

# Derive and conquer: sourcing and differentiating stem cells for therapeutic applications

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**Abstract** | Although great progress has been made in the isolation and culture of stem cells, the future of stem-cell-based therapies and their productive use in drug discovery and regenerative medicine depends on two key factors: finding reliable sources of multipotent and pluripotent cells and the ability to control their differentiation to generate desired derivatives. It is essential for clinical applications to establish reliable sources of pathogen-free human embryonic stem cells (ESCs) and develop suitable differentiation techniques. Here, we address some of the problems associated with the sourcing of human ESCs and discuss the current status of stem-cell differentiation technology.

## Pluripotent

In cell biology this term is usually used to indicate that pluripotent cells can develop into derivatives of all three germ layers.

## Organ-derived stem cells

These are often called 'adult' or 'somatic' stem cells; adult in this case meaning 'differentiated' or 'other than embryonic', because multipotent stem cells have also been isolated from extraembryonic and perinatal tissues.

More than 25 years ago, mouse embryonic stem cells (ESCs) were first established by two independent groups<sup>1,2</sup>, and extensive study in this field has allowed broad gains in our understanding of early developmental events and differentiation pathways. However, it was the subsequent derivation of human pluripotent cells in 1998 by James Thomson<sup>3</sup> and John Gearhart<sup>4</sup> that catalysed the huge recent increase in interest in the potential therapeutic applications of stem-cell research. Numerous reports since then have also described the similarities and differences between mouse and human ESCs, which are summarized in TABLE 1.

Stem cells, which are hallmarked by their ability to self-renew and differentiate into a wide variety of cell types, can be broadly classified as ESCs or organ-derived stem cells. ESCs can be differentiated into specialized cell lineages of all three embryonic germ layers — ectodermal, mesodermal and endodermal — *in vitro* in the presence of physical-inducing and biological-inducing factors.

So far, many promising studies have shown the therapeutic potential of differentiated derivatives of ESCs in ameliorating a range of disease in animal models. For example, ESC-derived neural progenitors transplanted into the brains of rats with Parkinson's disease generated functional dopamine neurons<sup>5,6</sup>; human ESC-derived cardiomyocytes have shown *in vivo* functional integration in rats with infarcted hearts and in a large-animal (pig) model of slow heart rate<sup>7–9</sup>; and retinal pigment epithelium derived from monkey and human ESCs appeared to preserve visual function in a rat model of macular degeneration<sup>10,11</sup>. However, such studies are

still mostly in the preclinical stage, and there are only a handful of companies that are conducting clinical trials with stem-cell derivatives (BOX 1).

In addition to their potential for application in regenerative medicine, ESCs have important roles in drug discovery. First, mouse ESCs can be used to create mice that have targeted mutations in particular genes. These mouse models have been used extensively, for example, to discover and evaluate the potential of drug targets<sup>12,13</sup>. Second, mouse ESCs can be differentiated to provide various types of cells for use in functional assays in hit and lead screening, and also to evaluate potential toxicity (reviewed in REF. 14). Traditionally, cells for such studies have been derived from animal or human tissues either as primary cultures — in which batch-to-batch variability is a common problem — or as immortalized lines, which often harbour uncharacterized genetic abnormalities. The benefits of using mouse ESCs include the ability to generate large and uniform cultures under good manufacturing practice/good laboratory practice conditions with minimal deviation of original characteristics. Even the cells that incur genetic abnormalities in culture can be eliminated from the final batches, therefore mouse ESCs can be used for drug screening and toxicity studies with high reproducibility. Efforts to extend this strategy to human ESCs, which could provide more realistic cellular models for drug discovery, are being actively pursued. Moreover, derivatives of human ESC lines can be established from genetically abnormal embryos<sup>15</sup>. Verlinsky and co-authors have derived human ESC lines with a wide range of genetic disorders, such as adrenoleukodystrophy, Duchenne and

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Table 1 | Comparison of mouse and human embryonic stem cells

Characteristic	Mouse*	Human†
Markers of pluripotency	Oct4 (also known as Pou5f1), Rex1 (also known as Zfp42), Nanog, SSEA1, alkaline phosphatase	Oct4, Rex1, Nanog, SSEA3, SSEA4 TRA-1-60, TRA-1-81, alkaline phosphatase
<i>In vitro</i> differentiation	Derivatives of all three germ layers	Derivatives of all three germ layers
Teratoma formation?	Yes, <i>in vitro</i> and in immunocompromised mice	Yes, <i>in vitro</i> and in immunocompromised mice
Germline transmission?	Yes	N/A
Derivation	No immunosurgery required, trypsin can be used for the first passage	Immunosurgery or microsurgery, or separation of the inner cell mass from the trophoblast at the earliest stages of outgrowth is required to prevent inner-cell-mass differentiation; propagation by mechanical dispersion is usually needed for several initial passages before the cells can be adapted to trypsin or collagenase
Transfection	Easy (can use conventional approaches)	Difficult (lentivirus required)
Feeder dependency	Can grow on gelatin in the presence of LIF; can be maintained feeder-free under defined conditions	Can be derived and maintained feeder-free under defined conditions; bFGF is required for self-renewal
Karyotypic stability	Aneuploidy can be acquired in culture and is permissible for successful germline transmission if 50–100% of cells have normal karyotype	Stability of karyotype through multiple passages is standard; acquired aneuploidy in chromosomes 12 and 14 has been reported for different lines that were passaged enzymatically
Passaging techniques	Trypsin	Mechanical dispersion, trypsin, collagenase

\*REFS 1, 2, 168–179. †REFS 3, 17–25, 69, 178–190. bFGF, basic fibroblast growth factor (also known as FGF2); LIF, leukaemia inhibitory factor; SSEA, stage-specific embryonic antigen; TRA, tumour rejection antigen.

**Feeder cells**

Cells of a different type and often different species that are used in a co-culture system to help maintain embryonic stem cells (ESCs) undifferentiated and mitotically inactivated to prevent overgrowing. Traditionally mouse embryonic fibroblasts were used as feeders for mouse and human ESCs, but in anticipation of using human ESC derivatives in the clinic, novel human ESC culture systems have been developed that use human cell lines as feeders or no feeder cells at all.

**Teratoma**

A rare tumour type that typically arises in the gonads and demonstrates mixed cellular populations of all three embryonic germ layers. Investigators can assess the differentiation capacity of stem cells by injection of pluripotent cells into laboratory animals and inducing the formation of teratomas *in situ*.

Becker muscular dystrophy, Fanconi anaemia, complementation group A, fragile-X syndrome, Huntington's disease, Marfan syndrome, myotonic dystrophy, neurofibromatosis type I and thalassaemia. According to the authors<sup>15</sup> these cell lines should be available on request from their group and can be used in *in vitro* models of these diseases.

The applicability of stem cells in regenerative medicine and drug discovery depends on obtaining suitable stem-cell sources and developing technologies for a reliable *in vitro* production of the appropriate stem-cell derivatives. This in turn relies on the ability to understand the differentiation cues that the cells send to each other in normal development and to reproduce these cues *in vitro*. In this Review, we discuss these challenges in the application of stem-cell technology and suggest approaches to address them.

**Sourcing embryonic stem cells**

The first criterion for ESCs intended for use in regenerative medicine is safety: unlike pharmacological agents, the cells cannot be sterilized at the final stage of production, and after transplantation their use cannot be discontinued if any undesirable effects occur. Therefore, as a minimum, the cells need to be free of pathogens and of any tumorigenic potential. Ancillary materials used for production of human ESCs may include animal sera, antibodies and matrix proteins. Most of the existing human ESC lines have been exposed to animal products, which could potentially contaminate them with animal

viruses, including those not yet known. The US Food and Drug Administration allows the use of such cells, but classifies them as xenotransplantation products, which will need to meet additional testing criteria and undergo additional clinical monitoring. In other countries, the requirements may be different<sup>16</sup>. The use of autogenic (differentiated from the same ESCs) feeders<sup>17</sup>, or human feeder cells that are pre-screened for pathogens<sup>18,19</sup>, is an area of active research. However, with the appropriate set of signals, ESCs can grow without feeder cells, and new technologies are being developed for deriving human ESCs using pathogen-free, feeder-free and animal-product-free culture systems<sup>17,18,20–22</sup>.

Although the culture conditions for the propagation of ESCs in an undifferentiated state are being refined<sup>21–25</sup>, the search for the perfect ESC remains an important challenge for the field. The enormous clinical potential of stem cells critically depends on their reliable behaviour (that is, the ability to retain their desired cell type) once implanted into a recipient. With the assumption that some intrinsic limitations on the fate of ESCs exist, great value is placed on finding 'right' human ESC lines that will be genetically stable, respond correctly to instructions from the surrounding environment and satisfy all the necessary criteria for effective restoration of tissue structure and function.

Tumorigenicity of undifferentiated or under-differentiated human ESCs is a serious concern. The ability of undifferentiated ESCs to form teratomas has been used as

**Bromodeoxyuridine**

(BrdU). 5-bromo-2-deoxyuridine is a synthetic analogue of the nucleoside thymidine. BrdU is used to label proliferating cells within a given time-frame *in vitro* or *in vivo*. It incorporates into the newly replicated DNA strands and can be detected with anti-BrdU antibodies.

**Blastocyst**

A blastocyst is a multi-cell structure formed by a developing mammalian embryo at the early stages of development that looks like a spheroid formed by an outer cell layer — trophoblast, a cavity — a blastocoel and an inner cell mass (ICM). In further development, a trophoblast gives rise to extraembryonic tissues, while the ICM develops into a new organism. Cells of the ICM are pluripotent and can also produce embryonic stem cells.

**Parthenote**

Parthenote, parthenogenesis is derived from the Greek 'parthenos', which means virgin. In this article, it pertains to activated unfertilized oocytes that are capable of undergoing the cleavage division and forming blastocysts. It is accepted that parthenote embryos in mammals are not capable of forming extraembryonic tissues and thus cannot complete normal development.

a test for their pluripotency, and this ability can backfire if a candidate derivative of ESCs is not completely free of pluripotent cells. Such cell products would need to be extensively evaluated for the presence of markers of pluripotency. However, even with differentiated derivatives of ESCs, certain concerns remain, as several reports have shown that transplanted derivatives of ESCs could also produce tumours<sup>6,26</sup>. When neurally selected derivatives of mouse ESCs that were positive for neuronal tubulin III, glial fibrillary acidic protein (GFAP) and Nestin were injected into the subretinal space of rhodopsin<sup>-/-</sup> mice<sup>6</sup> teratomas were formed, causing eye malformation within 2 months after transplantation. It is not clear from the authors' data whether there was a subpopulation of pluripotent cells after 7 days of selection, or whether the cells — seemingly to be of the neural lineage — still retained the capacity to induce teratomas. Other studies that used ESC-derived neural precursors for transplantation into fetal brains of mice<sup>27</sup> or dopaminergic neuron progenitors for transplantation into Parkinsonian rats<sup>6</sup> found that such ESCs of more differentiated neural lineage derivatives nevertheless showed signs of possible tumour formation. The first of these two studies discovered that in addition to functional integration of ESC-derived neurons, there were areas of neuroepithelial and even non-neural differentiation. The second study found that 70 days after transplantation, there was a mass of proliferating cells of neuroepithelial origin that could potentially be tumorigenic. There was no evidence of undifferentiated human ESCs being present, yet the grafts showed over 6% of bromodeoxyuridine (BrdU)-incorporating cells and over 1% of actively dividing cells. These are examples of different fates that progenitor cells can assume, and extreme care should be taken to exclude potentially tumorigenic cells from the transplantable population. Extensive *in vivo* studies need to be performed for each differentiated derivative that is considered for therapy before it can be regarded as safe.

The second criterion — close immune match with the recipient — is being addressed through several avenues, which we discuss below. FIGURE 1 illustrates the various ways to generate ESCs that can be used to tackle the issues associated with adverse immune reactions.

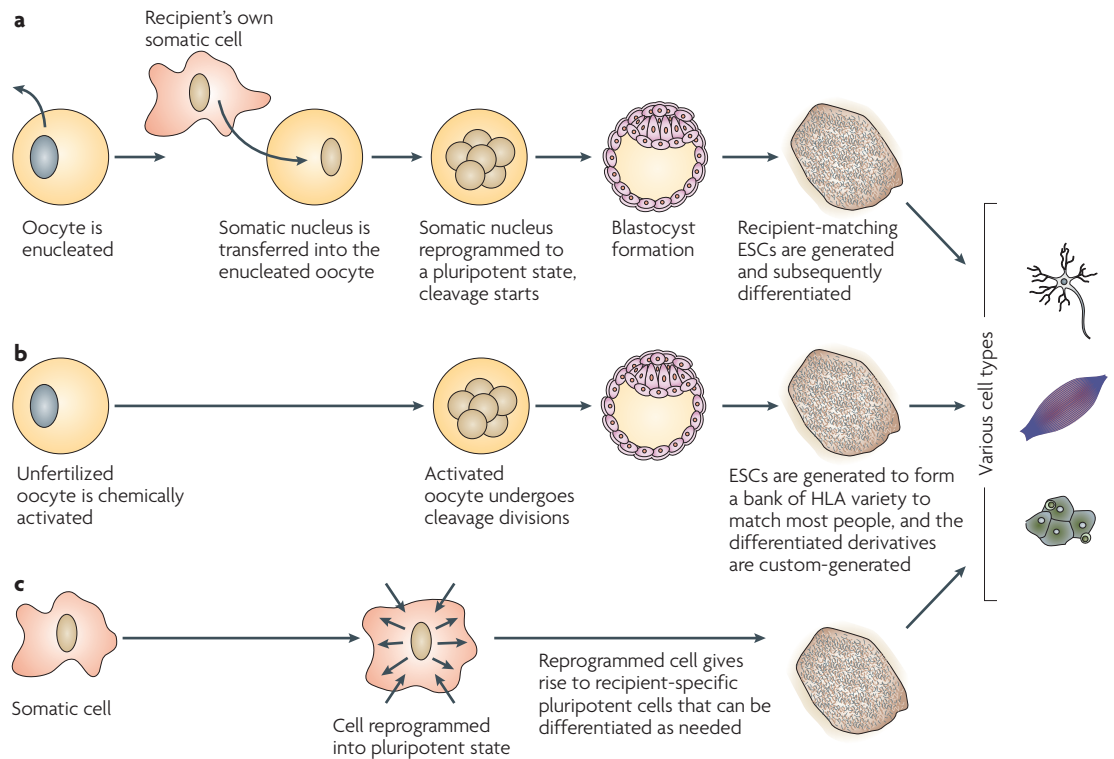
**Somatic cell nuclear transfer.** A highly publicized approach to generate stem cells that are immunologically matched to the recipient involves somatic cell nuclear transfer (SCNT). The past few years were distinguished by the rise and fall of hopes for these types of human ESCs to move into the clinic in the near future: primarily, a group claimed that they could generate multiple patient-specific human ESC lines by means of nuclear transfer technology<sup>28,29</sup>. Although the world initially applauded this group and numerous articles derived important conclusions from this breakthrough, the subsequent disclosure of fraud — which led to the retraction of the original publications — and the ensuing scandal set research in the field back. Now, a 'presumption of genuineness' was replaced with scrutiny of any new human ESC lines achieved with nuclear transfer. Nevertheless, the anticipation that SCNT human ESCs can eventually be developed has been fuelled by a study in which no major differences in transcriptional profiles were found between mouse ESCs derived by SCNT and fertilized embryo-derived ESCs<sup>30</sup> despite previous observations of gene dysregulation and defects in the first generation of cloned animals<sup>31–37</sup>. Earlier results had suggested that the epigenetic memory of the donor nuclei is frozen during subsequent embryonic development<sup>31,34,36</sup> and, therefore, raised serious concerns that SCNT ESCs could have potential abnormalities. If intrinsic epigenetic memory is indeed lost in ESCs derived from cloned blastocysts, it raises hopes that SCNT ESCs can be derived from a variety of donor cell types and be as safe for therapy as fertilized embryo-derived ESCs. The recent report of generating primate ESCs via SCNT<sup>191</sup>, although with low efficiency, confirmed the possibility of complete nuclear reprogramming of primate somatic cells to pluripotent cells through SCNT.

**Parthenogenesis.** Generating human ESC lines from activated oocytes without sperm fertilization, a process known as parthenogenesis, reduces immune (major histocompatibility complex; MHC) complexity. This could allow a match with most of the population with as few as a hundred or so lines derived from oocytes of pre-selected egg donors. In 2002, Cibelli and co-authors<sup>38</sup> reported derivation of pluripotent stem cells from parthenogenetically activated *Cynomolgus* monkey oocytes — the first primate parthenote ESC line. Lin and co-authors<sup>39</sup> were able to culture cells from the inner cell mass of parthenote blastocysts for only two passages. Recently a team have reported derivation of several donor-specific human ESC lines from parthenogenetically activated blastocysts<sup>40</sup>. If this work can be reliably reproduced and if parthenote human ESCs can form functional differentiated derivatives, this technique could become an important step towards generating patient-specific cells. An interesting study<sup>41</sup> applied genome-wide single nucleotide polymorphism (SNP) analysis to a "SCNT-hES-1" cell line, which was previously reported in a *Science* paper (that was later retracted) as SCNT-generated human ESC line<sup>28</sup>. The results of the study show that the originally presented data on the expression of two imprinted genes that would normally originate from paternally imprinted alleles probably resulted from epigenetic instability. The SNP

**Box 1 | Clinical trials of stem-cell therapies**

Some examples of companies conducting clinical trials with stem cells include Cellerix in Spain, which is conducting Phase II trials with its adipose tissue-derived stem cells for the treatment of complex perianal fistulas; clinical trials by Osiris Therapeutics (USA), using several adult stem-cell-based products for the treatment of graft-versus-host disease, Crohn's disease, and for regeneration of the joint cartilage; or the Israeli companies Teva Pharmaceutical Industries and Gamida Cell, which are now moving to Phase III clinical trials with haematopoietic cells as an alternative for bone-marrow transplants in patients with haematological malignancies.

However, no clinical trials have yet been initiated with derivatives of human embryonic stem cells (ESCs). Companies that could be the first to file for an investigational new drug license for human ESCs with the US Food and Drug Administration include Geron, with human ESC-derived oligodendrocytes for spinal-cord injury, and Advanced Cell Technology, with retinal pigment epithelium generated from human ESCs to treat retinal diseases.



**Figure 1 | Possible ways to generate immune-compatible derivatives of pluripotent cells. a** | Somatic cell nuclear transfer (SCNT). The nucleus of the fertilized oocyte is removed and the nucleus of a recipient's somatic cell is transferred into the enucleated oocyte. The somatic nucleus is reprogrammed by the competent cytoplasm and the oocyte with the donor nucleus starts cleaving and forms a morula that continues to develop into a blastocyst. Pluripotent embryonic stem cells with the recipient's genome are derived from either the morula or the inner cell mass (ICM) of the blastocyst. Their differentiated derivatives are immunologically identical to the recipient's own. **b** | Parthenote pluripotent cells. An unfertilized oocyte is activated and starts to cleave as a normal embryo. Pluripotent cells are derived from a morula or the ICM of a blastocyst. The absence of the paternal contribution greatly diminishes the human leukocyte antigen (HLA) variability and thus allows a bank of various HLA types of pluripotent cells and/or their derivatives to be built. **c** | Transdifferentiation. The recipient's somatic cell is turned into a pluripotent cell via cellular reprogramming using transfection of a defined set of transcription factors or loading the target cell with the cellular lysate of a pluripotent cell. The new pluripotent cell and its derivatives are immunologically identical to the recipient's and so can be used for transplantation.

analysis performed in this study provides evidence that rather than being SCNT-generated, human ESC lines such as SCNT-hES-1 represent the first human parthenote human ESC line.

**Cellular reprogramming.** Stem cells could also theoretically be generated using cellular reprogramming (also known as transdifferentiation or de-differentiation). This approach holds promise for generating pluripotent or multipotent stem cells without the need for human eggs or embryos. Cells from the recipient — for example, from a cheek swab or skin cells — can be reprogrammed into stem cells in the laboratory using various approaches. One strategy involves the use of agents such as bacterial protein streptolysin O, which can be used to permeabilize the somatic cell membrane in order to load it with cell lysates prepared from specific target cell types. The cocktail of factors from the donor cell lysate then reprogrammes the recipient cell. Lysates from a range of different cell types have achieved varying degrees — both short-term and long-term — of reprogramming<sup>42–45</sup>,

although the technology is not well enough developed yet to proceed to the clinic. A variation on this theme involves the fusion of somatic cells, such as fibroblasts, with human ESCs, thereby producing a hybrid cell type in which the ESC characteristics predominate, probably due to the overwhelmingly higher transcriptional activity of ESCs<sup>46,47</sup>. These studies are of obvious academic interest, but their clinical application depends on the development of methods to separate the chromosomes of ESCs and somatic cells. In a recent report<sup>48</sup>, the enucleated human ESCs were fused with various types of adult somatic cells. The resulting 'cybrids' had the genotype of the somatic cell and 'stemness' of human ESCs. This study, however, contradicts the earlier finding<sup>49</sup> showing that reprogramming cues reside in the nucleus, not the cytoplasm. Further studies will be needed to resolve these conflicting results.

As the search for the sorcerer's stone that would allow the conversion of somatic cells into pluripotent cells continues, a breakthrough in reprogramming was announced in 2006 (REF. 50), with subsequent studies

**Reprogramming**

Occurs naturally in regenerative organisms (dedifferentiation). Induced experimentally in mammalian cells by nuclear transfer, cell fusion or genetic manipulation of *in vitro* culture.

**Multipotent**

Can form multiple lineages that can give rise to several kinds of cells, tissues or structures, for example, haematopoietic stem cells.

**Cybrid**

Is a cell produced by the fusing of a eukaryotic cell with an enucleated cell or a cytoplasm.

published a year later<sup>51–53</sup>. The overexpression of four transcription factors — Oct3/4 (also known as Oct4 or Pou5f1), Sox2, Myc and Klf4 — in mouse tail fibroblasts reprogrammed them to a pluripotent state. Reprogrammed cells produced by these approaches acquired ESC-like morphology, formed raised ESC-like colonies, expressed markers of pluripotency, differentiated into the derivatives of all three germ layers and formed chimeric mice. Remarkably, these cells showed germline transmission<sup>51</sup> and reversed the somatic epigenome to an ESC-like stage<sup>53</sup>. Until recently, this technology remained a proof of principle, as it was not clear whether the same factors would allow reprogramming of human somatic cells. However, recent reports from two groups who successfully reprogrammed human somatic cells with defined transcription factors<sup>192,193</sup> catapults this technology into the realm of clinical relevance. Induced pluripotent stem cells were generated from human adult<sup>192</sup>, fetal and neonatal<sup>193</sup> dermal fibroblast using either the same set of genes<sup>192</sup> or with a core set of Oct4, Sox2, Nanog and Lin28, thus successfully eliminating Myc. The induction of human fibroblasts to pluripotency did not perturb their karyotype, but did induce ESC-like morphology, telomerase activity and epigenetic patterns, and imparting the potential to proliferate and differentiate into advanced derivatives of all three embryonic germ layers. Although these results represent a crucial advance in stem-cell biology, there are serious hurdles left to overcome before considering clinical applications, including the use of retroviruses or lentiviruses to genetically modify the cells. It is important to understand whether induced pluripotent stem cells and human ESCs can be differentiated using the same technology and whether their derivatives have similar functionality and behaviour *in vivo*. Understanding how newly integrated genes function in derivatives of the pluripotent cells, how they get silenced and if they can be reactivated are essential for determining the safety of this approach for future clinical applications. Years of additional research are likely to be required to resolve all these issues.

**Ethical issues and alternative approaches.** The most basic objection to ESC research is that it deprives embryos of any further potential to develop into a complete human being. Several alternative approaches for generating ESCs have been proposed that could potentially reduce or eliminate this ethical concern, and this is addressed in BOX 2.

### Stem-cell derivatives and cell therapy

Although there are high hopes for the use of stem cells in regenerative medicine, the reliable and robust production of ESC derivatives is still in its infancy. The number of publications reporting novel human ESC derivatives seems to be expanding rapidly, but most only show the presence of a small percentage of cells of a particular tissue type among other differentiating cells. If ESC differentiation *in vitro* mimics the patterning events of early development, it would also be dependent on the complex interplay of signals from a multilineage plethora of cells at various stages of differentiation. Combinations of soluble signalling molecules and extracellular matrix can

imitate the intercellular interactions to some extent, but it is hard to predict how closely the embryonic temporal and spatial organization must be recapitulated, and to what extent it can be controlled *in vitro*.

As the cells at early differentiation stages may be undecided, even the accurate reproduction of the embryonic microenvironment may result in a heterogeneous population of differentiation derivatives, as the same cues may cause different results in different cell targets. If the complex development of a human embryo is any guide, the success of any *in vitro* differentiation system may require a collaboration of various cell types originating from the same ESCs to produce a desired derivative. Separation of an ESC derivative from other diverse cell types after they have accomplished their role of supporting its differentiation would then be required by means of fluorescence-activated cell sorting or magnetic bead sorting, manual isolation by hand-picking or selective media and culture conditions.

To date, only a handful of human ESC derivatives have been successfully isolated, passaged and characterized to the extent that they can be considered similar to their *in vivo* counterparts or shown to be functional in animal models. The challenge for such isolation approaches is that immature or progenitor cells are produced, because fully differentiated somatic cells only have a limited lifespan and/or have poor growth characteristics in culture. Progenitor cells are often multipotent and still require further instructions to be directed to a specific target cell type. For instance, ESC-derived insulin-producing cells also had characteristics of neurons and neural progenitors formed teratomas in diabetic mice and did not reverse the hyperglycaemic state<sup>54</sup>.

At present, only a few reliably produced and well-characterized ESC derivatives have been reported, such as cells of neural lineage. Although independent reports have described the generation of sought-after insulin-secreting cells, cardiomyocytes, hepatocytes or haematopoietic lineages from ESCs<sup>55–58</sup>, specific protocols for producing these cells reliably in large quantities still need to be developed. Below we give examples of such original studies.

Much of the current work focuses on co-opting signals working in the embryo to deliver instructions that direct ESC fate. These signals can coerce ESC cultures to adopt characteristics derived from the three primitive embryonic germ layers (ectoderm, mesoderm and endoderm) (FIG. 2). For example, the bone morphogenetic protein (BMP) family of signalling factors is able to influence ESCs to choose a mesodermal rather than a neuroectodermal fate in culture<sup>59</sup>; however, Nanog binding to SMAD1 inhibits this pathway<sup>60</sup>. Treatment of ESCs with BMP4 shifts differentiation towards mesoderm<sup>59,61,62</sup>, inhibiting neural differentiation<sup>62</sup>. Under different conditions, BMP4 promotes the differentiation of ESCs and early primitive ectoderm-like cells towards surface ectoderm at the expense of neuroectoderm, which underscores the properties of BMP4 as a suppressor of neuroectodermal differentiation<sup>63,64</sup>; however, BMP antagonists promote the acquisition of an ectodermal fate by ESCs. Exposure of ESCs to recombinant Noggin, a signalling protein that counteracts the mesoderm-inducing

#### Totipotent

Sufficient to form an entire organism. Totipotency is seen in zygote and plant meristem cells, but has not demonstrated for any vertebrate stem cell.

#### Morula

A stage of early embryonic development when an embryo consists of a cluster of cells.

Box 2 | **Can human ESCs be produced without embryo destruction?****Human embryonic stem cells (ESCs) from single blastomeres**

One proposed solution to the challenge of producing human ESCs without destroying an embryo is to remove a single cell (blastomere) from an eight-cell-stage embryo using a biopsy procedure that is carried out at *in vitro* fertilization clinics throughout the world. The procedure — known as pre-implantation genetic diagnosis (PGD) — has been used for over a decade to remove one or two blastomeres for genetic testing without harming the embryo. Recent research indicates that ESC lines can be successfully generated from single blastomeres in both animals and humans<sup>130,131</sup>. Biopsied blastomeres, which were co-cultured with green fluorescent protein (GFP)-expressing ESCs provided a visual means to separate clumps of blastomere-derived cells from GFP-positive ESCs, and to select for GFP-negative cells. Several mouse and human ESC lines were produced that appeared to be identical to conventionally derived ESCs: they expressed ESC markers of pluripotency, differentiated into all three germ layers *in vitro* and in teratomas, and (mouse ESCs) showed germline transmission. Further research is underway to make this technique more robust, which would enable scientists to establish human ESC lines without embryo destruction, and could be used to produce matched ESCs for children and siblings born from transferred PGD embryos. However, concerns have been raised as to whether individual eight-cell-stage blastomeres, such as those used in these studies, are totipotent and could potentially generate a human being. Notably, however, individual morula (8–16 cell) stage blastomeres have not been shown to have the intrinsic capacity to generate a complete organism in most mammalian species.

**Altered nuclear transfer**

A second approach, referred to as altered nuclear transfer, is conceptually based on somatic cell nuclear transfer (SCNT). A somatic cell is genetically altered before being transferred to an enucleated oocyte, such that the resulting entity lacks the essential capacities of a human embryo. For instance, mouse embryos have been created from donor cells with a silenced gene encoding caudal-type homeobox 2 (Cdx2), a protein necessary for implantation<sup>132</sup>. Blocking its action would have successfully prevented the embryo from attaching to the uterine wall. Some consider such entities as 'biological artefacts', that is, ethically equivalent to tissue culture, teratomas or moles. Thus, derivation of human ESCs from the developing entity would not be killing or harming a human life. This proposal has garnered broad support from the pro-life community. However, questions have been raised as to whether this artefact is just a bundle of cells or a defective embryo. Others have questioned whether scientists should use cloning and genetic manipulation to deliberately create defective human embryos.

**Growth-arrested embryos**

Another suggestion is to derive stem cells from growth-arrested embryos. It is well known that some embryos (from *in vitro* fertilization) arrest at the early multicell stage and do not develop any further. Such embryos are often considered non-viable and discarded. Zhang *et al.*<sup>133</sup> recently used such growth-arrested embryos to derive human ESCs that met all the criteria for pluripotency and had normal karyotype. This work demonstrates that although an embryo may not be capable of further development, it may still retain enough viable blastomeres to generate an ESC line. Embryos often stop dividing, and then after a significant 'down' or 'resting' period, can go on to generate intact blastocysts. Unfortunately, the only certain way to know whether an embryo is not viable is to determine that its cells have also lost the potential to proliferate. Another opinion<sup>134</sup> is that even a low-grade embryo, which would most probably be non-viable if transplanted into a uterus but can nevertheless produce stem cells, can be seen as a special case of organ donation.

**Cellular reprogramming**

Cellular reprogramming would be an alternative to the derivation of ESCs from human embryos, and a solution to both the ethical and immune compatibility problems. There were several pioneering studies in this direction, including fusion of somatic cells with embryonic cell lysates, loading permeabilized cells with lysates of donor cells (somatic or pluripotent), or overexpression of a set of genes of pluripotency in target cells. These approaches are discussed in more detail in the main text.

effects of BMP4, or transfection with a Noggin-encoding plasmid promotes the differentiation of neuroectodermal cells<sup>62,65–67</sup>. Exposure to another signalling molecule, Chordin, results in reduced differentiation, with some cells retaining ESC characteristics and a small number differentiating to mesenchymal cell types<sup>67</sup>. Disruption of another signalling pathway involving SMAD4, BMP and transforming growth factor (TGF) enhances the ability of ESCs to adopt a neural fate<sup>68</sup>, which is consistent with the suppression of neural differentiation by BMP signalling. FIGURE 2 gives examples of how signalling molecules can direct ESC differentiation in culture.

More clinically directed research has concentrated on deriving therapeutically relevant cell types directly from ESCs, without necessarily passing through intermediate stages of programming that they normally experience in the three primitive embryonic germ layers. In general, reports of success are sporadic and results are often irreproducible. Small discrepancies in ESC maintenance conditions between laboratories can have dramatic effects on the outcome, and laboratory-specific modifications of the original protocols are often required. Differences in the human ESC lines used in published studies, combined with variations in the maintenance/differentiation conditions used in different laboratories, makes it even more difficult to compare the efficiency of various protocols. For instance, human ESCs traditionally require mouse embryonic fibroblast feeder layers for undifferentiated growth, but the safety requirements for production of therapeutic grade ESC derivatives are favouring xeno-free systems, and several alternative culture systems have been published<sup>21–25,69</sup>. Although all of them seem to allow derivation or maintenance of pluripotent human ESCs, it is not inconceivable that the cells grown on a particular cell type or feeder-free system using defined medium and extracellular matrix will gain certain intrinsic preferences as to what differentiation path to choose. In our own experiments (I.K. and R.L., unpublished observations) using certain feeder cell type or extracellular matrix to maintain human ESCs for several passages resulted in a faster and more robust production of retinal pigment epithelium (see below). The more important factor to consider, however, is whether a particular protocol can reliably produce cells in high numbers for characterization, expansion, safety evaluation and transplantation in animal models or clinical applications. The recent history of research into three specific kinds of ESC derivatives, which is described in detail below, gives an idea of how close we are to (or far from) this goal.

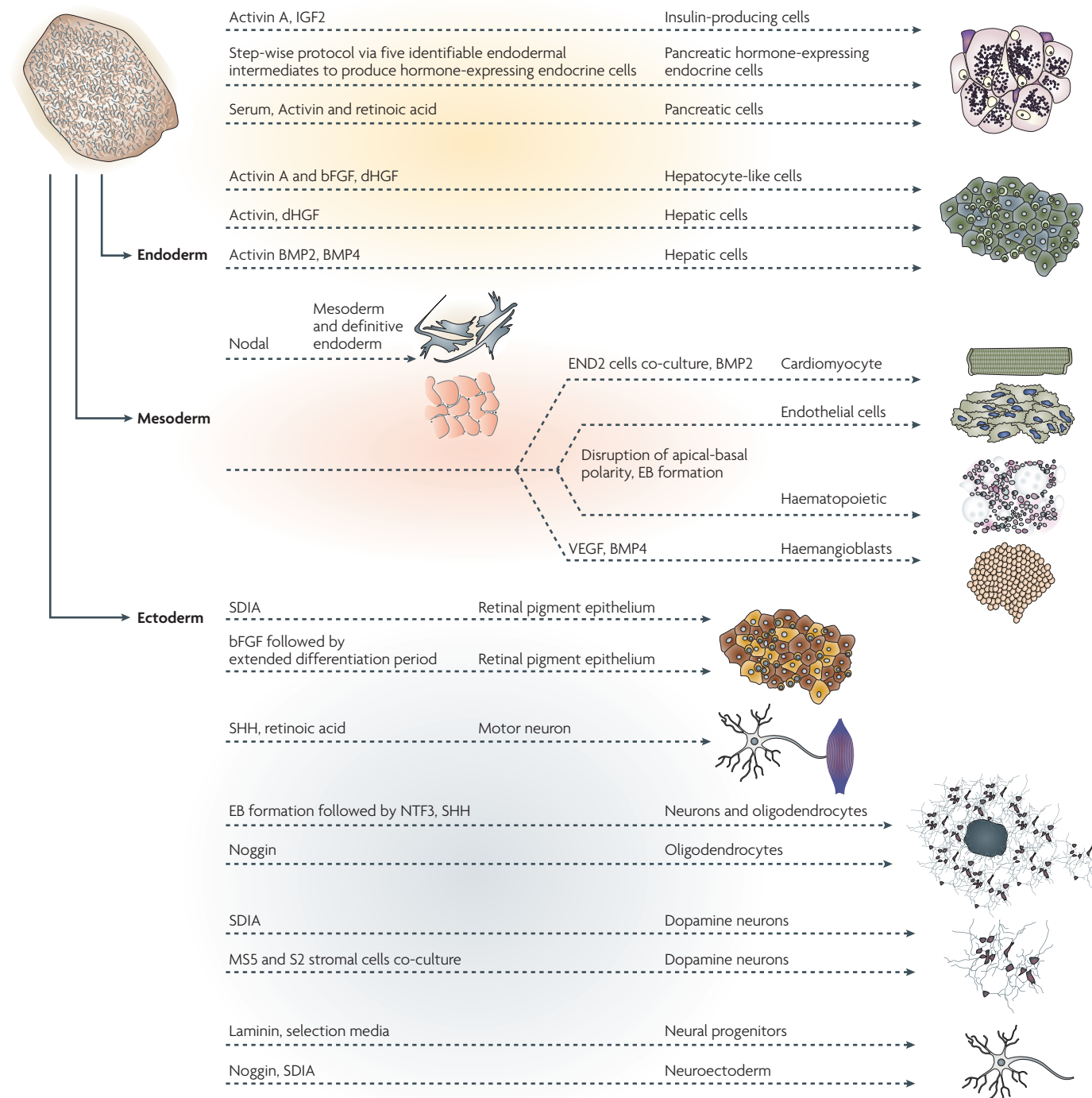
**Insulin-producing cells (endoderm).** The differentiation of human ESCs into insulin-producing cells has been fraught with controversy. Based on certain similarities in the development of the pancreas and the nervous system, researchers adapted the neuron-generating strategy to generate insulin-producing cells by selection of precursors expressing neural markers such as Nestin<sup>70–72</sup>. Others challenged this procedure and discovered that insulin staining in the cell aggregates was, in fact, co-localized with fluorescein isothiocyanate-labelled insulin from culture medium, probably because it was accumulated

**C-peptide**

C-peptide is a product of proinsulin when it is split, producing insulin and C-peptide after release from the pancreas.

by cells undergoing apoptosis inside high-density cell clusters<sup>73</sup>. Nevertheless, insulin-coding transcripts have been found in such cultures, which stained positively for insulin C-peptide<sup>54,55,74,75</sup>. It is likely that cell types that both produce and take up insulin from the medium exist in such differentiation models. This ambiguity was addressed by additional studies<sup>56</sup> that reproduced the Lumelsky–McKay protocol and found that C-peptide and

proinsulin staining was localized to Nestin-positive and neuron-like cells, and the transcription factors involved in pancreatic and neural development, such as ISL1, hepatic nuclear factor 3 (HNF3), pancreatic-duodenal homeobox 1 (Pdx1), neurogenic differentiation (NeuroD), neurogenin 3 (Ngn3; also known as Neuro3), were expressed at early stages of differentiation. Moreover, when a subset of cells expressing enhanced GFP under an insulin



**Figure 2 | *In vitro* avenues to differentiation of embryonic stem cells (ESCs).** This illustrates several examples of *in vitro* ESC differentiation protocols and show how a defined microenvironment can enhance various differentiation pathways by partially imitating early embryonic development. Data used to compile this figure were taken from REFS 156–167.

bFGF, basic fibroblast growth factor (also known as FGF2); BMP2/4, bone morphogenetic protein 2/4; dHGF, deleted variant of hepatocyte growth factor; EB, embryoid body; IGF2, insulin-like growth factor 2; NTF3, neurotrophin 3; SDIA, stromal-derived inducing activity method; SHH, sonic hedgehog homologue; VEGF, vascular endothelial growth factor.

promoter were isolated and characterized, they failed to express mature insulin or insulin-producing cell determinants such as Pdx1. This may be a peculiar example of differentiation analogy rather than homology in that ESC progeny with neuronal features also appear to be insulin-positive without showing other features of  $\beta$ -cells, and it remains to be determined whether they can release insulin in a dose-dependent manner. A study by D'Amour and co-authors<sup>76</sup> demonstrated that by taking human ESCs through the stages mimicking natural organogenesis, cells similar to  $\beta$ -cells can be produced. Such cells had insulin content similar to adult  $\beta$ -cells and released C-peptide in response to stimuli but not to glucose, thus resembling fetal pancreatic cells. Use of the endoderm differentiation pathway as a first step towards pancreatic differentiation was used recently in two independent studies that differentiated human ESCs towards the pancreatic lineage<sup>77,78</sup>. The authors induced endoderm differentiation stepwise using an embryoid bodies culture system that was sequentially treated with serum, Activin, retinoic acid and Bmp4 (REF. 77) or Activin and Bmp4 followed by several growth factors in the semi-solid matrix<sup>78</sup>. Both studies produced Pdx1-positive cells, indicative of pancreatic progenitor formation, and in the first study the cells further differentiated to more mature insulin-producing cells *in vivo*<sup>77</sup> and *in vitro*<sup>78</sup>. Both studies showed the ability of such cells to secrete insulin upon transplantation into streptozotocin-treated<sup>77</sup> or diabetic<sup>78</sup> mice and normalized blood glucose levels. Further investigation of the properties of such transplantable progenitors of  $\beta$ -cells will be necessary to evaluate their ability for long-term reversal of diabetes in the animal models.

**Cardiomyocytes (mesoderm).** To date, numerous studies have reported the derivation of cardiomyocytes from human and mouse ESCs. The first observations of ESC differentiation *in vitro* described the formation of spheroid structures called embryoid bodies<sup>79</sup>, which have been conventionally used for studying the differentiation of mouse — and then human — ESCs. Differentiation patterns of embryoid bodies are rather stochastic, but by modulating media, growth factors and time after embryoid-body formation, the equilibrium can be shifted towards the desired cell type. Heart-muscle cells, or cardiomyocytes, are usually found in the walls of embryoid bodies next to visceral endoderm, and spontaneously beating cells can be seen as early as 1 day after plating embryoid bodies for outgrowth on gelatin or collagen I; some embryoid bodies begin to contract without being plated (reviewed in REFS 56,80).

Human ESCs can also differentiate into beating cardiomyocytes in a similar system but at a slower rate, possibly reflecting the different timing of embryonic development in humans and mice<sup>81</sup>. The efficiency of cardiomyocyte differentiation of human ESC cultures is also about ten-times lower than that of mice, comprising only about 8% of the human cells versus 80–90% in mouse cultures. A serum-free differentiation protocol has been reported for mouse ESCs, raising the yield of cardiomyocyte-like cells sixfold by using insulin, transferrin and platelet-derived growth factor-BB or sphingosine-1-phosphate<sup>82</sup>.

However, in this study the original yield in the presence of more conventional media was below 1%, so it is difficult to argue whether the system as described will prove highly efficient.

Developmental markers of embryonic heart formation have been used to promote cardiogenic differentiation in both human and mouse embryoid bodies. Modulation of BMP and WNT signalling, addition of retinoic acid, dimethyl sulphoxide (reviewed in REF. 83), other small molecules<sup>84</sup> or even reactive oxygen species<sup>85</sup> have been variously successful in enriching cardiac-specific ESC differentiation. Genetic manipulation using muscle-specific microRNAs that are involved in embryonic cardiogenesis<sup>86</sup> or cardiac hypertrophy<sup>87</sup> could be an additional route to initiating or sustaining the cardiogenic programme in ESC cultures.

The low efficiency of human ESC-to-cardiomyocyte differentiation has also been augmented by co-culturing human ESCs with a visceral endoderm mouse cell line (End-2) or with a liver parenchymal cell line (HepG2), both of which might generate similar signalling profiles, thereby mimicking the signals coming from the endoderm in embryonic development (reviewed in REF. 88). The protocol appeared to be robust: cardiomyocytes produced in this study were contractile after embryoid bodies were dissociated to single cells and even after freeze-thaw cycles<sup>89,90</sup>. However, the authors admit that they were not able to obtain spontaneous cardiomyocyte differentiation or even embryoid-body formation by human ESCs. These problems could reflect particular features of the human ESC line used (hES2). Alternatively, deviations in culture conditions for the routine maintenance of human ESC and embryoid-body induction that usually vary among laboratories could lead to different responses of human ESCs to the same differentiation induction procedures.

Both mouse and human ESC cardiac derivatives share the same physiological, morphological and molecular characteristics; for example, they can continuously beat at a rate of 30–130 beats per minute for up to 5 weeks, express cardiac-specific genes such as those for transcription factors GATA4 and NKX2-5, sarcomeric proteins, myosin heavy chain, atrial myosin light chain,  $\alpha$ -actinin, cardiac troponin I, cardiac ion channels and sarcomeric markers. The cardiac derivatives can also respond to stimulation with  $\beta$ -adrenergic and cholinergic agonists<sup>80,81,89</sup> (reviewed in REFS 91,92). Although reports claim that human ESC-derived cardiomyocytes were different from adult heart cardiomyocytes, possibly owing to immature function of the sarcoplasmic reticulum<sup>93</sup> (reviewed in REF. 94), it is possible that such immature cells could develop into fully functional cardiomyocytes when placed in a proper physiological environment. In some animal studies, ESC-derived cardiomyocytes were shown to survive and provide functional benefits to the infarcted myocardium<sup>95–97</sup>. An encouraging study was performed by Laflamme and co-authors<sup>8</sup> whose stepwise Activin A–BMP4 differentiation protocol turned more than 30% of human ESCs into high numbers of cardiomyocytes of 71–95% purity after Percoll isolation. These cardiomyocytes engrafted into infarcted rat hearts and partially remuscularized them, improving their function.

#### Embryoid bodies

Spherical cell clusters observed after spontaneous or induced differentiation of embryonic stem cells in culture. Embryoid bodies show differentiation that recapitulates the early stages of mammalian embryonic development, including cell types derived from endoderm, mesoderm and ectodermal lineages.

## Box 3 | Non-embryonic stem cells

Harvesting therapeutic stem cells from the adult body would circumvent the ethical difficulties of stem-cell work that involves destroying human embryos. However, the sourcing of adult stem cells is not free of hurdles either. Although most adult tissues undergo self-renewal through cell turnover and replacement, they use local, tissue-specific progenitors that give rise to specific types of mature cells, rather than calling on a common pool of stem cells that undergo multilineage differentiation. If a stem-cell pool could be isolated and propagated from a patient, it would eliminate the problem of immune rejection. Adult stem-cell pool sizes are tightly regulated, and their safe manipulation *in vivo* will require a better understanding of the controls that maintain them.

The relative scarcity of stem cells residing in the adult tissues has prompted a search for a renewable source of circulating somatic progenitor cells that might home in to damaged areas. The existence of such cell populations has gained credibility from observations of sex-mismatched cardiac human transplants in which a female heart is transplanted into a male host. In these patients, the presence of the Y chromosome marks host-derived cells in the transplanted heart. The presence of differentiated Y-chromosome-positive myocytes and coronary vessels in the transplanted tissues<sup>135,136</sup> proved the existence of migratory precursor cells that are induced to differentiate by the cardiac milieu. Although the migration of host cells into the donor tissue could merely be a response to organ transplantation, it may also be part of a normal homeostatic process that could be exploited if the origin of these cells and how to call on this reserve in more effective ways is understood<sup>137</sup>. When isolated and cultured, these cells have been assigned different identities, which are likely to be dependent on the culture conditions used<sup>138–140</sup>.

Among human adult stem cell types, bone-marrow-derived cells have been most extensively tested for their ability to contribute to tissue repair. Well studied as they may be, human stem cells are still a contentious source of stem cells for therapeutic use outside of the immune system. Numerous recent studies have attempted to capture human stem cells in the act of transdifferentiating into other cell types in injured or diseased tissues, thereby contributing to the repair process through nuclear reprogramming<sup>140</sup>. Despite these exciting cases of human stem-cell plasticity, their potential for participating effectively in functional tissue regeneration remains to be proven.

Perhaps not surprisingly, the most promising pool of reprogrammable and renewable stem cells may be found in the adult testes, that is, derived from sperm-producing stem cells<sup>142,143</sup>. When harvested from mice, these cells shared similarities with ESCs in that they were able to spontaneously differentiate into derivatives of the three embryonic germ layers *in vitro*: differentiating into heart, brain and skin cell derivatives. After extensive culture and re-injection into early embryos, the adult testes-derived cells contributed to various organs of the resulting mice. Most importantly, they contributed to the mouse germline, thus proving their integrity and pluripotency. Although the principle has yet to be extended in humans, at least for male patients this would constitute a rich source of autologous cells for therapeutic use, bypassing rejection by the immune system. A California-based company, PrimeCell Therapeutics, claims to have developed the technology to isolate pluripotent cells from adult human testes. If the derivatives of such cells prove to be functional in animal models, this could deliver a practical solution to the controversy of using human ESCs for therapeutic benefits and could even solve the immune compatibility problem for male patients.

Other possible sources of adult stem cells with limited differentiation potential could be hair follicles<sup>144–147</sup>, adipose tissue<sup>148,149</sup>, teeth<sup>150–152</sup> or menstrual blood<sup>194</sup>.

Extraembryonic tissues could also be a source of multipotent stem cells. Umbilical-cord blood cells have been extensively researched and have been undergoing multiple clinical trials, mostly for haematological disorders. Moreover, stem cells with some characteristics of ESCs (such as the markers of pluripotency OCT4, stage-specific embryonic antigen 3 (SSEA3), SSEA4, TRA-1-60 and TRA-1-81) were isolated from cord blood<sup>153</sup>. Amniotic fluid stem cells<sup>154</sup> were shown to express some markers of pluripotency (OCT4, SSEA4), retain normal karyotype and long telomere for over 250 population doublings. Stem cells derived from amniotic membranes of term pregnancies were shown to have markers of pluripotency and differentiated *in vitro* into derivatives of the three germ layers<sup>155</sup>. Such alternative sources of pluripotent or multipotent stem cells could be attractive, if their derivatives show the same characteristics and functionality in animal models as derivatives of ESCs or, ultimately, as their *in vivo* counterparts.

However, most advances in this field still remain primarily a proof of principle, and a better understanding of heart formation pathways and development of efficient differentiation systems along with cell delivery methods is needed before this can provide an ESC-based course for the regeneration of the infarcted or failed heart.

**Neural lineage (ectoderm).** It is widely accepted that ESCs favour a neuronal fate by default in the absence of other instructive cues<sup>98,99</sup> (reviewed in REF. 100), and many neural progenitors and specific cell types have been isolated from ESCs and characterized<sup>5,65,99,101–105</sup> (reviewed in REF. 106). Most of these cells show the presence of neuronal markers such as tubulin III, Nestin, neural cell adhesion molecule (NCAM), tyrosine hydroxylase (TH), microtubule-associated protein 2 (MAP2) and glial fibrillary acidic protein (GFAP). Among neuronal lineage derivatives, dopamine neurons have been reliably derived from mouse and human ESCs by various groups<sup>71,104,107–111</sup> and were shown to improve the behaviour of Parkinsonian mice after transplantation into the midbrain<sup>112–116</sup>. Several studies also showed the *in vivo* functionality of motor neurons derived from ESCs<sup>117,118</sup> as well as oligodendrocytes and neural progenitors in animal models of spinal-cord injury (reviewed in REFS 119–122). The potential of neural progenitors derived from ESCs to repair brain injury due to ischaemia have also been reported<sup>120–125</sup>. In addition, others have demonstrated that ESC-derived neural progenitors can form functional neurons, which integrate synaptically into the host brain circuitry<sup>27</sup>.

A very specific cell type of neural lineage is retinal pigment epithelium (RPE), a highly specialized tissue of the eye that supports the function of the photoreceptor. RPE shares the same progenitor with the neural retina — neuroectoderm — and its specification is thought to be induced by the signals coming from surrounding extraocular tissue, in particular Activin A<sup>126</sup> (reviewed in REF. 127). Kawasaki and co-authors showed that co-culture of primate ESCs with stromal cells PA6 — that produce stromal (cell)-derived inducing activity (SDIA) and were previously used to generate dopamine neurons and astrocytes from ESCs<sup>105,128</sup> — also produced RPE. It is unclear what the critical factor of this SDIA cocktail is that induces this specification; especially as RPE can also be robustly generated from human ESCs without any need for co-culture. In our own experiments<sup>11,129</sup> more than 20 human ESC lines have currently produced RPE cells in over 100 independent experiments over the course of 6–8 weeks of differentiation. Judging from the rather stochastic differentiation of various lineages and cell types, cross-signalling from mesoderm-like cells could be involved in specification of the RPE neuroectodermal fate that is frequently found in differentiating ESC cultures. These cells expressed the RPE markers Bestrophin, cellular retinaldehyde-binding protein (CRALBP), retinal pigment epithelium-specific protein 65 kDa (RPE65), pigmented epithelium-derived factor (PEDF), and transdifferentiated into neural progenitor type cells in culture. They also re-established RPE phenotype upon reaching confluency, phagocytosed latex beads and showed a higher similarity to human RPE tissue than existing human RPE cell lines

by gene-expression profiling analysis<sup>129</sup>. Our group's recent animal studies show that these cells are capable of photoreceptor support in a model of RPE degeneration (dystrophic Royal College of Surgeons rats)<sup>11</sup>. Because of the ease and reliability of RPE generation, purification and expansion in culture, they may become one of the first ESC-based therapies of the future.

### Concluding thoughts

As stem-cell therapy begins to move from the laboratory to the clinic, it will be essential to learn how to generate a wide range of vital differentiated cell types under well-defined and reproducible conditions. This will require an understanding of the many developmental signals that cells use to organize into higher ordered structures, including blood vessels, bone and even entire organs such as kidneys or hearts. Techniques such as SCNT and cell reprogramming could potentially be used to minimize or eliminate the immune responses associated with the transplantation of these various tissues, and thus the requirement for immunosuppressive

drugs and/or immunomodulatory protocols that carry the risk of a wide range of serious and potentially life-threatening complications.

Although there is much hope for the clinical future of ESCs — with all the controversy and accompanying challenges — there are other stem-cell types that retain developmental capabilities that resemble those of ESCs that could become valuable therapeutic products. The adult body maintains populations of stem cells that participate in tissue maintenance and repair, which are governed by a combination of intrinsic regulatory mechanisms and extrinsic signals coming from the body's microenvironments. As well as circumventing the ethical issues with ESCs (BOX 2), immunogenicity is not as critical an issue with patient-derived adult stem cells, but there remain significant hurdles before their full clinical potential can be realized (BOX 3). Perhaps once we understand the molecular basis underlying pluripotency, we will discover how to coax them to differentiate with more versatility such that they approach the pluripotency of their embryonic counterparts.

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**Competing interests statement**  
The authors declare competing financial interests: see web version for details.

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