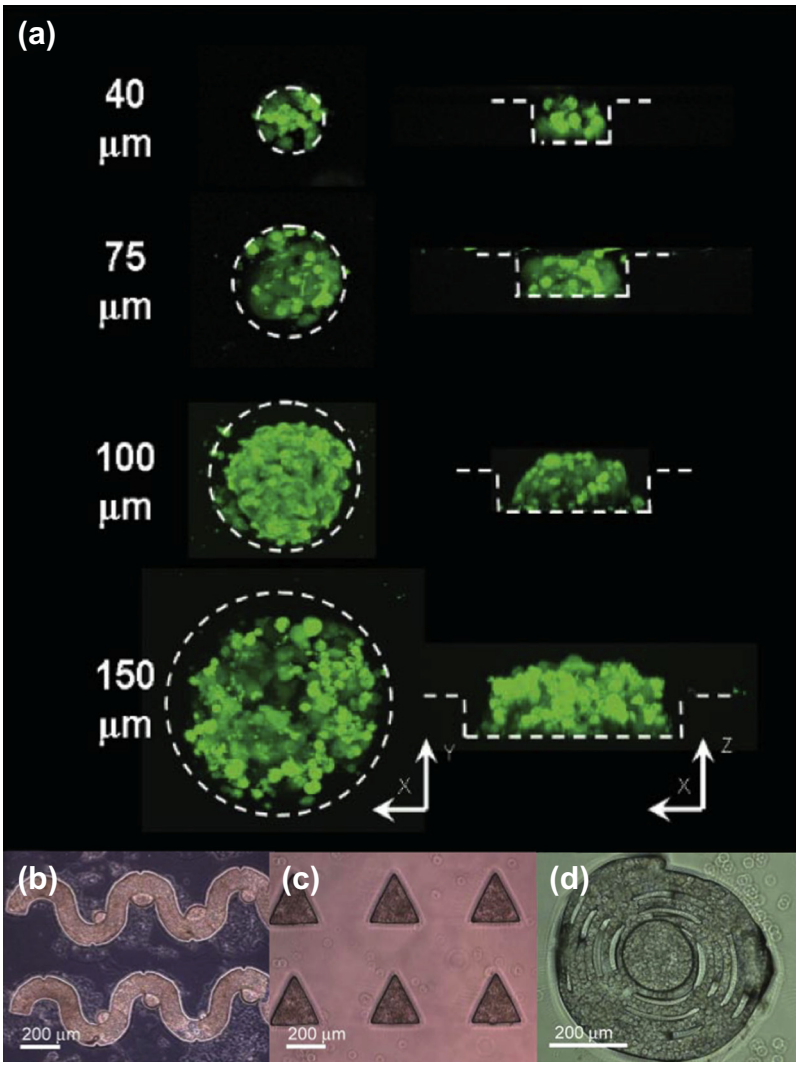

FIGURE 32.6
Microwells for the formation of EBs with controlled size and shape. (a) Confocal images of fluorescently labeled EB cell aggregates within microwells with different diameter raging from 40 μm to 150 μm on day 5 of culture; formation of EBs with different shapes including (b) curves, (c) triangles, and (d) swirls. *(Adapted from reference [148].)*

shape and direct the differentiation of EBs, RA-loaded PLGA microspheres were used to deliver morphogenic factors within EB microenvironments in a spatiotemporally controlled manner [149]. Homogenous differentiation of cystic spheroids with a bi-epithelial morphology was obtained when EBs were cultured on the fabricated microspheres [149].

In another study, the effect of EB aggregate size on its differentiation was investigated by seeding ESC on PEG microwells of various diameters [150]. It was found that larger microwells (450 μm diameter) induced differentiation of ESC to cardiogenesis through the expression of Wnt11. However, EBs formed in small (150 μm) microwells differentiated to endothelial cell by increased expression of Wnt5a [150].

Cell arrays have been also used to pattern stem cells on substrates. Arrays of cells can be used to localize and track individual cells, enabling the clonal analysis of stem cell fates. For example, clonal populations of neural stem cells were immobilized within microfabricated structures and their progeny were tracked using real-time microscopy, yielding information about cellular kinetics and cell fate decisions in a high-throughput manner [151]. Using this approach, it is possible to study the response of individual stem cells to various micro-environmental signals.

Cell patterning on geometrically defined shapes has been used to study the effects of cell shape on cell fate decisions. As cells adhere onto a micropatterned substrate, they align themselves to

the shape of the underlying adhesive region. A change in shape induces changes in the cell cytoskeletal features, which in turn influence cell apoptosis, proliferation [152] and differentiation [153]. Co-culturing ESCs with secondary cells can promote their differentiation into specific cell lines [154]. For this purpose, Fukuda et al. developed a technique to fabricate micropatterned co-cultures of ES with secondary cell lines on surfaces containing three different layers of hyaluronic acid, fibronectin, and collagen [155]. First, the hyaluronic acid was micropatterned on a glass substrate. Then, fibronectin was then deposited on the areas of exposed glass to create cell adhesive regions. After the cell attachment on fibronectin-coated areas, a layer of collagen was added to hyaluronic acid patterns to switch surface properties and facilitate the adhesion of the second cell type. Using this system, the patterned co-cultures of ECs with NIH-3T3 and AML12 cells could be obtained [155]. Further elucidation of the molecular mechanisms indicated that cell shape regulated the activation of the RhoA pathway demonstrating that mechanical stresses can be crucial for directing stem cells differentiation. Therefore, controlling cellular microenvironment using micropatterning may be used for directing cell fate for tissue-engineering applications.

Physical signals

Mechanical forces affect the differentiation and functional properties of many cell types, and are being increasingly used in tissue engineering. For example, functional autologous arteries have been cultured using pulsatile perfusion bioreactors [156]. Although it is known that mechanical stimuli (such as cyclic stretching and fluid shear stress) may be required to direct the differentiation of ESCs, understanding their effects is still in its infancy [157].

In one study, fluid shear stress was applied to induce Flk-1-positive ES differentiation into vascular endothelial cells through the activation of Flk-1. The expression of vascular endothelial cell-specific markers such as Flk-1, Flt-1, VE cadherin, and PECAM-1 enhanced in the presence of shear stress; however, shear stress had no effect on markers of epithelial or smooth muscle (keratin, or α-SMA) [157]. In another study, Shimizu et al. demonstrated that cyclic uniaxial stretching on Flk-1-positive ES cells for 24 hrs significantly increased the expression of VSMC markers α-SMA and smooth muscle-myosin heavy chain (SM-MHC), decreased the expression of EC marker Flk-1, and had no effect on the other EC markers (Flt-1, VE cadherin, and PECAM-1) [158]. Platelet-derived growth factor (PDGF) receptor beta kinase inhibitor blocked cell proliferation and VSMC marker expression that were induced by applying mechanical stimulation [158]. Mechanical stretching and fluid shear stress have been also used to direct ES cell differentiation into cardiovascular lineages [159,160]. Taken together, these studies demonstrate that mechanical stimulation can enhance the ability of ESCs to respond to exogenous signals, and promote their differentiation into a specific lineage. In one study, the hESC differentiation on deformable elastic substrates was inhibited by applying a 10% cyclic stretch [54]. The expression of Oct4 and SSEA-4 was promoted in the presence of mechanical stimulation, demonstrating an increase in hESC self-renewal. It was also found that mechanical stretch inhibited hESC differentiation when the cells were cultured in a mouse MEF-conditioned medium. However, differentiation of hESCs was not affected by mechanical stimulation when an unconditioned medium was used [54].

Other environmental factors that may be required include electrical signals. For example, it was found that electrical field stimulation could affect cardiac differentiation and reactive oxygen species (ROS) generation in hESC-derived EBs [161]. Hopefully, with time, such techniques will allow for the development of ESC-based tissue-engineering applications. The design of bioreactors that control the spatial and temporal signaling that induce ESC differentiation requires further collaborative efforts between engineers and biologists.

Microfluidic systems can be also used to investigate the effect of growth factor and chemical environments on stem cell differentiation in a high-throughput manner. For example, a microfluidic device was developed to generate a concentration gradient of growth factors for

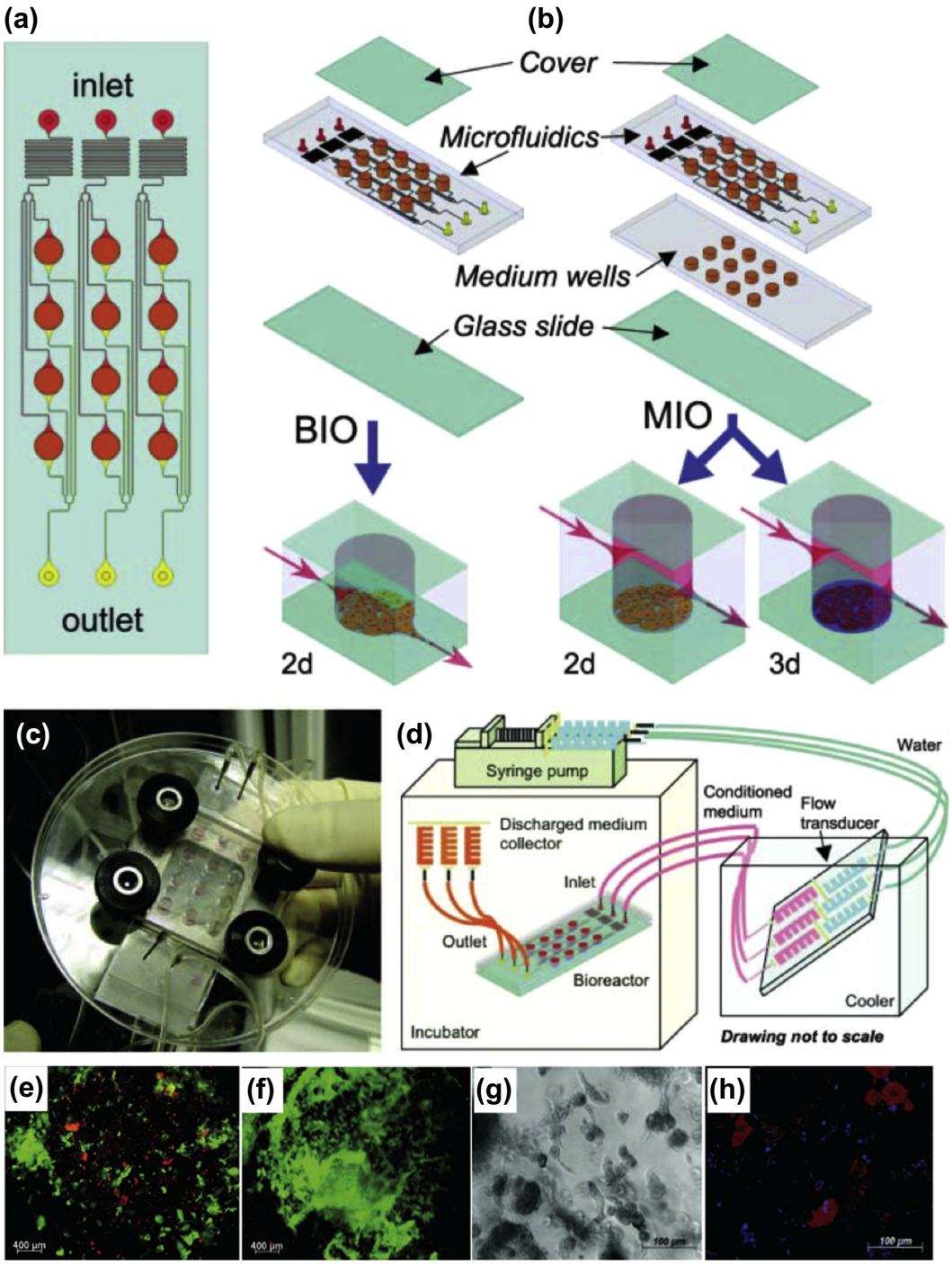

FIGURE 32.7

Microarray bioreactors. (a) The micro-bioreactor wells with 3.5 mm in diameter are arranged in an array. The medium (red) is delivered using three inlets through the flow transducers to four wells (orange) via microfluidic channels (100 mm wide) and waste medium removes from each bioreactor through a separate set of channels (yellow). (b) Two configurations were used: a bottom inlet/outlet (BIO) configuration, and a middle inlet/outlet (MIO) configuration (right) that allows for 3D cultivation. (c) Image of a single MBA with compression frame and fluidic connections. (d) Experimental setup. MBAs and medium collectors are placed in an incubator, and the medium reservoirs are maintained external to the incubator in an ice bath; (e—f) Representative images of hESCs on day 4 of culture (e) without and (f) with perfusion of culture medium (live cells are red and deal cells are green); (g) bright field image of differentiated hESCs, demonstrating that hVEGF addition to culture media resulted in hESC sprouting and elongation outside the colonies. (h) Confocal image of vascular differentiated hESC indicating the expression of a-SMA (shown in red). *(Adapted from reference [55].)*

optimizing the proliferation and differentiation of stem cells. The developed platform enabled rapid optimization of media compositions by exposing the cells to a continuous gradient of various growth factors within the microfluidic environment to induce proliferation and differentiation in a graded and proportional manner, depending on growth factor concentration [162]. In another study, micro-bioreactor arrays (MBAs) system composed of a microfluidic platform and an array of micro-bioreactors was designed to investigate the effect of culture microenvironments on hESCs differentiation both in 2D and 3D culture conditions [55,163] (Fig. 32.7a–d). Medium perfusion promoted the viability of encapsulated hESCs within hydrogels (67% viability in perfused culture compared to 55% in static culture) (Fig. 32.7e,f). In addition, using this system, it was possible to induce the vascular differentiation of hESCs through the addition of vascular growth factor (hVEGF) to the culture media [163] (Fig. 32.7g,h).

ISOLATION OF SPECIFIC PROGENITOR CELLS FROM ESCs

Although hESCs can generate specific functional cell types from all three germ layers, it is typically not possible to directly differentiate the cells in culture and obtain pure cell populations. Isolation of a specific differentiated population of cells for transplantation will eliminate the presence of undifferentiated hESCs which have tumorigenic potential, and allow for efficient use of the various cell populations for therapeutic purposes. With the exception of few cases where the enrichment of cells of interest was almost fully achieved [83,88,90], the protocols adopted for the differentiation of hESCs do not yield pure cell populations. Therefore, there is a need for suitable techniques to isolate desired cells from heterogeneous cell populations (Table 32.1). One approach for achieving this is to isolate specific cells by using cell surface markers and FACS. In this case, the initial population of cells is immunostained by a single or a combination of different markers, and the desired cell type is isolated by FACS. Part of the initial population of cells is also labeled with isotype controls to gate the populations. The use of FACS yields a pure population of cells, and allows one to select cells using different markers [113], but the limitations of this technique may hamper the final cell survival.

Magnetic immunoselection has been used very often to isolate specific differentiated cells [84,114,164]. Initially, the cells are labeled with relevant cell surface antibodies conjugated with magnetic beads. The magnetically labeled cells are then separated from the other ones by a magnetic column, to purities that are generally higher than 80% [164]. Although these purities are slightly lower than the ones obtained by FACS, the magnetic selection is less harmful to the cells than FACS. Recently, different surface markers specific for cardiomyocytes have been identified: Emilin2 [165] and later SIRPA [166,167] and VCAM [167,168]. These findings allow the prospective isolation of live cardiomyocytes, from ESCs or iPSCs-differentiated mixed cardiac population, with purities above 95%.

Another potential method for cell isolation is through reporter gene knock-in modifications [169]. For example, to trace hepatic-like cells during differentiation of hESCs in culture, a reporter gene expressed under the control of a liver-specific promoter was used [169]. For that purpose, hESCs underwent stable transfection with eGFP fused to the albumin minimal promoter sequence. This methodology allowed one to follow the differentiation pattern of hESCs into hepatic-like cells and to isolate those cells by FACS using the fluorescence of eGFP. Similarly, hESCs genetically manipulated to carry the Nkx2.5-eGFP reporter construct allow the isolation of cardiac cells [167]. Since Nkx2.5 is an early cardiac transcription factor, it allows the identification and isolation of early cardiac progenitors.

Isolation of a specific differentiated population of cells may also be accomplished by mechanical/enzymatic separation of cells exhibiting specific morphology, functional activity or adhesion to a substrate. For example, cardiomyocytes have been isolated by dissecting contracting areas in embryoid bodies and dissociating those areas using collagenase [170].

TABLE 32.1 Summary of methodologies to enrich specific lineages from hES cells

Cell type	Methodology followed to enrich specific lineages	Cell lines	Reference
Cardiomyocytes	Flow activated cell sorting	hES2	[165]
Cardiomyocytes	Flow activated cell sorting, Magnetic immunoselection	hES2, 3	[166]
Cardiomyocytes	Introduction of a reporter gene and cell selection by flow activated cell sorting	hES3Nkx2.5^{eGFP}	[167]
Cardiomyocytes	Flow activated cell sorting	KhES1	[168]
Cardiomyocytes	Flow activated cell sorting	cmESC, KhESC1, 2, 3	[171]
Cardiomyocytes	Flow activated cell sorting	hES2	[165]
Cardiomyocytes	Discontinuous percoll gradient	H1,H7,H9	[117]
Cardiomyocytes	Enzymatic and mechanical dissociation	N/A	[170]
Cardiomyocytes	Enzymatic dissociation	HES2	[116]
Hematopoietic progenitor cells	Magnetic immunoselection	H1, H1.1, H9.2	[114]
Hematopoietic progenitor cells	Flow activated cell sorting	H1, H9	[206]
Hematopoietic progenitor cells	Magnetic immunoselection	H1, H9	[164]
Leucocytes	Selective adhesion of cells	H1	[207]
Endothelial cells	Flow activated cell sorting	H9	[111]
Endothelial-like cells	Flow activated cell sorting	H1, H9	[113]
9 Ectoderm			
Neurons and glia	Magnetic immunoselection	H1, H7, H9	[84]
Neurons and glia	Enzymatic dissociation and selective adhesion of cells	H1, H9, H9.2	[85]
Oligodendrocytes	Selective adhesion of cells	H7	[208]
10 Endoderm			
Hepatocyte-like cells	Introduction of a reporter gene and cell selection by flow activated cell sorting	N/A	[169]

Oligodendroglial cells were isolated from stem cell aggregates that adhered to a specific substrate [90]. In addition, neuroepithelial cells were isolated from embryoid bodies attached to a tissue culture-treated flask by using dispase [85], an enzyme that selectively detached neuroepithelial islands from the embryoid bodies, leaving the surrounding cells adhering. It is also possible to take advantage of the cell body content of specific cell types. For example, cardiomyocytes have a higher mitochondrial density than their progenitors and other cardiovascular cells, such as smooth muscle and endothelial cells. It is thus possible to isolate cardiomyocytes by flow cytometric sorting using the mitochondrial dye tetramethylrhodamine methyl ester percholate without genetic modification or surface antigen staining [171].

TRANSPLANTATION

The first application of stem cells as a cellular replacement therapy is associated with bone marrow transplantation and blood transfusion in which donor hematopoietic stem cells repopulate the host's blood cells [172]. Today, modalities are being developed for cell-based therapies of numerous diseases, including diabetes, Parkinson's disease, spinal cord injury, liver failure, muscular dystrophy, bone and cardiovascular disease, among others. Despite the advances in the development of disease models [173], only a few studies have reported the *in vivo* functionality of hESC-derived cells. In most cases, the cells are injected into a disease area and their functionality is evaluated by immunohistochemistry and functional tests. Using such methods, partial functional recovery of a mouse model of Parkinson's disease after hESC-derived neural progenitor cells has been reported [88]. Also, transplantation of hESC-derived oligodendroglial progenitor cells into the *shiverer* model of dysmyelination resulted in myelin formation [174].

Studies of neural regeneration in animal models have given very promising results [175–177]. In particular, hESC-derived oligodendrocytes have been shown to repair injured spinal cord in animal models with rebuilding myelin sheets [178]. Based on this system, in 2010 Geron started the first clinical trial for the treatment of patients with spinal cord injury. In the same year, a clinical trial also started for the treatment of the Stargardt's macular dystrophy [179], a pathology characterized by the death of photoreceptor cells in the central part of the retina (called the macula). This trial was based on the promising observation that hESCs are able to differentiate into RPEs (retinal pigmented cells) [180].

A new important step towards the clinical application of hESCs for infarct therapy is the very recent finding that hESC-derived cardiomyocytes electrically couple and are protective against arrhythmias in the recipient heart when transplanted into guinea-pigs [181], an animal model with a much closer heart physiology to humans than that of rodents.

Despite the ability of stem cells to differentiate into cells with desired phenotypic and morphological properties, there has been very few scaffold-based tissue-engineering studies that use ESCs, by differentiating these cells in culture, selecting desired cell types and seeding these into scaffolds. Ideally, scaffolds provide cells with a suitable growth environment, facilitated transport of oxygen and nutrients, mechanical integrity and suitable degradation. The scaffold brings the cells into close proximity and thereby enhances the formation of tissue structures.

Tissue-engineering scaffolds are comprised of either synthetic or natural materials, or a composite of the two. Scaffolds are commonly made of synthetic materials such as hydroxyapatite, calcium carbonate, PLA, poly(glycolic acid) (PGA), PLGA, poly(propylene fumarate), and natural materials such as collagen, Matrigel or alginate. Natural materials typically have better biocompatibility, while synthetic materials provide better control of various properties such as degradation rate, biomechanics, and structure [7]. hESC-derived endothelial progenitors were seeded onto highly porous PLGA biodegradable polymer scaffolds to form blood vessels that appeared to merge with the host vasculature when implanted into immunodeficient mice. These endothelial progenitor cells were also able to support the formation of vascularized skeletal muscle [182]. Osteoblast-like cells derived from hESCs were also transplanted into an animal model by using a poly(D,L-lactide) scaffold. After 35 days, regions of mineralized tissue could be identified within the scaffold by Von Kossa staining and expression of human osteocalcin [123]. For cardiac tissue engineering, synthetic materials were used in the form of injectable hydrogels and surfaces that can be treated to get detached cardiomyocyte layers [183,184].

Transplantation and immune response

One of the major obstacles for successful transplantation of hESC-derived differentiated cells is their potential immunogeneity. As long-term immunosuppressive therapy would limit clinical applications, the creation of immunologic tolerance would enable stem cell-derived therapy. Methods currently under development include:

1) The establishment of hESC line banks large enough to represent the majority of tissue types;
2) Nuclear reprogramming of the cells to carry patient-specific nuclear genome (therapeutic cloning);
3) Creation of 'universal cells' by manipulating the major histocompatibility complex (MHC) [185];
4) Deletion of genes for immune response proteins using homologous recombination (as mentioned above); and
5) The generation of hematopoietic chimerism, to create the required tolerance for tissues or cells derived from it [186].

The latter method was demonstrated using rat embryonic-like stem cells that permanently engrafted when injected into full MHC mismatched rats [187].

Although the rejection of ESC-derived tissues is triggered by minor histocompatibility antigens, simple host conditioning with monoclonal antibodies against CD4 and CD8 could be sufficient to induce transplantation tolerance of ESC-derived donor tissue, but not of primary animal tissue [188]. It was recently observed that hESCs-derived mesenchymal progenitors have strong immunosuppressive properties resulting, similar to bone marrow mesenchymal stem cells, in inhibiting CD4+ or CD8+ lymphocyte proliferation and being more resistant to natural killer cells [189].

As an alternative to the novel approach of iPSC generation for autologous transplantation or disease-specific drug screening, therapeutic cloning or somatic cell nuclear transfer (SCNT), the process through which Dolly the sheep was cloned in 1997, might be an important tool to create hESCs from patient-specific genome, and thus preventing immunorejection [190] (Fig. 32.8).

This is important for the application of hESCs in tissue engineering, where transplantable populations of cells can be generated with genes that are derived only from the patient. Studies to date have demonstrated that cells derived by SCNT can be expanded in culture and will organize into tissue structures if transplantated with biodegradable scaffolds. However, before SCNT research can be translated into human therapies, the reliability of the overall process needs to be improved, including prevention of the alterations in gene expression.

Immunoisolation systems may help overcome the problems with the immunological incompatibility of the tissue. Thus, immunoisolation of cells may prove to be particularly useful in conjunction with ESCs to overcome the immunological barrier associated with the ESC-based therapies. Cells may be immobilized within semi-permeable polymeric matrices that provide a barrier to the immunological components of the host. Membranes can be designed to be permeable to nutrients and oxygen while providing a barrier to immune cells, antibodies and other components of the immune system, by adjusting the cutoff size of membrane pores [191,192]. Within these systems, the engineered tissues can either be implanted or used as extra-corporeal devices. Such closed tissue-engineering systems have been used for the treatment of diabetes [193–195], liver failure [196–198], and Parkinson's disease [199–202]. For example, ESC-derived β-cells that can respond to insulin or dopamine-producing neurons can be used in clinics without rejection. In addition, closed systems can protect the host against potentially tumorigenic cells.

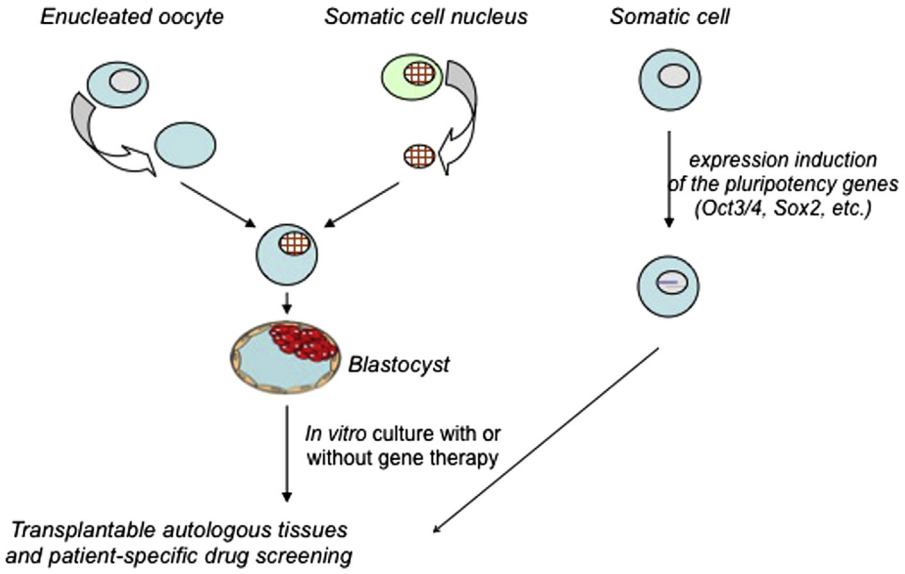

FIGURE 32.8
Schematic diagram of therapeutic cloning.
A somatic cell nucleus is transferred into enucleated oocyte to form a cell capable of giving rise to a blastocyst, which can be used to derive therapeutic cells. Alternatively, somatic cells are reprogrammed into iPS cells that can also be differentiated into therapeutic cells. In both cases, the resulting cells are used to engineer tissues for regenerative medicine and drug screening applications.

Currently, engineering and biological limitations such as material biocompatibility, molecular weight cutoff and immune system reaction to shed antigens by the transplanted cells are some of the challenges that prevent these systems from widespread clinical applications.

FUTURE PROSPECTS

Despite significant progress in the field of tissue engineering and ESC biology, there are a number of challenges that provide a barrier to the use of ESCs for tissue engineering. These challenges range from understanding cues that direct stem cell fate to engineering challenges on scale-up, to business questions of feasibility and pricing.

Although the derivation of hESCs from the ICM of preimplantation blastocysts has become a standard procedure and has been performed in a variety of laboratories, live human embryos must be destroyed in the process, which is ethically unacceptable. However, recent reports show that embryonic stem cells can be isolated without destroying blastocysts [203]. The generation and use of iPSCs require no embryo at all, overcoming the ethical issues associated with ESCs.

Since 2001, federal funding can be used for research using the existing 60 lines of embryonic stem cells, but not for the creation of new cell lines, even from surplus embryos normally discarded in fertility clinics. The existing federally approved lines are not adequate for human therapies, as they have been prepared using mouse cells and thus pose a risk of contamination. Major advancements since 2001 have established methods to culture hESCs without mouse feeder layers. It has recently been proposed that a common ground for pursuing hESC research may exist through assessing the death of a human embryo in the ethical context surrounding organ donation. Specifically, Landry and Zucker argue that a significant fraction of embryos generated for *in vitro* fertilization undergo irreversible arrest of cell division and thus can be considered as organismically dead, yet can still be used to harvest cells [204]. Donation of these embryos could ethically be considered analogous to the donation of essential organs from cadavers. Although criteria for determining the irreversible arrest of cell division have yet to be defined, it will certainly be interesting to see if these theories can be experimentally established and how these arguments will fare with those who currently oppose hESC research. In support of the therapeutic promises held by hESCs, in 2009 the limitations of the use of federal funding for research on hESCs have been reduced, encouraging research in this field.

Stem cells and their progeny reside in a dynamic environment during development, thus a scaffold should be designed to mimic the signaling and structural elements in the developing embryo. The use of 'smart' scaffolds that release particular factors and/or control the temporal expression of various molecules released from the polymer can help induce differentiation of ESC [205]. For example, by dual delivery of vascular endothelial growth factor (VEGF)-165 and PDGF, each with distinct kinetics, and from a single polymer scaffold, resulted in the formation of stable vascular networks [205]. An alternative approach to modifying the surface exposed to the cells is to immobilize desired ligands onto the scaffold. For example, RGD peptides, the adherent domain of fibronectin, can be incorporated into polymers to provide anchorage for adherent cells.

Another difficulty with the currently used materials is limited control over the spatial organization of the scaffold. Spatial patterning is necessary to create tissues that resemble the natural structure of biological tissues. In the direct cell patterning system, cells can be seeded into the scaffold at particular regions within the cells. For example, the direct attachment of two different cell types in different regions of the scaffold has been used to generate cells of the bladder. Cell patterning was critical for the effective co-culture of hepatocytes and fibroblasts.

CONCLUSIONS

A number of challenges are still ahead of us before ESC-based therapy can become clinically viable. These include directing the differentiation of ESCs (i.e., using controlled microenvironments or genetic engineering), ensuring their safety (i.e., by eliminating tumorogenicity), functionally integrating differentiated cells into the body, achieving long-term immune compatibility, and improving the cost and feasibility of cell-based therapies. Each of these challenges is currently being addressed. In particular, since ESCs can give rise to many different cell types, solving these challenges for the various possible tissue types will be a major undertaking. Further research is required to control and direct the differentiation of ESCs, in parallel with developing methods to generate tissues of various organs, to realize the ultimate goals of tissue engineering. We might be getting close to a day when ESCs can be manipulated in culture to produce fully differentiated cells that can be used to repair specific organs. Clearly, our ability to overcome these difficulties is not confined within any single scientific discipline but rather involves an interdisciplinary approach. Solving these challenges could lead improved quality of life for a variety of patients that could benefit from tissue-engineering approaches.

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