

## Engineering tissue from human embryonic stem cells

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### Abstract

Recent advances in human embryonic stem cell (hESC) biology now offer an alternative cell source for tissue engineers, as these cells are capable of proliferating indefinitely and differentiating to many clinically relevant cell types. Novel culture methods capable of exerting spatial and temporal control over the stem cell microenvironment allow for more efficient expansion of hESCs, and significant advances have been made toward improving our understanding of the biophysical and biochemical cues that direct stem cell fate choices. Effective production of lineage specific progenitors or terminally differentiated cells enables researchers to incorporate hESC derivatives into engineered tissue constructs. Here, we describe current efforts using hESCs as a cell source for tissue engineering applications, highlighting potential advantages of hESCs over current practices as well as challenges which must be overcome.

**Keywords:** tissue engineering • cell culture • human embryonic stem cells • differentiation • ectoderm, mesoderm • endoderm

The derivation of human embryonic stem cell (hESC) lines in 1998 has generated great excitement in the fields of regenerative medicine and tissue engineering, for these cells possess the potential to provide a limitless supply of virtually all somatic cell types from a clonal source [1]. Culture practices that expand undifferentiated hESCs and methods to direct differentiation toward specific lineages are improving at a rapid pace, and researchers have begun to incorporate hESC deriva-

tives into tissue engineering strategies. Here, we summarize the use of hESCs in tissue engineering applications, highlighting specific advantages hESC-derived cells provide over current practices that predominantly utilize primary cells. We also discuss potential pitfalls that may arise and must be addressed to successfully use hESCs in tissue engineering. In addition, progress toward the generation of specific tissues from hESCs or human adult progenitors will be described.

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## Stem cell tissue engineering

Tissue engineering can be broadly defined as the generation of biological substitutes that reproduce one or more functions of a particular tissue or organ [2]. Engineered tissue constructs can be used therapeutically or for diagnostic purposes, and examples range from acellular nanofibre peptide scaffolds [3] to fully cellularized skin grafts containing cells expanded from autologous skin [4]. The use of synthetic and biological materials and the complex, multi-step processes required to generate such tissues emphasize the importance of engineering concepts in this field.

Any engineered tissue must incorporate some or all of the following: cells to carry out biological functions, chemical factors to present cues to cells or the surrounding tissue, and a matrix to provide physical support and/or chemical stimulation (Fig. 1). The functional requirements of specific tissue types dictate which components are included; however, the most effective engineered tissues are likely to contain cells that can perform more complex functions than materials alone. Inclusion of stem cells or progenitors may enable the tissue to regenerate and withstand the rapid cell turnover rates observed in many biological systems. Therefore, the production and isolation of tissue-specific progenitors is a key step in the generation of stem cell engineered tissues.

## Potential cell sources

Current, cell-based tissue therapies utilize post-natal cell sources, but most cannot be expanded efficiently and cost-effectively *in vitro*. Many efforts have focused on improving methods of expanding somatic cell populations, including mesenchymal [5] and haematopoietic progenitors [6], though most approaches require costly growth factors and/or undefined components. Autologous cells are often preferential due to the risk of immunological rejection of allogeneic cells, but patients in need often lack sufficient quantities of healthy cells to donate. The finite amount of obtainable adult progenitors makes large-scale implementation of tissue engineering strategies difficult; in fact, the limited commercial and economic success of this industry has been recently attributed to a lack of well-developed processes [7].

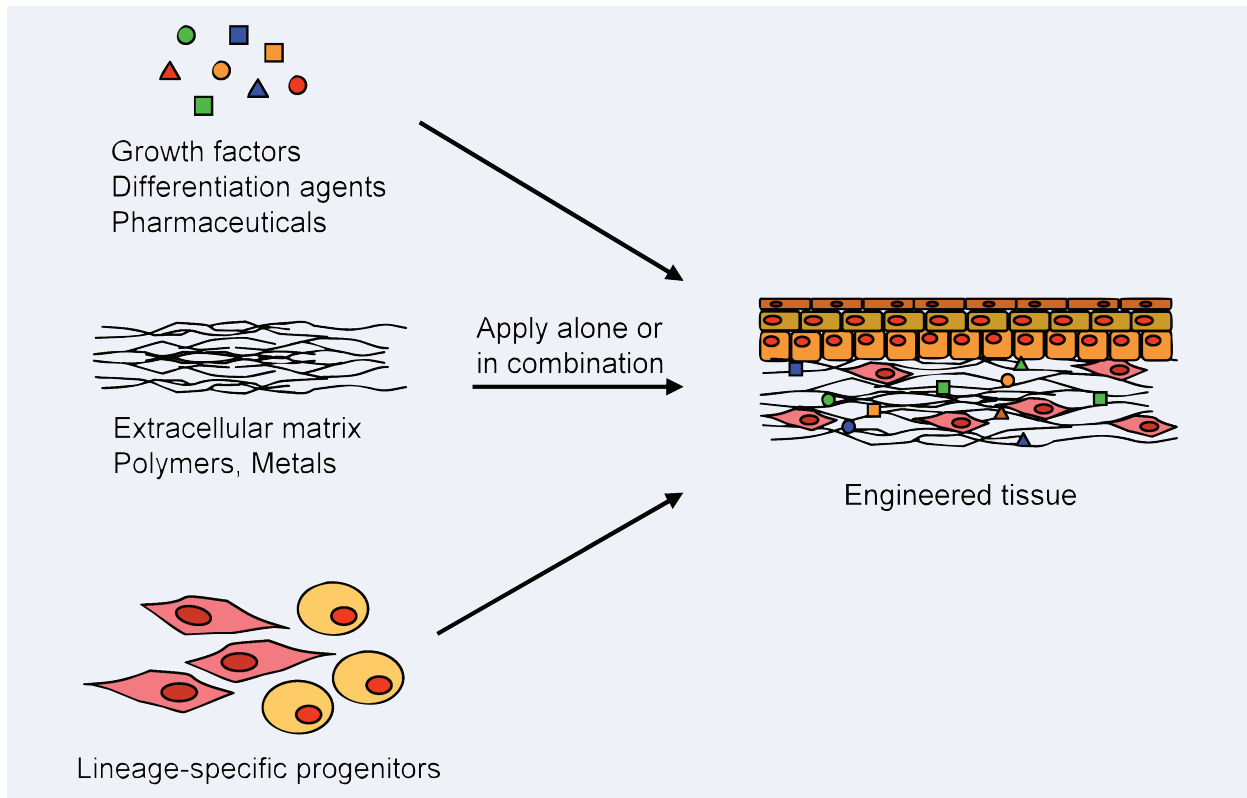
In contrast to adult progenitors, hESCs can be maintained almost indefinitely in the undifferentiated

state [1], which could allow for the scaleable expansion of cells at an early stage of the tissue engineering process. Large quantities of clonally derived undifferentiated hESCs can then be induced to differentiate to generate an adequate supply of functional cells, avoiding the need to harvest and expand lineage restricted cells from donors (Fig. 2). Directed differentiation methods for hESCs are rapidly improving for many therapeutically relevant lineages, whereas other cell types can be efficiently purified from heterogeneous differentiated populations based on surface marker expression. The specific strategy employed can be tailored to particular lineages given the distinct challenges of differentiation and purification of the desired cells.

## Incorporation of hESCs

Substantial advances in hESC culture and differentiation processes must be made before hESC-derived tissues can be successfully implemented in the clinic. The genetic stability of these cell lines during extended culture must be assessed with greater scrutiny, and any undifferentiated cells must be removed to preclude teratoma formation. Additionally, the functionality of any somatic cell type derived from hESCs must be established *in vitro* or in animal studies prior to use in the clinic. Other tissue engineering applications, however, are likely to benefit greatly through the inclusion of hESC derivatives. Engineered human tissues can serve as diagnostic tools in pharmaceutical research as well as model systems for developmental and cell biology. Advanced techniques in the genetic manipulation and clonal isolation of undifferentiated hESCs may soon enable researchers to engineer and study human tissues containing specified genetic changes (knockout or knock-in systems) [8].

Incorporation of hESCs into engineered tissues will require a detailed understanding of how the cells interact with the various components that comprise engineered tissues. The complex interactions between hESCs and their microenvironment must be characterized to better control stem cell growth and differentiation [9]. While previous studies using adult progenitors and murine embryonic stem cells (mESCs) can serve as an initial template for knowledge-based approaches, random methods such as directed evolution and combinatorial screening will



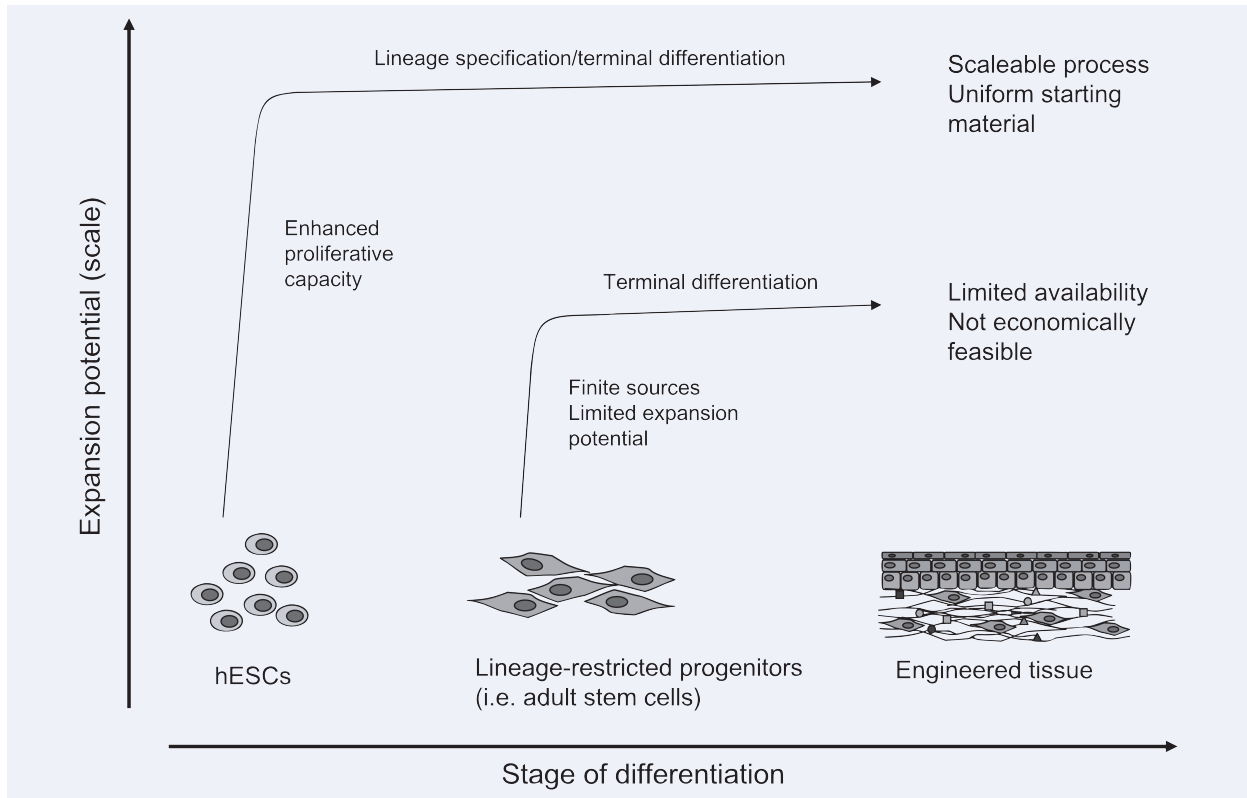
**Fig. 1** Components included in engineered tissues. Any combination (one or more) of the following components may be included in engineered tissue constructs. Tissue-inducing factors (*i.e.* growth factors) mediate endogenous or exogenous cell growth and differentiation, often aiding in vascularization of complex tissues. Physical supports or scaffolds are often present to provide a 3D microenvironment that allows for cell integration or differentiation. Synthetic materials (*i.e.* polymeric hydrogels) or biologically derived substances (*i.e.* extracellular matrix [ECM] proteins) are also effective. Incorporation of exogenous progenitors endows engineered tissue with a regenerative capacity, improving the *in vivo* lifespan of the construct.

provide additional insight into the mechanisms of hESC growth and differentiation. Early advances in embryonic stem cell tissue engineering are described below; these include general hESC culture process improvements, methods of efficiently obtaining pure populations of lineage-restricted cells, and applications of traditional tissue engineering techniques to induce terminal differentiation. Table 1 provides a summary of defining markers and differentiation factors used to derive and characterize the hESC-derived tissues included in this review. Biomaterials used as scaffolding in the engineering of such tissues are also included. Since the results and progress vary significantly with the stage of differentiation and specific lineage, engineering tech-

niques to improve hESC culture and tissue generation have been grouped accordingly.

### Undifferentiated hESC culture engineering

Incorporation of hESCs in tissue engineering technologies will require marked advances in undifferentiated hESC culture practices, including cell expansion, genetic manipulation and process robustness. The initial derivation and characterization of hESCs was performed using irradiated murine embryonic fibroblast (MEF) feeder layers and culture medium containing undefined components such as Knockout



**Fig. 2** Comparing the scalability of human embryonic stem cell (hESCs) and adult progenitors in tissue engineering processes. Adult progenitors (autologous and allogeneic) are most often used for production of cellular-engineered tissues. Donor sources are finite and must be screened extensively for biological contaminants to address potential safety risks. Most somatic cells cannot be efficiently expanded and require high concentrations of growth factors or undefined medium components (*i.e.* serum). These factors have contributed to limiting the economic viability of tissue engineering companies. hESCs exhibit an enhanced proliferative capacity and greater pluripotency than lineage-restricted adult cells. Large-scale culture conditions, differentiation protocols, and purification techniques are improving rapidly as hESC research progresses. These properties may be exploited to more efficiently generate cells for tissue engineering processes.

Serum Replacer (KSR) [1]. Since that time researchers have identified more defined, robust culture conditions capable of supporting undifferentiated self-renewal. Feeder-free culture has been facilitated by the use of Matrigel [10], a complex matrix secreted by mouse sarcoma cells, as an extracellular matrix (ECM). Basic fibroblast growth factor (bFGF or FGF2) has been identified as a key factor in hESC self-renewal [11], and various other pathways have been shown to regulate hESC growth and differentiation, including the Activin/Nodal/transforming growth factor- $\beta$  (TGF- $\beta$ ) pathway, which promotes undifferentiated growth [12], and bone morphogenet-

ic protein (BMP) signalling, which induces differentiation [13]. Wnt/ $\beta$ -catenin signalling, a pathway often activated in cancer, has also recently been implicated in hESC proliferation and self-renewal [14]. Researchers have derived hESC lines using completely defined conditions, though these initial lines acquired karyotype abnormalities [15]. More recently Crook *et al.* have generated six hESC lines using current good manufacturing practices (cGMPs) which are suitable for clinical use [16].

Advancements have also been made in the genetic engineering of hESCs. Although efficiencies are quite low, researchers have successfully obtained

**Table 1** Tissue engineering strategies for specialized cell types from hESCs

Tissue	Key functional cells included	Defining markers used	Key regulators of differentiation	Biomaterials/scaffolds used
	hESCs	Oct4, Nanog, SSEA-4, Tra1-60 [1,13]	Activin A [12], bFGF [11,13], Wnt [14]	Agarose gels [21], polymeric hydrogels [26], microwells [27, 28]
Skin	Keratinocytes	p63, K5/K14 [42]	RA, BMP [46]	GAG [35], collagen gel, organotypic culture [36, 41, 44]
Cornea	Corneal epithelia	p63, K3/K12 [51]	Not defined	Nanotopography [48], organotypic culture [50]
Neural	Neuroepithelia	Pax6, Sox1 [58]	Noggin [53]	PLGA [59, 99]
	Dopaminergic neurons	TH, AADC [53, 54]	FGF8, SHH [54]	
	Motor neurons	HB9, ChAT [58]	RA, SHH [57, 58]	
	Oligodendrocytes	Sox10, O4, MBP [60, 61]	RA, EGF, bFGF [60, 61]	Peptide nanofibres [3]
Bone marrow	Mesenchymal progenitors	CD73 [80]	Not defined	
	Haematopoietic progenitors	CD43 [92], CD34 [91–93]		
Heart	Cardiomyocytes	GATA-4, Nkx2.5 [72, 73]	Ascorbic Acid [69], 5-aza-2'-deoxycytidine [73]	PLLA /PLGA [71], Fibrin-coated dishes [66]
Bone	Osteoblasts	Cbfa1/Runx2, osteocalcin, bone sialoprotein [81–83]	$\beta$ -glycerophosphate, ascorbic acid, dexamethasone [82]	PDLLA [82]
Cartilage	Chondrocytes	Sox9, GAG [84]	BMP-2 [85], TGF- $\beta$ 1 (mESCs) [86]	PEG hydrogel [86]
Blood vessels	Endothelial cells	VE-cadherin, CD31, vWF [91, 92, 96, 97]	VEGF [92]	Alginate [98], PLGA [99]
Pancreatic	$\beta$ -islets	Pdx1 [111], Ngn3 [112], Nkx6.1 [112]	Activin A, RA [110]	
Liver	Hepatocytes	HNF-3 $\beta$ , indocyanine green uptake [117]	FGF-4, hepatocyte growth factor [117]	2D and 3D collagen scaffolds [118]

Abbreviations: Stage-specific embryonic antigen-4, SSEA-4; basic fibroblast growth factor bFGF; glycosaminoglycan, GAG; L-amino acid decarboxylase, AADC; tyrosine hydroxylase, TH; sonic hedgehog, SHH; poly(lactic-co-glycolic acid), PLGA; choline acetyltransferase, ChAT; myelin basic protein, MBP; retinoic acid, RA; epidermal growth factor, EGF; bone morphogenetic protein, BMP; stem cell factor, SCF; interleukin, IL; granulocyte colony stimulating factor, G-CSF; poly(L-lactic acid), PLLA; poly(DL-lactic acid), PDLLA; transforming growth factor, TGF; polyethylene glycol, PEG; von Willebrand factor, vWF; vascular endothelial growth factor, VEGF.

genetically modified hESC clones *via* homologous recombination [17]. Chemical and viral methods have been used to introduce signalling molecules and short interfering RNA (siRNA) gene knockdown sequences into hESCs [18, 19]. Recently a Rho-associated kinase (ROCK) inhibitor has been shown to increase the survival of hESCs grown at low density, improving hESC cloning efficiency to as high as 27% (compared to 1% for controls) [8]. These meth-

ods may enable researchers to identify the specific roles of genes in hESC self-renewal and differentiation, and to incorporate gene reporters driven by lineage-specific promoter sequences as a means of purifying differentiated populations that lack unique surface markers. In the future, genetic manipulation of hESCs may also be used to mitigate clinical problems that arise, such as immunological rejection of hESC-derived engineered tissues [20].

The large quantity of cells required for tissue engineering applications underscores the importance of improving hESC culture robustness and scale. Initial attempts to culture hESCs in stirred suspensions have employed encapsulation methods or growth as embryoid bodies (EBs, also a common differentiation technique). Dang *et al.* have demonstrated that E-cadherin mediates agglomeration of murine embryonic stem cell (mESC) and hESC EBs, which can reduce yields of suspension cultures; encapsulation of ESC aggregates in agarose gels mitigates aggregation while allowing cells to proliferate and emerge from capsules [21]. Uncoated EBs have also been cultured in traditional stirred vessels [22] as well as rotating wall bioreactors [23]. Perfusion has been shown to enhance cell yield in adherent hESC cultures [24], though much work must be done to implement these processes at the larger scales required for manufacturing processes.

Novel culture methods capable of more precisely presenting physical and chemical stimuli in the stem cell microenvironment are also emerging. Studies in our laboratory have demonstrated that hESCs are responsive to mechanical signals, as application of cyclic strain prevents spontaneous differentiation of hESCs [25]. Synthetic polymer hydrogels have been successfully used to cultivate undifferentiated hESCs over short periods of time [26]. Conjugation of functional arginine-glycine-aspartic acid (RGD)-containing peptides promotes cell adhesion, and adjustment of the hydrogel cross-linking density allows modulation of matrix porosity and stiffness. Furthermore, the chemical structure of these hydrogels permits cellular reorganization of the matrix *via* matrix metalloproteinases (MMPs). Three-dimensional (3D) scaffolds offer a better representation of the *in vivo* microenvironment and have also been employed in the culture of various stem cell derivatives.

Our group and others have generated systems that can exert spatial control over undifferentiated hESC growth [27, 28]. hESCs can be cultured for extended periods of time ( $\geq 21$  days) in microwells patterned with protein-resistant self-assembled monolayers (SAMs) without appreciable differentiation, in contrast to the weekly passaging required when using traditional unconstrained adherent culture methods. Furthermore, these microwell cultures can be used to produce hESC aggregates of near uniform size and morphology, which may subsequently prove to facilitate differentiation toward par-

ticular lineages. Microwell cultures may also be used to generate uniform arrays of undifferentiated cells for quantitatively screening the effect of factors and/or compounds. Two-dimensional (2D) SAM-based arrays have been used to screen hESC-substrate interactions. In one embodiment of this method, peptides conjugated to SAMs were spotted onto an array containing a hydrophobic background. hESCs were cultured on the patterned surface for 2 days and stained for alkaline phosphatase activity to identify peptides that promote adhesion without inducing differentiation [29]. An array of different cell adhesion peptides or cell signalling molecules could easily be screened for functionality with respect to hESC growth and differentiation. These technologies should enable design of more effective strategies that achieve the desired stem cell phenotype *via* precise regulation of the stem cell microenvironment; however, the duration required for hESC expansion and differentiation will likely require a dynamic microenvironment. Further characterization of material interactions with hESCs and their progeny is necessary before these practices can be implemented in tissue engineering applications.

Future incorporation of hESCs in clinical and diagnostic tissue engineering processes will certainly exploit their vast potential for expansion; however, efficient differentiation and purification processes must be developed for all lineages of interest. Differentiation protocols established for mESCs may be particularly informative, though recent studies have identified key differences in the effects of the BMP signalling pathway on undifferentiated mESCs and hESCs [13, 30]. An emphasis should be placed on obtaining high purities of lineage-specific cells given the cost and difficulties associated with live cell purification. Advances in the generation and isolation of particular cell types and tissues from hESCs are described below; the emergent nature of this field requires, in some cases, discussion of applications which utilize adult progenitors or ESCs from other species, though an emphasis is placed on hESC technologies.

## Ectodermal tissues

Ectodermal tissues comprise the outer epithelial structures as well as cells of the nervous system; the most therapeutically relevant tissues include the



skin, cornea, and various neural lineages. Studies of embryo development in a variety of species and ESC lines suggest that ectodermal specification occurs early during development [31], and the epithelial phenotype of ESCs (*i.e.* E-cadherin expression) provides evidence to support this concept. Culture of mESCs or hESCs in serum-free medium induces expression of neuroectoderm markers, and these precursors can later be specified to various cell types of the nervous system. Activation of phosphatidylinositol 3-kinase (PI3K) signalling *via* high insulin concentrations (often present in chemically defined media) has been shown to block mesendoderm differentiation, implying a role for this pathway in ectodermal specification [32], and TGF- $\beta$  superfamily members such as Activin and Nodal seem to inhibit neuroectodermal specification [33]. However, the specific factors required for ectodermal differentiation are unclear given that both PI3K and TGF- $\beta$  signalling promote maintenance of hESCs in the undifferentiated state [12, 34]. While the exact mechanisms of differentiation are yet to be identified, researchers have had success obtaining epithelial and neural precursors from hESCs and have begun to gauge their ability to form ectodermal cell types and tissues.

## Skin

Skin is the largest organ of the body and acts as the primary barrier between bodily tissues and the *ex vivo* environment; the main components include the dermis and epidermis. The dermis contains hair follicles, blood vessels, and a variety of glands as well as fibroblasts and ECM. Keratinocytes are the most abundant cell type in the epidermis, providing barrier function by stratifying to form a keratinized surface layer. As terminally differentiated cells slough off the skin, tissue homeostasis is maintained by a population of epidermal progenitors present in the basal layer (in contact with the dermis). The well-characterized structure of skin makes it a model system for tissue engineering applications, and the relative ease of propagation and accessibility of adult epidermal progenitors has enabled many advances in skin tissue engineering.

Over the past 30 years a wide variety of cellularized and acellular skin replacement materials have been generated using tissue engineering techniques,

and some have been successfully used for clinical treatment of burns and chronic wounds. Early development of acellular engineered skin focused on the fabrication of dermal substitutes capable of promoting healing *via* endogenous cells; the most widely used materials were cross-linked collagen precipitates containing glycosaminoglycans (GAG) [35]. Optimization of component concentrations and production methods improved mechanical strength and porosity, promoting fibroblast integration and vascularization while providing some form of barrier function [36]. These constructs continue to act as a basal substrate for cellularized full-thickness skin equivalents.

Inclusion of keratinocytes provides skin grafts with a regenerative capacity while establishing an epidermal barrier. Advances in keratinocyte culture methods in the 1970s enabled researchers to expand these cells *in vitro* and generate epithelial sheets for coverage of large wounds [37]. More recently full thickness tissue engineered skin has been reconstructed using autologous keratinocytes and fibroblasts with collagen-GAG substrates [4]. Further improvements have been made to these systems *via* incorporation of melanocytes to provide pigmentation [38], addition of endothelial cells to promote vascularization [39], and genetic modification of keratinocytes to induce expression of vascular growth factors [40]. There are now several tissue engineered skin products available for treatment of burns and/or chronic wounds (reviewed in [41]).

While fabrication of cultured skin grafts has proven to be technically feasible, the industry faces several challenges. Safety concerns exist regarding the use of donor cells, murine fibroblast feeder layers and animal-derived components, which may contain biological contaminants, such as viruses or prions. Allogeneic keratinocytes are also susceptible to immunological rejection, and costly procedures (especially in the case of autologous grafts) have hindered economic success in this area. hESC-derived keratinocytes are a potential alternative source of cells, as their enhanced proliferative capacity could provide a safer, more consistent and cost-effective supply of cells for tissue engineered skin.

Specification of the keratinocyte lineage from hESCs was first demonstrated by Green *et al.* *via* EB differentiation, producing cells positive for the basal epithelial markers p63 and Keratin 14 (K14) [42]. While these differentiation schemes have been reproduced, quantitative studies of this system have to date

demonstrated only moderate efficiencies, and the presence of contaminating cell types makes expansion difficult [43]. Experiments using mESCs have identified factors such as BMP-4 that may improve differentiation efficiency [44], though this method may not be applicable to human lines given their propensity to generate trophoblasts in response to BMP-4 [30]. The expansion potential of hESC-derived keratinocytes has also been questioned [45], but these studies have relied upon adult keratinocyte culture protocols, and it is unclear whether those methods are optimal for hESC derivatives. Recently, we have been able to generate relatively pure populations of keratinocytes from hESCs under defined conditions using retinoic acid (RA) and BMP signalling [46]. These cells can express terminal differentiation markers (Fig. 3B and D) and form epithelial sheets upon enzymatic detachment from the substrate (Fig. 3E). While further characterizations of this system are required, these results demonstrate the potential use of hESCs in skin tissue engineering. In the future, the use of clonally derived genetically modified hESCs may enable researchers to generate human knockout (or knock-in) skin equivalents to study the basic biology of these tissues. Additionally, the 'embryonic' origin may grant these hESC-derived keratinocytes plasticity with respect to their ability to generate various epithelia (*i.e.* cornea or hair follicles) when presented with different mesenchymal substrates.

## Cornea

Self-renewal of the corneal epithelium depends upon stem cells located in the limbal region of the cornea. When trauma or disease depletes this cell population, transplantation of corneas or tissue-engineered grafts can prevent visual impairment caused by vascularized scar tissue [47]. As a result, many efforts have focused on identifying culture conditions to enable engineering of functional corneal tissues. When cultured on synthetic nanotopographic substrates that mimic basement membrane structure, human corneal epithelial cells exhibited specific cellular responses (*i.e.* alignment) depending on the topographical pitch and culture media [48]. The ability to manipulate cellular functions and tissue development through surface patterning is an emerging tool that may be of value for tissue engineers.

Novel materials have also been employed in the clinical application of engineered corneal tissues.

Primary human corneal epithelial cells were expanded to form multi-layered sheets on a temperature-responsive polymer substrate (at 37°C); reducing the temperature of this material (below 32°C) resulted in hydration and swelling, causing detachment of the cell sheets [49]. The absence of proteolytic enzymes leaves cell-cell junctions and deposited ECM intact, promoting rapid adhesion of the epithelium during transplantation. More recently this process has been performed using autologous oral mucosa epithelial cells, which also express the corneal epithelial marker K3; tissue-engineered epithelial sheets were transplanted to patients and remained transparent at least 14 months after treatment [50].

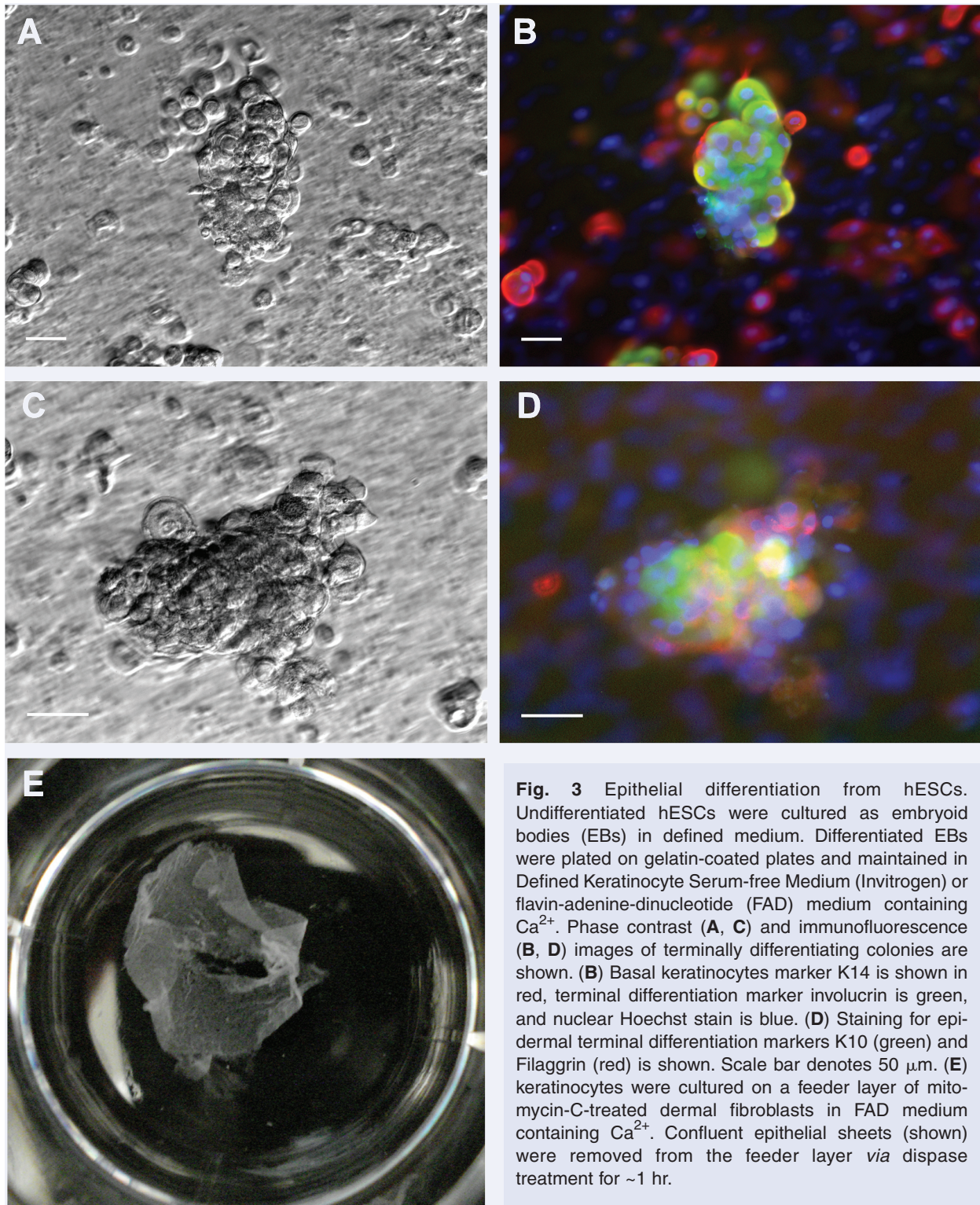
hESCs may also be a potential source of corneal cells used to generate tissues for transplantation or diagnostic studies. Corneal epithelial cells have recently been produced from hESCs, although serum-containing conditioned medium was used and the differentiated cultures contained K3/K12 expressing corneal cells as well as putative skin cells (K10 expressing) [51]. Nevertheless, as hESC culture and differentiation technologies improve, these systems may provide a more efficient means of obtaining epithelial progenitors for corneal tissue engineering.

## Neural lineages

Many debilitating diseases are associated with the nervous system, and given the limited accessibility of adult neural progenitors, substantial effort has been devoted to the generation of these lineages from hESCs. The neuroectoderm gives rise to several cell types, including neurons, astrocytes and oligodendrocytes. While *in vitro* synthesis of complex, functional, neural tissues is unlikely, isolated populations of these cells or their progenitors may be used for clinical treatment of degenerative disorders, spinal cord injury or as diagnostic tools for specific disease models. Some investigations have employed foetal-derived neural progenitors [52], though hESCs hold the greatest potential as a scaleable source of neural cells. Current progress on the derivation of clinically valuable neural cell types is described below.

In the absence of BMP signalling, hESCs readily differentiate into neural precursors, and continued culture of these cells on a neural substrate such as laminin stimulates generation of neuronal cells [53]. Of particular interest are tyrosine hydroxylase





**Fig. 3** Epithelial differentiation from hESCs. Undifferentiated hESCs were cultured as embryoid bodies (EBs) in defined medium. Differentiated EBs were plated on gelatin-coated plates and maintained in Defined Keratinocyte Serum-free Medium (Invitrogen) or flavin-adenine-dinucleotide (FAD) medium containing  $\text{Ca}^{2+}$ . Phase contrast (**A**, **C**) and immunofluorescence (**B**, **D**) images of terminally differentiating colonies are shown. (**B**) Basal keratinocytes marker K14 is shown in red, terminal differentiation marker involucrin is green, and nuclear Hoechst stain is blue. (**D**) Staining for epidermal terminal differentiation markers K10 (green) and Filaggrin (red) is shown. Scale bar denotes 50  $\mu\text{m}$ . (**E**) keratinocytes were cultured on a feeder layer of mitomycin-C-treated dermal fibroblasts in FAD medium containing  $\text{Ca}^{2+}$ . Confluent epithelial sheets (shown) were removed from the feeder layer via dispase treatment for ~1 hr.

(TH)-expressing dopaminergic neurons, which become depleted in the degenerative disorder Parkinson's disease [54]. Many studies have successfully used stromal co-cultures to generate TH<sup>+</sup> neurons, reflecting the localized nature of these neurons, though stromal cell contamination complicates clinical applications [55]. More recently, researchers have identified FGF8 and sonic hedgehog (SHH) as inducers of dopaminergic differentiation in hESC-derived neuroepithelia [56]. Interestingly, appropriate temporal application is required to direct cells to specific phenotypes (early induces midbrain, late induces forebrain), highlighting the similarities between *in vitro* differentiation and *in vivo* development. These findings demonstrate the benefits of using chemically defined, specific differentiation methods over less defined, 'black-box' approaches to investigate lineage specification.

Severe trauma to the spinal cord often results in permanent paralysis due to the inability of motor neurons to regenerate after injury. Mechanistic studies using mESCs initially identified several necessary components for spinal progenitor specification, including the caudalizing factor RA and SHH [57]. Similar methods have proven successful in hESC differentiation, though the necessity of applying factors (RA) to early neuroepithelia in this process was again emphasized [58]. hESC-derived motor neurons expressed HB9 and choline acetyltransferase, formed synapses with neighbouring neurons, and responded to electrophysiological signals. However, quantitative comparisons must be made with primary neurons to ensure proper functionality of these hESC-derived cells. Recently researchers have used self-assembling nanofibre scaffolds to promote reconnection of axons in a severed optic tract hamster model; injection of the peptide matrix alone enhanced the endogenous healing process [3]. Similar tissue engineering approaches could potentially be combined with hESC-derived neurons to improve recovery after spinal cord injuries [59].

Another problem associated with spinal cord injury is axon demyelination resulting from oligodendrocyte losses, which causes further degeneration of the nervous system. Using a 6-week differentiation protocol, oligodendrocyte progenitors have been generated at high efficiencies from hESCs. Upon transplantation into the *shiverer* mouse model of dysmyelination, these cells proliferated and expressed myelin basic protein (MBP) [60]. This method has been employed

more recently in pre-clinical strategies to re-myelinate damaged axons in rat spinal cord injuries [61]. Rats receiving hESC-derived oligodendrocyte progenitors 7 days after injury showed substantial improvements in motor function compared to those receiving undifferentiated hESCs or oligodendrocytes 10 months after injury. These results highlight the exciting potential of hESC derivatives to improve long-term function in damaged tissues.

hESC-derived progenitors are the most feasible means of generating scaleable cellular therapies for neural tissues; however, potential hurdles must still be overcome. As is the case for most other cell lineages, transplanted cells must be immunocompatible with the host to avoid rejection. In addition, differentiation to particular neuronal phenotypes has only been demonstrated at moderate efficiencies, and most neural lineages lack known specific markers that would facilitate purification. Combinatorial array-based methods have been employed to screen for phenotype inducing factors in human foetal neural precursors [52], and incorporating hESCs into these systems is a promising approach for the development of specific, well-defined differentiation protocols. Regardless, hESCs remain a promising source of neural progenitors, and their future utility in clinical neural tissue therapies is likely.

## Mesodermal tissues

Mesodermal cells comprise skeletal, dermal, muscle and connective tissue, as well as components of the circulatory and excretory systems. During gastrulation, as cells migrate through the primitive streak, mesendoderm cells diverge to the endoderm and mesoderm germ layers. Mesoderm derivatives of particular therapeutic interest include cardiac muscle, bone, cartilage, blood, and vascular endothelial cells. Few early human mesodermal markers or induction factors have been identified to date. However, studies of mouse embryogenesis have shown that both Wnt and nodal signalling are required for formation of the primitive streak [62], and BMP-4 signalling is necessary for gastrulation and mesoderm formation [63]. Finally, the transcription factor brachyury (T) is specific to early mesoderm cells and is commonly used to identify and characterize these cells as they undergo an epithelial-to-mesenchymal transition [64].



## Heart

Heart disease and myocardial infarction can cause an irreversible loss of cardiomyocytes, resulting in heart failure, a leading cause of death. The current standard for treatment of damaged or diseased cardiac tissue is organ transplantation, but insufficient availability of donor organs and immunological rejection limit the availability of this option. Implantation of cardiomyocytes into damaged regions of the heart is one potential approach to restoring cardiac function. Transplanted cardiomyocytes have been shown to form cardiac-like tissue in rats [65], and fibrin-coated dishes can be used to culture cardiomyocyte sheets that can be transplanted with minimal cell loss [66].

Various progenitor cell types, including bone marrow cells [67] and adult cardiac progenitors [68] have been shown to differentiate into cardiomyocytes. However, hESC culture could potentially be scaled up to yield significantly greater quantities of cardiomyocytes. Development of serum-free cultures supplemented with ascorbic acid has increased the efficiency of cardiomyocyte differentiation [69]. Cardiomyocytes derived from hESCs and implanted into swine with complete atrioventricular block, which prevents conduction between the atria and ventricles, were able to integrate with native cardiac tissue and pace the heart [70]. One obstacle impeding development of tissues for transplantation is the need for vascular networks to transport oxygen and nutrients to the implanted cells, an especially important consideration for cells with high metabolic rates, including cardiomyocytes. Caspi *et al.* recently developed a vascularized 3D cardiac tissue containing endothelial cells, embryonic fibroblasts and hESC-derived cardiomyocytes [71], which expressed both late and early markers for cardiomyocyte differentiation. These findings illustrate the potential hESCs possess for creation of functional tissue-engineered cardiac constructs.

Following plating and culture, hESC-derived EBs often develop rhythmically contracting regions [72, 73]. These spontaneously beating cells express certain cardiac-specific genes, including the cardiac transcription factors GATA-4 and Nkx2.5 (Nkx2 transcription factor related locus 5), as well as contractile and structural proteins (reviewed in [74]). Functional analysis of beating cell action potentials revealed the presence of three cardiomyocytes types in the EBs: nodal-like,

embryonic atrial-like and embryonic ventricular-like [75]; however, cells with adult-like action potentials were not found, suggesting further cell maturation must occur prior to use in cellular therapeutics.

One major challenge facing implementation of hESC-derived cardiomyocytes in tissue engineering is the need for increased differentiation efficiency and purification methods to improve functional cardiomyocyte yields. Cardiogenesis requires precisely timed and oriented inductive signals *in vivo* and *in vitro* [76–78]. Thus, culture methods to spatially and temporally regulate presentation of soluble factors and cell position will likely improve cardiomyocyte generation. Recently Percoll-based separation strategies have been employed to purify cardiomyocytes from EB outgrowths, though it is unclear if the achieved enrichment is adequate for clinical usage [79]. Regardless of the induction method used, the resulting culture will likely contain a significant population of contaminant cells, so development of robust purification strategies will be crucial to the generation of a homogenous population of cardiomyocytes.

## Bone and cartilage

Bone contains two main cell types: osteoclasts, which are responsible for bone resorption, and osteoblasts, which promote bone growth. Both cell types are embedded in a matrix consisting primarily of hydroxyapatite and type I collagen. Cartilage cells, or chondrocytes, secrete ECM proteins and maintain ECM structure. Cartilage is not a vascularized tissue, and therefore it has poor healing properties; however, this lack of vascularization simplifies efforts to construct implantable, engineered cartilage.

Both bone and cartilage are derived from the mesenchymal lineage of the mesoderm germ layer. hESCs can be induced to form mesenchymal precursors expressing the surface marker CD73; these cells have been shown to differentiate into osteoblasts and chondrocytes [80]. hESCs cultured on Matrigel can differentiate down the osteogenic lineage, as characterized by expression of osteogenic markers and deposition of a hydroxyapatite-like mineral, upon induction with ascorbic acid,  $\beta$ -glycerophosphate and dexamethosone [81]. When implanted into immunocompromised mice using poly(DL-lactic acid) (PDLLA) scaffolds, hESC-derived osteoblasts were

capable of producing mineralized bone [82]. A recent study demonstrated that monolayer differentiation of hESCs enhances the generation of cells exhibiting osteoblastic characteristics, including production of mineralized matrix, compared to standard EB methods [83].

Vats *et al.* utilized co-culture with mature chondrocytes to differentiate hESCs to chondrocytes that express the transcription factor Sox 9 and secrete GAG. The mature chondrocytes were placed in porous inserts such that the only interactions between hESCs and primary cells were mediated through diffusible signals [84]. More recently BMP-2 has been shown to induce chondrogenic differentiation of hESCs, though KSR was present in the differentiation medium [85]. Matrix composition and architecture also affect chondrocyte differentiation. 3D poly(ethylene glycol)-based hydrogel culture resulted in improved chondrocyte generation from mESC-derived chondrocytes upon induction with TGF- $\beta$ 1 [86]. Presumably, 3D tissue engineering scaffolds presenting appropriate matrix cues will also enhance chondrocyte formation from hESCs. Additionally, substrate stiffness and application of uniaxial strain have both been shown to affect differentiation of mesenchymal stem cell down osteogenic and chondrogenic lineages [87, 88]. The biophysical environment will therefore play a critical role in the production of osteocytes and chondrocytes from hESCs.

The current standard for regenerating both bone and cartilage tissue involves autologous cell transplantation. However, the procedural manipulations frequently render the cells unstable, and primary cells may disperse from the implantation site. One alternative to cell transplantation uses bone marrow-derived mesenchymal stem cells (MSCs) to generate an implantable population of osteoblasts or chondrocytes through *in vitro* culture. A major drawback to this approach is that MSCs need to be expanded significantly given that only small populations can be obtained from the donor, and MSCs exhibit a limited self-renewal potential *in vitro*. One of the more promising areas of bone and cartilage tissue engineering is the design of constructs that use functionalized biomaterials to mimic the *in vivo* microenvironment (reviewed in [89, 90]). As advances continue to be made in this area, incorporation of hESC derivatives will likely enhance the ability of tissue engineers to produce functional bone and cartilage replacements for clinical use.

## Circulatory system

Blood cells and the endothelium arise from a common progenitor during embryogenesis, and similar environmental stimuli have been shown to stimulate haematopoietic and endothelial differentiation from hESCs [91, 92]. While the diverse number of cell types complicates haematopoietic differentiation systems, progenitors and lineage-specific cells can be identified and sorted using cluster of differentiation (CD) antigens. Haematopoietic stem cells (HSCs) have tremendous clinical applications as they are easily transplanted and can generate all blood cell types. However, current sources of HSCs, such as bone marrow or umbilical cord blood, are limited and contain risks associated with allogeneic tissue transfer and biological contamination. Producing haematopoietic progenitors from hESCs may offer a scaleable alternative to the clinical use of primary cells and could provide a new means of studying HSC differentiation.

Various protocols have been used to generate haematopoietic progenitors from hESCs, including EB differentiation in the presence of cytokines (*i.e.* BMP-4) and induction using stromal co-cultures [91–93]. Recently a scaleable suspension culture process using stromal induction was developed for the production of red blood cells from hESCs [94]. Application of similar methods to generate platelet-producing mega karyocytes could have a significant clinical impact, as platelets have a limited shelf life and cannot be efficiently preserved.

Dendritic cells are another haematopoietic population of clinical interest; these antigen-presenting cells can potentially be used as vaccines to mediate specific immune responses in transplant recipients. OP9 stromal co-culture and cytokine cocktails have successfully induced dendritic cell differentiation from hESCs through the myeloid lineage. These cells exhibited dendritic morphology, marker expression, and functionality with respect to antigen processing and presentation [95]. The use of hESCs as a cell source demonstrates a more scaleable method of generating dendritic and other haematopoietic cell types for biological research and clinical applications, potentially offering a cost effective solution to the problems associated with distributing these therapies.

The prevalence of cardiovascular disease has drawn substantial attention to the problem of engineering blood

vessels *in vitro*. In addition, most engineered tissues must be vascularized to survive and function after implantation. Endothelial cells line the inner wall of the vasculature, mediating nutrient transport and communication between the haematopoietic system and surrounding tissues and initiating angiogenesis. Several groups have demonstrated production of endothelial cells from hESCs, and results using both 2D and 3D differentiation protocols indicate that hESC-derived endothelial cells can be readily obtained [91, 92, 96, 97].

Culture of EBs or early differentiating hESCs in an *in vivo* microenvironment induces vascular network formation in response to angiogenic factors. Biologically derived materials, such as alginate [98] or synthetic, biodegradable, polymeric scaffolds [99] support vascular development from hESCs. Isolation of precursor populations, based on cell surface marker expression, and subsequent culture in a 3D matrix has been employed to generate implantable blood vessels, resulting in formation of vascularized skeletal muscle [100] and durable blood vessels that can survive over the long term [97]. These recent findings have demonstrated successful incorporation of hESC derivatives into tissue engineering applications; however, the expression of endothelial markers and vascular formation alone do not demonstrate full vascular functionality. The capability of these cells to detect and respond to environmental changes must also be examined prior to clinical use. Recently, researchers have induced endothelial differentiation of mESCs using fluid shear stress, indicating that ESCs and their derivatives can detect and respond (by differentiation) to biophysical cues [101]. Nevertheless, generation of blood vessels using a scalable cell source may greatly improve the ability of engineers to more efficiently produce transplantable tissues.

## Endodermal tissues

During embryogenesis, the definitive endoderm forms the gastrointestinal and respiratory tracts as well as the organs derived from the primitive gut. The endodermal derivatives which hold the most therapeutic promise include the pancreas and liver. Studies of vertebrate embryogenesis have shown that the definitive endoderm forms during gastrula-

tion when epiblast cells migrate through the primitive streak. At this embryogenic point, common mesoderm and endoderm progenitor cells (mesendoderm) segregate, forming their respective germ layers [102]. In mice, high levels of Nodal preferentially select for endoderm over mesoderm [103]. A recent study demonstrated that Activin A, another a member of the TGF- $\beta$  superfamily, commits hESC differentiation to definitive endoderm in low-serum conditions [104]. In addition, the temporal sequence of gene expression during hESC differentiation to definitive endoderm mimics the vertebrate gastrulation process. Subsequent work has determined that repression of PI3K signalling is also necessary to generate definitive endoderm from hESCs [32].

General challenges in deriving definitive endoderm include identifying and purifying the resulting cells. Although Sox17 [105] and HNF3 $\beta$ /FoxA2 [106] are often used as markers for definitive endoderm, these genes are also expressed in visceral and parietal endoderm, which compose the extraembryonic endoderm. Thus, a complex analysis of transcription factors and expression markers is necessary to demonstrate that the differentiated cells are indeed definitive endoderm [107]. Finally, the cell-surface chemokine receptor CXCR4 can be used to purify definitive endoderm cells from visceral and parietal endoderm, since it is only expressed in cells that migrate through the primitive streak during gastrulation (mesoderm and definitive endoderm) [108].

## Pancreas

The pancreas plays an important role in both the exocrine and endocrine systems, secreting enzymes into the digestive tract and hormones into the bloodstream. The main disorder that affects the pancreas is diabetes mellitus, which is caused by autoimmune destruction of  $\beta$ -islet cells (type 1 diabetes) or glucose resistance leading to death of  $\beta$ -islet cells (type 2 diabetes). Current efforts to treat diabetes consist mainly of insulin injections to manage blood glucose levels, but in practice blood glucose levels would be more effectively regulated by functional  $\beta$ -islet cells, reducing incidence of complications that result from glucose fluctuations.

One option for introducing functional  $\beta$ -islets is a whole pancreas transplant, but the limited number of donor organs is not adequate to treat a sufficient



amount of patients. A more promising alternative is allogeneic  $\beta$ -islet cell transplantation. It was previously thought that the body possessed pancreatic stem cells that served as progenitors to maintain the endocrine cells. However, Dor *et al.* found that  $\beta$ -islet cells are formed by symmetric proliferation of  $\beta$ -islet cells, suggesting that there may not be adult progenitors that can be used to develop large populations of  $\beta$ -islet cells for transplantation [109]. Thus, hESCs are excellent candidates for development of therapeutic  $\beta$ -islet cells.

Early pancreatic development is marked by RA signalling and inhibition of the SHH pathway [110]. hESCs overexpressing pancreatic and duodenal homeobox factor-1 (Pdx1) have been shown to differentiate to cells expressing further pancreatic markers [111]; however, these pancreatic cells have not committed to either exocrine or endocrine lineages. The transcription factor neurogenin-3 (Ngn3) is necessary to specify the endocrine precursor to the four cell types in the Islets of Langerhans. Various signals downstream of Ngn3 determine the specific fate of the endocrine cells; for example, the homeobox gene Nkx6.1 has been implicated in  $\beta$ -islet differentiation [112]. D'Amour *et al.* produced endocrine cells from hESCs using a differentiation pathway mimicking *in vivo* pancreatic organogenesis [110]. Although the differentiated cells exhibited insulin production levels comparable to adult islets, C-peptide release was not very responsive to glucose, indicating that glucose-sensing mechanisms were not active. This behaviour is characteristic of foetal  $\beta$ -islet cells, so it is possible that further maturation will be necessary to develop fully functional adult  $\beta$ -islet cells. Recently, a serum-free method was developed to produce insulin-producing islet-like clusters from hESCs without the use of feeder cells [113].

Preventing immune rejection of the implanted cells is a key consideration in islet cell therapies. Encapsulation of islet cells within polymers containing semi-permeable membranes that isolate the transplanted cells from the immune system but permit glucose and insulin transport may mitigate the need for immunosuppressive drug therapies [114]. With further advances in encapsulation technology or development of non-immunogenic hESC lines, as well as increased efficiency in differentiating hESCs to  $\beta$ -islet cells, hESCs hold great potential in generating the large quantity of  $\beta$ -islet cells needed for cellular therapy.

## Liver

Hepatocytes are the most abundant cell type in the liver, and the ability of these cells to proliferate is an enabling factor in liver regeneration. Therefore, hepatocyte production is the primary focus of liver engineering development. In bioartificial liver support systems, hepatocytes are seeded on an extracorporeal device which can be connected to the patient's circulatory system. However, this system is primarily a means of maintaining some temporary level of liver function until a transplant organ becomes available. Additionally, hepatocyte transplantation has shown limited success in treatment of various metabolic disorders in small clinical trials [115]. Development and transplantation of engineered hepatic tissue containing primary human hepatocytes has shown promise in treating a murine model of acute liver failure [116]. These engineered tissues permitted engraftment of the transplanted cells and limited migration of the hepatocytes to other organs, an improvement over previous methods of transplantation. Although these findings are promising, primary hepatocytes are difficult to isolate and grow *in vitro*, and thus hESCs may be a better alternative in the development of large populations of hepatocytes for cell transplantation systems.

hESCs have been shown to differentiate to functional hepatic cells in fully defined conditions upon induction by FGF-4 and hepatocyte growth factor [117]. In addition, 3D collagen scaffolds may spatially replicate the *in vivo* microenvironment for differentiation of hESCs to hepatic cells [118]. Hepatocytes can be identified by expression of transcription factors such as hepatocyte nuclear factor-3 $\beta$  (HNF-3 $\beta$ ), and hepatic functionality can be assessed using indocyanine green uptake, glycogen storage, ALB/CK-18 co-expression and urea production, among other factors [117]. However, the differentiation efficiency must be improved in order to develop clinically usable hepatocytes from hESCs. Recently, progress has been made in the development of a human hepatocyte-like cell line from hESCs containing GFP driven by the  $\alpha$  1-antitrypsin promoter [119]. mESCs have recently been used to develop implanted bioartificial liver devices which improved liver function in mice [120]. The success of cell transplantation methods with hepatic cells formed from mESCs suggests that it may eventually be possible to use hESCs for treatment of liver disorders.

**Table 2** Progress on the generation of specialized cell types from hESCs

Germ layer	Cell Lineage/Tissue	Differentiation efficiency†	Chemically defined process‡	Surface markers for lineage purification §	References
Ectoderm	Keratinocyte	High	Defined	Not demonstrated	[46]
	Corneal epithelium	Not defined	Serum/CM	Not demonstrated	[51]
	Dopaminergic neurons	Moderate	KSR	Not demonstrated	[55, 56]
	Motor neuron	Low	KSR	Not demonstrated	[58]
	Oligodendrocyte	High	KSR	Not demonstrated	[60, 61]
Mesoderm	Osteoblasts	Not defined	Serum/feeders	Mesenchymal precursors (CD73)	[80, 81]
	Chondrocytes	Not defined	KSR	Mesenchymal precursors (CD73)	[80, 85]
	Cardiomyocytes	Low	Serum/feeders	Not demonstrated	[69, 73, 75, 79]
	Haematopoietic cells	Moderate	Serum/feeders	Various available	[91–93]
	Endothelial cells	Moderate	Serum/feeders	Various used	[91, 92, 96, 97]
Endoderm	$\beta$ -islets	Moderate	Defined	Definitive endoderm only (CXCR4)	[113]
	Hepatocytes	Not defined	Yes	Not demonstrated	[117, 118]

† Efficiency is reported as the percentage of cells staining positive for a lineage specific marker. High denotes >90%, moderate denotes <90% and >30%, and low denotes <30%.

‡ Chemically defined processes use serum-free medium in the absence of feeder layers for all differentiation steps. Conditioned medium, CM; knockout serum replacer, KSR

§ Surface markers available for live cell sorting (*i.e.* magnetic or fluorescence based).

## Future challenges

Efficient hESC culture techniques were reported only 9 years ago, and while much of the excitement regarding the potential of hESCs is warranted, many obstacles must be overcome before these cells can be effectively utilized in the clinic. Defined conditions for the derivation and expansion of two hESC lines have only recently been identified [15]. The robustness of this method and genetic stability of lines cultured under defined conditions must be characterized in multiple labs and at larger scales. In addition, one cannot assume that hESCs cultured under defined conditions will respond similarly to differentiation protocols established using older methods of propagation.

Given that many lineages lack unique surface markers that might be used to isolate pure populations, highly efficient, well-defined differentiation processes must be developed using chemically defined media. These properties are described for specific lineages of clinical relevance in Table 2, although it must be noted that research groups often use different criteria in identifying particular cell

types. A diverse set of markers determined by experts in each field should be used for quantification. Furthermore, the functionality of differentiated cell types must be evaluated both *in vitro* and *in vivo*; for example, neurons must mediate electrical and chemical signals properly, and  $\beta$ -islets must release insulin in response to changing blood glucose concentrations. The ability to genetically manipulate hESCs must also be improved. Early (reported) efforts using various methods have been successful, but low efficiencies of recombination and clonal isolation impede implementation of these methods. Genetic modification of undifferentiated cells could be employed to improve differentiation and/or purification of specific lineages and will enable the generation of disease models from hESCs.

Finally, the immunocompatibility of hESC lines must be addressed. Immunological rejection of transplanted cells is expected if human leukocyte antigens (HLAs) expressed in hESC lines do not match those of recipients. Techniques, such as somatic cell nuclear transfer may enable the generation of patient-specific cell lines, though even if this method can be

executed successfully, implementation at a reasonable scale would be impossible [121, 122]. Genetically altering hESC lines to improve immunocompatibility could be an alternative, but it is unclear how many clones would need to be generated. A large cell bank containing diverse hESCs lines to cover the distribution of HLAs has also been proposed [123]. Alternatively, other sources of immunocompatible pluripotent cells may arise, such as amniotic fluid-derived stem (AFS) cells [124], or induced pluripotent stem (iPS) cells [125, 126]. While these alternatives avoid the ethical problems associated with using viable human embryos to generate pluripotent cells, these cells (iPS cells in particular) must be studied over the long-term to adequately characterize their robustness and eliminate any potential safety hazards (*i.e.* disruption of genes by viral insertion).

The above issues are also compounded by the fact that researchers as of yet maintain a 'narrow' perspective on hESC behaviour. The slow propagation of robust hESC practices has severely limited the distribution of hESC technology to the biomedical research community. As these issues are resolved, researchers must continue to integrate the best hESC practices into tissue engineering processes, for stem cell and biomaterials technologies are evolving at a rapid pace. Although many challenges must be overcome, the incorporation of hESCs is a pivotal step in the advancement of human tissue engineering.

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