Chapter 13
Use of Combinatorial Screening to Discover Protocols That Effectively Direct the Differentiation of Stem Cells

Yen Choo

Abstract Embryonic stem cells (ESCs) have the rare ability to differentiate into all cell types that comprise the human adult, offering an unprecedented opportunity to perform developmental studies *in vitro* and promising unlimited supplies of somatic cells for numerous biomedical applications including transplantation medicine. Reliably controlling the differentiation of ESCs *in vitro* by conventional methods requires an understanding of complex developmental pathways, the availability of a series of phenotypic markers and involves technically demanding and time-consuming empirical determination of cell culture conditions. Directed differentiation of ESCs has thus proved a robust challenge and is likely to become the first major bottleneck in the stem cell field, particularly when the time comes to substitute costly recombinant growth factors by small molecule functional mimetics. This chapter briefly examines the benefits and shortcomings of various approaches currently used to differentiate stem cells, from mechanism-based rational approaches to the emerging systematic and higher throughput methods, and ultimately describes a novel 'directed evolution' method called 'Combinatorial Cell Culture' which has the potential to increase the throughput of directed differentiation experiments by orders of magnitude.

Keywords Stem Cell; Differentiation; Combinatorial; Screening

13.1 Introduction

A major driver of biomedical industry growth this century is predicted to be the availability of high quality stem cell-derived human tissue for which there is currently an increasing and unmet need.

Plasticell Ltd., Imperial Bioincubator, Bessemer Building (RSM), Prince Consort Road, South Kensington, London SW7 2BP, UK

Y. Shi, D.O. Clegg (eds.) *Stem Cell Research and Therapeutics*,

First, increased demand for organ transplantation from an aging population combined with a severe shortage of healthy organ donors, means that amongst those patients currently on hospital waiting lists only one in five will eventually receive treatment [1]. It is thought that stem-cell derived tissue could functionally substitute the whole organs currently used in transplantation and thus form the basis of numerous cell-replacement therapies to treat a remarkable variety of serious degenerative conditions.

Secondly, the pharmaceutical industry is clearly suffering from a lack of simple *in vitro* pharmacological models that can accurately predict the body's response to drug candidates. Over-dependence on unreliable animal models and costly human clinical trials, both of which often fail to detect ineffective or toxic compounds at a sufficiently early stage, has driven the average cost of developing a single drug almost to the billion dollar mark. The availability of quality controlled stem-cell derived human tissue for use in drug development would go a long way towards increasing efficiency and reducing the cost of medicines [2, 3].

13.2 Stem Cells

An increasing number of embryonic-, foetal- and adult- derived stem cell preparations and lines are readily available today through various cell banks and commercial suppliers. Stem cells constitute a virtually inexhaustible supply of raw material from which to derive the specific differentiated cell types required for current biomedical research and future cell replacement therapy. Pluripotent stem cells are of the widest applicability and by their nature the most challenging stem cell types to differentiate reliably. This chapter is thus focused on these cells and makes repeated reference to ESCs; but the reader should note that much of what is described is not limited to ESCs but also applies to induced pluripotent stem cells (iPS) [4], as well as foetal and adult stem cells.

13.2.1 Embryonic Stem Cells

ESC lines are indefinitely self-renewing and pluripotent [5–7]. Over 400 human ESC (hESC) lines from laboratories in more than 20 countries are available [8]. The International Stem Cell Initiative has characterised 59 of these lines [9], a subset of which are available from centralised cell banks (e.g. UK Stem Cell Bank; www.ukstemcellbank.org.uk) that ensure the lines are ethically derived and properly characterised using accredited quality control systems.

It is generally agreed that the immediate challenges in the stem cell field are to develop: (i) industrial scale culture of research and clinical grade undifferentiated stem cell lines [10] and (ii) effective, reproducible and economical differentiation protocols that generate the particular, specialised cell types required for a given application [11].

13.2.2 Scale Up of hESC Culture

While scale up of hESC culture currently presents a variety of technical challenges, these are expected to yield to near term advances in cell handling, laboratory automation and substrate and/or media formulation [12–16]. It is beyond the scope of this chapter to examine this topic in any detail, however it is worthwhile citing certain recent developments in passing. For example, original growth conditions for hESC required coculture with a feeder layer of mitotically inactivated mouse embryonic fibroblasts to maintain cell pluripotency and self-renewal [7]. Since use of feeders is cumbersome, there has been much progress in providing functional substitutes such as cell-free lysates or combinations of defined growth substrate and media formulations, and also in humanising the sources of these reagents for future clinical use [17–19]. Furthermore, hESC lines which previously required laborious manual mechanical dissection for efficient propagation can be passaged with automated protocols [20] or have been adapted for passage as enzymatically-dissociated single cell cultures [21], and compounds are emerging which are capable of increasing the survival of dissociated hESCs [22].

The handling of stem cells has been automated, increasing the ease of culture and also reproducibility. Robotic liquid handling systems adapted from machines originally designed to carry out cell-based drug screens on microtitre plates now support fully automated culture at least of mouse ESCs (mESCs). A typical reconfigured system such as Hamilton's CellHost consists of a liquid handling robot in a sterile housing flanked by tissue culture incubators which provide plates and media to the robot using transfer units [23].

Historically, many different cell lines besides hESC have been 'domesticated' through technical advances or via adaptation/selection while maintaining the essential characteristics that underpin their utility. Assuming researchers maintain the impressive rate of progress in improving culture conditions for hESC, it is reasonable to expect large-scale production of these cells to be available in the foreseeable future. Production of clinical grade hESC, which is more cumbersome owing to regulatory (and not merely practical) concerns, may take a little longer but is also anticipated [24, 25].

13.3 Stem Cell Differentiation

While large quantities of undifferentiated hESCs may soon be at hand, reliably directing their differentiation towards the mature cellular phenotypes found in adult tissues continues to be an extremely thorny problem, essentially because we know relatively little about the precise pathways involved in early human development and organogenesis (despite the power of comparative developmental biology) and even less about how to replicate the process *in vitro* using the hESC model.

13.3.1 Multilineage Differentiation of ESCs

hESCs spontaneously differentiate in the absence of a feeder layer (which provides surrogate pluripotency signals), when dissociated in the absence of optimised conditions or when overgrown in culture. Indeed a common and heretofore useful method of precipitating spontaneous differentiation is to culture ESCs as three-dimensional multicellular aggregates referred to as embryoid bodies [26]. In this system, spontaneous differentiation occurs mainly through a process of intercellular signalling akin to that in the early embryo: thus it cannot be placed under full experimental control. It generally gives rise to a large number of differentiated cell types (i.e. a large degree of cell heterogeneity) but yields relatively few if any cells of a given lineage of interest. This multilineage differentiation is of little practical use, other than as an indication that ESCs are indeed pluripotent: i.e. capable of differentiating into the various cell types found in the three major embryonic lineages (ectoderm, mesoderm and endoderm).

13.3.2 Directed Stem Cell Differentiation

Directing cell differentiation towards any one phenotype can be achieved through one of two generally complementary means: (i) by expression of transcription factor or other transgenes (genetic modification) and more importantly (ii) by manipulating the culture conditions (sometimes called epigenetic methods).

13.3.2.1 Directing ESC Differentiation by Expression of Transgenes

Examples of the genetic modification approach include the use of Hoxb4 in hematopoietic differentiation [27]; constitutive expression of Pax4 [28] or inducible expression of Pdx1 [29] in the differentiation of mESC to pancreatic islet-like cells; and the transient expression of Nurr1 [30] or a combination of Nurr1 and Pitx3 [31] in mESC differentiation to dopaminergic neurons. In these instances the transfected genes are transcription factors that provide instructive differentiation signals by either up- or down- regulating expression of developmental genes. Alternatively, this genetic modification approach can be used to increase the purity (but not necessarily the yield) of a differentiated cell type, for instance by enabling lineage-specific positive or negative selection – a strategy which is discussed further below.

Obvious drawbacks of genetic modification include the low efficiency of transfection (and therefore differentiation) of hESCs or partly differentiated developmental intermediates and the difficulty of obtaining or maintaining transgene expression at the correct level. Over and above these practical issues, genetic modification of stem cells for therapeutic applications introduces complicating regulatory and safety considerations which are well known in gene therapy; most obviously

the risk that transfected cells may have (or may acquire in future) some altered characteristics as a result of hosting the transgene [32].

Genetic modification is likely to be a valuable tool in directing differentiation of ESCs, though its full potential awaits better understanding of the gene networks which regulate various aspects of development [33] and improved methods of gene delivery, stable transfection and inducible expression in hESCs.

13.3.2.2 Directing ESC Differentiation by Culturing Under Specific Conditions

By far the most common and important method of directing the differentiation of ESCs is to manipulate the conditions under which they are cultured. Development involves a series of cell fate decisions that are largely controlled by a cascade of extrinsic signals and this process can be recapitulated *in vitro* through the use of appropriate instructive or selective cell culture conditions applied in the correct sequence, at the right time and for a suitable duration.

Many examples of ESC differentiation by this method are reviewed by Keller [11, 34] and Trounson [35] and include differentiation into lineages such as definitive endoderm [36], haematopoietic cells [37], cartilage [38], mesenchymal precursors [39], photoreceptors [40], cardiomyocytes [41–44] and skeletal muscle [45] to name but a few.

The most commonly manipulated extrinsic variables are the basal cell culture media and their supplements, in particular soluble signals such as morphogens, growth factors, hormones, organic compounds (e.g. nutrients, lipids, vitamins), synthetic small molecules (e.g. DMSO) and even ions (e.g. Li^+). Distinct cell types respond differently to these variations in the media, so while some cells in a culture interpret a given signal as instruction to differentiate, other cells may be induced to proliferate, become quiescent or die, leading to a selective enrichment of certain cell types in a population. With some imagination one might picture how consecutive waves of differentiation, phenotypic selection and proliferation of particular cell populations lead to the appearance of progressively specified cell lineages.

Another common variable in the control of stem cell behaviour is the extracellular microenvironment, in particular insoluble factors present in/on the growth substrate or on adjacent cells. The extracellular matrix (ECM), which is comprised of proteins such as the collagens, fibronectins and laminins, can directly affect cell signalling, attachment and activity. It is well known that stem cell differentiation can be affected by the composition of the culture substrate and the ECM, examples of which are the longstanding use of polylysine or laminin to support neural differentiation or conversely the use of matrigel in maintenance of hESC pluripotency [17]. Of particular interest in this field is the testing of combinatorial mixtures of ECM components [46] and the development of synthetic biomaterials [47, 48] that can be used to affect the differentiation of stem cells either alone or more likely in conjunction with soluble factors.

13.3.2.3 Examples of Directed Differentiation Through Cell Culture Under Specific Conditions

Unlike conventional cell culture, in which cells are usually grown in a single medium, directed differentiation of ESCs requires culture in a number of media, each of which has a different composition and must be applied in the correct series over a period of time.

Embryonic neural progenitors exhibit considerable developmental plasticity and neuronal fate depends on the timing and dose of different morphogens to which cells are exposed during development. This principle was applied by Jessell and colleagues to produce motor neurons from mESCs by differentiation through a pathway which recapitulated the physiological process, giving rise to the term 'directed-differentiation' [49]. mESCs were first neuralised by culturing embryoid bodies with stromal cell conditioned medium, after which these cells were caudalised by retinoic acid treatment and finally ventralised by Sonic hedgehog agonist treatment to produce the required motor neurons. Selective neuronal differentiation of hESCs was first shown by Thomson and by Reubinoff and colleagues [50, 51], and it was subsequently shown that the protocols devised by Jessell and coworkers to direct motor neuron differentiation could be adapted for use with human cultures [52].

The principles of directed differentiation have been extended to a number of other neuronal subtypes. In further examples of stepwise cell culture, a number of groups have adapted serial culture protocols for the derivation of midbrain dopaminergic neurons from mESCs [30] to achieve the same using hESCs [53–55]. Once again, regional specification is achieved by the sequential application of defined patterning molecules (such as FGF8 and Sonic hedgehog) that direct midbrain development *in vivo*. Dopamine neurons are heterogeneous in character and elicit different functions with distinct physiologies; in further studies it was shown that manipulation of *in vitro* neural patterning cues could selectively generate hypothalamic as opposed to midbrain DA neurons [56].

Examples of differentiation to other neuronal lineages include cerebellar neurons [57], neural crest cells with the potential for subsequent differentiation to sensory neurons, autonomic neurons, smooth muscle cells and glial cells [58–60]. Both mouse and human oligodendrocytes can also be derived from ESCs and in both cases have been shown to exhibit major restorative function by remyelinating damaged neurons in models of disease [61, 62].

Outside of the neural lineage a particularly clear example of serial cell culture to direct differentiation is the conversion of hESCs into hormone secreting cells similar to those found in the pancreas. D'Amour et al. devised a relatively effective protocol based on thorough knowledge of pancreatic organogenesis and the presence of a substantial body of literature describing the importance of such factors as activins, Wnt3a, BMPs, TGFs, FGFs, HGF, IGFs and others. These were assayed in various concentrations and combinations, in a background of different basal media with and without serum supplementation and at variable time intervals. Optimisation of the protocol was performed in a stepwise fashion and at each stage known markers of differentiation were assayed by RT-PCR and immunocytochemistry, to validate

progression of the cells through predetermined stages of lineage commitment. Eventually, a five-step method was devised in which hESCs undergo six media changes over a 2–3 week period! The protocol yielded a mixed population of cells, around 7% of which contained insulin and could release it in response to treatment with various secretagogues, but not in response to glucose challenge.

The above works are important milestones in stem cell research, not only as definitive validation of the serial cell culture approach to directed differentiation, but as clear illustration of how a rationale based on detailed understanding of lineage development is normally required in this process. However, these papers also demonstrate that despite profound knowledge of natural developmental processes there is no *a priori* method of deducing the conditions that will lead to the differentiation of one cell type into another *in vitro*. These must be discovered empirically, entailing lengthy and costly experimentation – research that unfortunately is all too often rewarded only by low efficiency differentiation into phenotypes which approximate but do not fully resemble the desired cell type.

13.4 General Strategies for Achieving Stem Cell Differentiation

Directed differentiation of ESCs is technically challenging and threatens to form a robust bottleneck in an otherwise rapidly advancing field. The key to this problem is to adopt novel, generally applicable methods that can accelerate progress by increasing experimental throughput whilst reducing cost and the well-known day-to-day variability and tedium that are burdensome hallmarks of cell culture.

13.4.1 Genetic Lineage Selection of Differentiated Cells

One of the earliest, generally applicable techniques for 'directing' stem cell differentiation was the use of selectable genetic markers to purify differentiated lineages. This approach requires genetic modification of ESCs to place an antibiotic resistance gene (or other marker, e.g. green fluorescent protein (GFP) [63]) under the control of a tissue-specific promoter. Following differentiation, whether spontaneous or directed, selective pressure is applied to yield cultures of differentiated cells that express the selectable marker. Field and colleagues described a mESC line stably transformed with a construct in which aminoglycoside phosphotransferase was driven by the α–cardiac myosin heavy chain promoter, enabling G418 selection of essentially pure cardiomyocytes [64]. An alternative strategy demonstrated by Smith and colleagues using the Sox2 gene [65], was to integrate antibiotic resistance directly into the gene locus by homologous recombination, thus bringing it under specific control of the endogenous promoter.

Genetic lineage selection is a widely applicable technique. Though it is dependent on knowing tissue specific markers (and their specific promoter elements which can be elusive or complex), these are not limited to cell-surface antigens as is the case for selection by e.g. immunopurification or fluorescence activated cell sorting (FACS), and moreover the selection step is facile and effective. An important point to note is that genetic selection does not solve the problem of directed differentiation *per se*, but rather is a purification technique that is applied post differentiation to ablate non- or wrongly-differentiated cells. If differentiation of ESCs into the desired lineage is highly efficient then it is possible to isolate relatively large numbers of target cells, however if this is not the case then the yield will be minimal. Thus, even for relatively 'easy-to-obtain' lineages such as cardiomyocytes [66] and neuronal precursors [67–70], lineage selection must be used in conjunction with serial cell culture methods that direct large numbers of ESCs towards defined cell types.

13.4.2 Guided Walks Along Differentiation Pathways

The great difficulty with directing ESC differentiation in general is that (unlike some of the better studied neural lineages) most of the 200 or so basic cell types in the human body arise through cellular intermediates bearing phenotypic markers which are largely unknown at present, or which are common and therefore shared between different lineages. Indeed, progress towards devising instructive culture conditions towards endodermal lineages such as pancreas was held up considerably while there were no unique markers capable of distinguishing visceral and definitive endoderm, only the latter of which ultimately gives rise to the pancreas, liver and other organs [71, 72]. Without such a cell type-specific marker, it was in turn impossible to devise assays for cell culture media capable of directing differentiation.

The lack of specific lineage markers for stem cell differentiation is a serious obstacle in the field, however if a series of highly specific markers does exist, such that a fairly informative developmental roadmap can be assembled, the biotechnologist can attempt a guided walk from marker to marker towards a defined endpoint (i.e. A $\rightarrow$ $\rightarrow$B $\rightarrow$ C $\rightarrow$ $\rightarrow$ $\rightarrow$ Z). Nevertheless, guessing the composition of the appropriate tissue culture medium for each step is still a complicated process that normally involves testing a multitude of factors in various combinations in order to arrive at an optimised formula. In principle, this process might be simplified (though not necessarily hastened) by screening the cells for the presence of cell surface growth factor receptors which signify the ability to respond to cognate soluble factors. An example of this approach was the RT-PCR screen of 5-day-old embryoid bodies comprising hESCs for expression of eight different growth factor receptors [73] and subsequent screening of the corresponding ligands to determine their effect on differentiation. The approach of screening for cellular receptors to home in on putative differentiating agents is potentially advantageous but the benefits may be outweighed by the fact that it is laborious and time consuming, and technically

dependent on having a sufficiently pure population of cells at hand. Since in practice cell culture homogeneity is greatly reduced as ESC culture is progressed towards a terminally differentiated fate, it may be necessary to purify marked cells in order to carry out receptor analysis [63]. Finally, in the example above, all eight receptor types screened were found to be present in embryoid bodies, thus the screen was not particularly instructive and the experiment might have been quicker and just as instructive if the factors had been tested blindly.

13.4.3 Systematic Screening of Differentiation Conditions

Systematic screens of differentiation conditions are possible using current methods but are extremely complex owing to the large number of interacting variables (e.g. timing of addition, concentration and combination of multiple factors).

The pharmaceutical industry has recently embraced cell-based screens, together with related automation such as the high content screening systems of Cellomics, Evotech, GE Healthcare, Becton Dickinson et al., that allow highly parallel differentiation assays and quantitative analysis of results. A few groups to date have exploited the pharmaceutical screening approach to search libraries of synthetic small molecules and natural products for their effect on the self-renewal and differentiation of ESCs [74]. Takahashi et al. [75] and Wu et al. [76] performed chemical screens using mESCs bearing reporters for cardiomyocytes and identified known and novel compounds (including some from chemical libraries produced by combinatorial synthesis) that increased the efficiency of differentiation to spontaneously beating cardiomyocytes. Conversely, high throughput chemical screening has been used to identify a compound that promotes mESC self renewal in serum- and feeder- free culture [77].

Systematic tests of interacting variables can be performed using 'factorial' experimental designs in which two or more factors, each with discrete levels (e.g. concentrations), are tested in all possible combinations and the outputs subjected to statistical analysis. An example of this approach is Chang and Zandstra's quantitative screen of mESC differentiation to endoderm in which an initial experiment involving five factors tested at two levels revealed the interactions between the various media supplements, and a further experiment in which two factors tested at three levels allowed limited testing of concentration effects [78].

The number of experiments required for complete factorial designs rapidly gets out of hand as the complexity of a process increases. The vast majority of these experiments are carried out in two levels (i.e. factor present or absent) and the number of variables that can be tested is limited even when using automated cell culture and screening systems. In practice, factorial experiments to determine conditions for stem cell differentiation are possible for individual steps in guided walks along differentiation pathways, but probably not for substantial leaps (i.e. A $\rightarrow \rightarrow$ F) nor the entire journey (i.e. A $\rightarrow \rightarrow \rightarrow$ Z) both of which would require massive screens over a prolonged period. Large screens are expensive (the cost of growth

factors alone is prohibitive) and the behaviour of biological cultures in the long-term often becomes unstable, making it difficult to correlate results from well to well. Thus factorial experiments are likely limited to testing individual steps in a differentiation pathway, for each of which a separate assay must be devised and validated (this is very frequently the most challenging part of the workflow). The screen itself must be repeated (typically three times on separate days) to obtain statistically relevant data and well content should be replicated and randomised on each plate to eliminate position-specific effects. Statistical analysis of results can also be prolonged depending on the complexity of the screen.

An important additional consideration is that each screen requires large numbers of quality-controlled input cells, which is a relatively simple matter during early stages of differentiation (i.e. A $\rightarrow$ B $\rightarrow$ C) but later becomes progressively harder. It is therefore no coincidence that differentiation factor screens have been performed predominantly on mESCs (which are easier to amplify than hESCs) and that the endpoints of these screens have been either ESC self-renewal or differentiation into readily obtained lineages.

In conclusion, conventional cell-based screening approaches, even if facilitated by robotic systems, are not easily adapted to the study of the directed differentiation of ESCs which is dependent on unusually large numbers of variables. The state of the art enables testing of a modest number of interacting variables per run, and is confounded by the stepwise aspect of the differentiation process that brings to play temporal variables such as the timing of addition of and duration of exposure to each medium.

13.5 'Directed Evolution' and Directed Differentiation

The remainder of this chapter is essentially concerned with the practical application of protein engineering principles to stem cell differentiation: these are seemingly disparate fields but in fact have interesting parallels. In ESC differentiation, cellular phenotypes with desirable functions (e.g. β-islet cell, dopaminergic neuron etc.) are created from a common building block (i.e. stem cell) that is changed through a combination of signals. In principle this process is highly analogous to protein engineering, in which protein phenotypes with desirable functions (e.g. antigen binding, DNA recognition or catalytic activity) are created from a common building block (e.g. antibody, zinc finger or enzyme motif) that is changed through a combination of mutations. Since the two disciplines are faced with precisely the same problem – namely finding effective combinations of variables that impinge on a biological process – they may also have solutions in common. This is a valuable insight because protein engineering is a mature field in which a number of practical approaches, ranging from rational design to random mutagenesis, have been tested. Interestingly, for all but the simplest cases of protein engineering it has emerged that the strategy of selecting or screening functional clones from a randomised

combinatorial library of variants is the method of choice both in terms of ease and efficacy. Examples include panning large phage display libraries for novel antibodies and zinc fingers, or screening libraries created by DNA shuffling for improved enzyme activities. These methods are commonly referred to as combinatorial biology or 'directed evolution' [79] approaches because like the natural evolutionary process they sample random permutations of variables (typically amino acids or polypeptides) followed by phenotypic screening in order to identify combinations that result in improved or novel function.

13.5.1 Features of Directed Evolution Techniques: Example of Phage Display

A number of directed evolution techniques have been developed for protein engineering, notably phage display [80] and more recently *in vitro* compartmentalisation [81]. These techniques have a certain rationale in common: (i) a naïve protein scaffold is randomly mutated to create a large library of variants; (ii) selection or screening is used to isolate those variants which have improved function; and (iii) there is a coding mechanism allowing sequence information to be extracted from the system (typically the protein is somehow linked to its coding gene).

Phage display is the best-known directed evolution technique and is commonly used to engineer highly specific binding proteins such as antibodies or zinc finger proteins. Protein variants are cloned as fusions to the coat protein of a bacteriophage and therefore exposed (or 'displayed') on the outer surface of phage. Combinatorial libraries encoding millions of random variants are created in which key amino acids or peptides are present in many, if not all, possible permutations. Phage displaying variants with high binding affinities for a ligand of interest are affinity purified on a column of immobilised ligand, and non binding phage washed off. The binding phage are subsequently eluted, amplified by infection of bacteria and used in further rounds of selection to enrich for the tightest binders. Finally the genomes of the phage that have survived this 'natural selection' are sequenced to deduce the amino acid sequence of the tightest binding protein.

One can only imagine what a protein engineer of the old school must have thought when originally presented with such nonsense! At first it seems counterintuitive that such a haphazard approach could be more effective than an orthodox rational design strategy. But in fact combinatorial biology is a highly effective protein engineering tool and by far the method of choice when large numbers of trial and error experiments need to be performed. Notably, the approach of engineering proteins using a purely rational or knowledge-guided strategy has consistently performed unfavourably relative to the comparatively random evolutionary approach described above.

Highly complex biological problems such as protein folding, intermolecular interactions and very probably embryonic development can be tackled using high throughput empirical experimentation and combinatorial selection techniques are

designed to test large numbers of interacting variables. Given the above, it is worthwhile exploring whether a 'directed evolution' or 'combinatorial selection' technique can be applied to a complex cellular engineering problem such as the directed differentiation of ESCs.

13.5.2 *A Combinatorial Screen for ESC Differentiation Conditions: Combinatorial Cell Culture*

Combinatorial Cell Culture is a technology which allows screening of multitude combinations of cell culture conditions to identify complex protocols that result in rare cell biological events [82].

A schematic overview of a simple Combinatorial Cell Culture experiment is provided in Fig. 13.1 (an animated version of which is available at http://www.plasticell.co.uk/technology_animated.php). In the first step of this process stem cells are seeded on specialized microscopic beads (microcarriers) to yield groups of 'cell units'. Using a 'split-pool' process, cell units are shuffled systematically through all pre-determined combinations of various differentiation conditions (e.g. media that contain growth factors, drugs etc.) and labeled with unique tags that attach to the

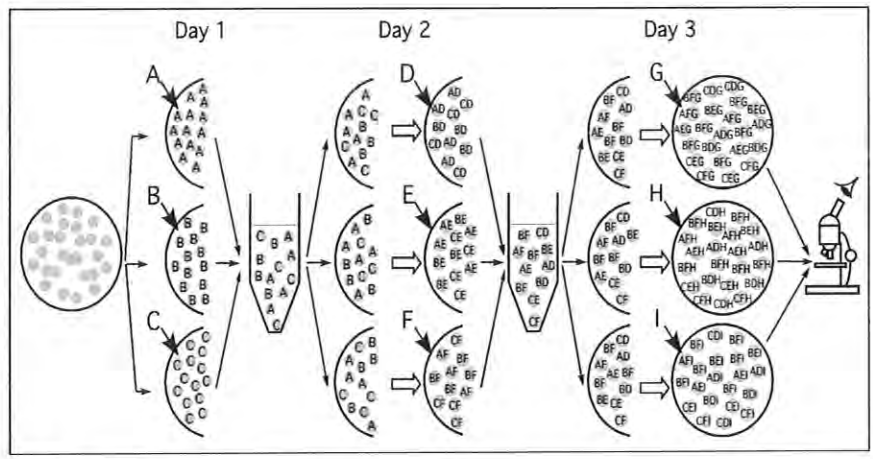

Fig. 13.1 Example of combinatorial cell culture. Cells are seeded on microcarrier beads (green). On day 1 these are split equally into three sets that are cultured under different conditions and the beads labeled using corresponding tags (A, B and C). On day 2 beads are mixed and re-partitioned into three sets that are cultured under different conditions and the beads again labeled using corresponding tags (D, E and F). On day 3 the procedure is repeated and further unique tags are added (G, H and I). At the end of the experiment, beads are screened for the desired cell type (shown as magenta) using an appropriate assay. The tags present on these 'positive' beads provide a record of the cell culture history (in this case tags C, D and H indicate cell culture in condition C on Day 1, D on Day 2 and H on Day 3) (Figure courtesy of J Green) (*See Color Plates*)

microcarrier substrate. Cell units that undergo directed differentiation as a result of fortuitous passage through appropriate conditions are later identified using a suitable cell-based screen. Finally, these are isolated and the associated tags are analysed to infer the cell culture history.

This method has been used to discover novel protocols for the directed differentiation of ESCs and very likely will find numerous other applications throughout cell biology. The various aspects of the Combinatorial Cell Culture workflow are discussed in more detail below.

13.5.2.1 Formation of Cell Units Through Microcarrier Cell Culture

Microcarriers are commonly used in basic research and industry for the scale up of adherent cell culture and in particular for commercial production of biological molecules and viruses in fermenters of up to 4,000 l [83]. Many systems have been developed in which carriers range in shape, size and porosity, and are manufactured using different processes and using a variety of materials including polystyrene, collagen and dextrans (see Table 13.1). In addition to solid carriers a number of porous systems featuring a network of open, interconnecting pores are available with greatly increased surface area for cell growth. These carriers are well characterised in terms of physical properties such as the specific gravity of the beads, the diameter and the surface area available for cell growth. Some of the carriers described and many besides are available as dried products that can be weighed accurately, and subsequently prepared by swelling in liquid medium.

Table 13.1 Examples of commercially available microcarriers for the formation of cell units

Microcarrier	Supplier	Size (μM)	Material
Cytodex 1	GE healthcare	180	Dextran
Cytodex 3	GE healthcare	175	Dextran and collagen
Cytopore1	GE healthcare	250	Cellulose
Cytopore2	GE healthcare	250	Cellulose
Cellagen	MP biomedicals	100–400	Bovine corium collagen
Fact102	Hyclone(SoloHill)	125–212	Polystyrene and porcine collagen
Cgen 102	Hyclone	125–212	Polystyrene and porcine collagen
ProF102L	Hyclone	125–212	Polystyrene and recombinant fibronectin
P102L	Hyclone	125–212	Polystyrene
Pplus 102	Hyclone	125–212	Polystyrene
Hlx 112	Hyclone	90–212μM	Polysteryne and trymethyl ammonium
Polybeads	Polysciences	100–600	Polystyrene
Spherobeads	Spherotech	90–249	Polystyrene
Cultispher G	Percell Biolytica	50–400	Porcine gelatin
Cultispher S	Percell Biolytica	50–400	Porcine gelatin

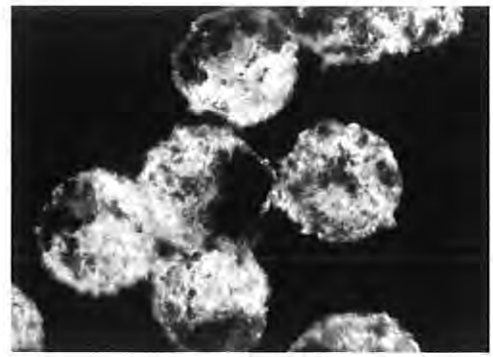

Fig. 13.2 Example of a cell unit.

Mouse or human ESCs seeded onto most of these microcarriers will readily form cell colonies on the surface of the carrier (Fig. 13.2). In general, healthy adherent cells are not readily dissociated from their growth substrate, and so the integrity of each cell unit largely persists despite mechanical manipulation of the microcarrier, agitation of the culture medium, or transfer of the carriers from one vessel to another.

Growing cells in integral units that are suspended in the liquid phase allows individual units to be removed at will and conveniently transferred to a different culture vessel. By successively transferring cell units through a set of different tissue culture media, all cells in the unit are exposed to the same series of culture conditions, in the same order and for the same period of time. Importantly, passaging of cells in different media can be achieved without perturbing any niche that has formed on the carrier and which may be important in the process of differentiation.

Since not all cells are adherent, it is worthwhile noting that cells may also be grouped into units by immurement, i.e. confined within a medium permeable barrier or by cell encapsulation in semi-solid polymer matrices comprised of e.g. gelatin, polylysine, alginate or agarose.

13.5.2.2 Split-Pool Microculture of Cells

Forming cell units is useful for sampling multiple tissue culture conditions as each cell unit constitutes an easily handled element that can be exposed to a variety of cell culture conditions. 'Split-pool microculture' is an efficient, systematic method for testing multiple combinations of cell culture conditions. In this process, large numbers of cell units are divided randomly (but equally) into a number of sets and grown separately for a given time under different culture conditions and in the presence of unique tags which label the cell units (see section 13.5.2.3). The segregated cell units are subsequently washed to remove excess media and tags, then pooled and mixed thoroughly, and once again split randomly into equal sets that are cultured under further conditions. This split-pool procedure can be repeated for any number of cycles. In many respects the principle of this procedure resembles that of split synthesis

of large combinatorial chemical libraries (known as combinatorial chemistry), which samples all possible combinations of chemical building block groups [84, 85].

Cell units subjected to this iterative split-pool process systematically sample all possible combinations of conditions in a predetermined experimental matrix (see Fig. 13.1). The number of different cell culture protocols sampled in an experiment where beads are split T times into N number of sets is equal to N^T (the 'complexity' of the experiment). However, this experiment can be carried out conveniently and cost effectively using only (N.T) number of different culture vessels and tag types. It is thus feasible to sample many thousands of combinations of conditions in a single run.

In theory, the split-pool procedure can be repeated over any number of rounds, and any number of conditions can be sampled at each round, so long as the number of cell units is greater than or equal to the number of different conditions sampled. In practice, the number of cell units used in the experiment should be approximately tenfold greater than the complexity of the experiment to compensate for losses of cell units in transit and to provide statistical confidence that each putative differentiation pathway has been sampled by at least one cell unit.

The practical complexity of a Combinatorial Cell Culture experiment is limited by the number of available tags, by the bead numbers (or culture volume) that can be conveniently handled and by the availability of starting cell material and/or the cost of reagents such as recombinant growth factors. In this latter respect the experimental schema is highly efficient because multiple cell units share a common vessel (i.e. are multiplexed) resulting in huge savings in reagents. The complexity of experiments that can be performed today is easily in reach of the hundred thousand range and with technical improvements will probably be in the millions.

The variables which can be sampled using this technique are not limited to the growth factors and morphogens present in different culture media but may include growth substrate (e.g. fibronectin on microcarrier), cell type, cell grouping (e.g. microcarrier culture, cell encapsulation, organotypic culture), duration of cell culture round, temperature, infection with viruses, addition of transgenes or antisense molecules, etc.

13.5.2.3 Use of Tags to Track Cell Culture History

Combinatorial Cell Culture necessarily involves mixing cell units, so to avoid confusing the chronology and the exact nature of the culture conditions to which any one cell unit has been exposed the process is performed in conjunction with labelling of the cell units using tags (Fig. 13.1).

A variety of tagging systems comprising distinctive chemical or non-chemical labels have been developed for use in combinatorial chemistry, but the vast majority of these are better suited to tagging chemical reactions rather than biological assays [86]. The ideal tag for Combinatorial Cell Culture should (i) exist in a large number of unique varieties ('flavours') which are easily distinguishable, preferably using an automated method; (ii) attach easily and permanently to cell units (and not transfer

Fig. 13.3 Tagging of cell units and isolation of tags for analysis.

or 'hop' between them); (iii) be physically stable, non-toxic and otherwise biologically inert; and (iv) be easily detected in a background of cells and biomolecules.

Cell unit substrates such as microcarriers can be derivatised or coated with substances that facilitate tagging and do not interfere with cell growth, and it may be possible to selectively elute tags from colonised beads or to strip off the cells from tagged beads using selective conditions to facilitate detection (Fig. 13.3).

There are two general strategies for tagging cell units. If a different tag is stably associated with (the growth substrate) of each cell unit at the beginning of the experiment and the tags are read following each split, this would provide for a log of the series of cell culture conditions to which every cell unit was exposed. Alternatively, if tags are sequentially associated with the cell units as they are exposed to each different culture condition, one could infer the movement of any cell unit at the end of the experiment by analyzing the associated tags. Both strategies have advantages and disadvantages. For example, the first allows the tags to be very stably associated with the growth substrate under harsh chemical conditions but obviously requires an enormous number of tag 'flavours' (one for each cell unit) and repeated reading of the tags after each split; the second method requires repeated tagging of cell units but uses a relatively smaller number of tag 'flavours' (one for each cell culture condition sampled).

In practice, the tagging strategy used in Combinatorial Cell Culture will likely depend on the state of the art of various alternative tagging technologies. For instance, an attractive tagging strategy would be to use radio-frequency identification (RFID), where electronic memory is used to record the history of a sample and where information is read remotely via radiofrequency transmission [87, 88]. RFID for Combinatorial Cell Culture ideally would comprise a very large number of tags each with a unique binary code, and cell unit tracking would be carried out remotely during the cell culture steps. However, currently available RFID technology is practically unsuitable for this application: tags are still relatively expensive and much bulkier than microcarriers, and there are problems reading large numbers of tags when these are in close proximity owing to mutual interference of signals.

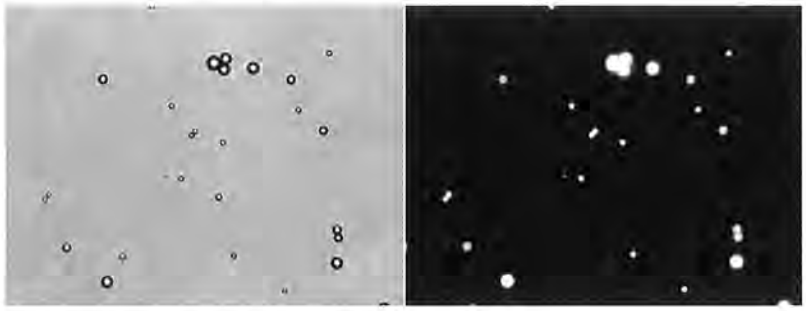

Fig. 13.4 Example of the analysis of coded microsphere tags. Fluorescent microsphere tags arrayed on a glass surface viewed using phase (left) and eplifluorescence (right) microscopy. The different tag flavours are coded by size (Ø 2–10 microns) and by loading with various concentrations of a given fluorophore

Alternatively, a number of optical or visual methods of tagging have been described [89], some of which are easily adapted for Combinatorial Cell Culture. In a number of these a pattern or bar code is encoded onto a substrate and recognised using pattern recognition technology [90–92], however most of these systems are not yet widely available and are often too complicated for large numbers of tags to be synthesised in the typical life science laboratory.

More common visual tagging systems comprise populations of monodisperse beads produced from a variety of polymeric materials and loaded or externally labeled with fluorescent organic dyes to produce distinct populations. Fluorescent microsphere arrays are in widespread use for multiplexed analysis of biomolecules [93] and such encoded beads can be easily adapted for the labeling of cell units. A well-known system is that produced by Luminex Corporation (Austin, TX) in which beads are loaded with different ratios of two dyes, producing an assortment of 100 distinguishable bead types. A further example is the QuantumPlex system (Bangs Laboratories, Fishers, IN) in which a single dye is loaded in microspheres of varying size and at different concentrations, producing a different type of array in which members are distinguished by bead size and fluorescence intensity (Fig. 13.4).

13.5.2.4 Screening of Differentiated Cell Units

Following each round of Combinatorial Cell Culture, or after a defined number of rounds, the cell units are assayed to determine whether there are members bearing large numbers of correctly differentiated cells.

This can be achieved by a variety of techniques, most obviously by detecting a marker product that is characteristic of the differentiated cell, such as a cell surface antigen that is recognised by a ligand or antibody (Fig. 13.5). Alternatively, stem cells can be engineered with an exogenous marker, such as GFP under the control

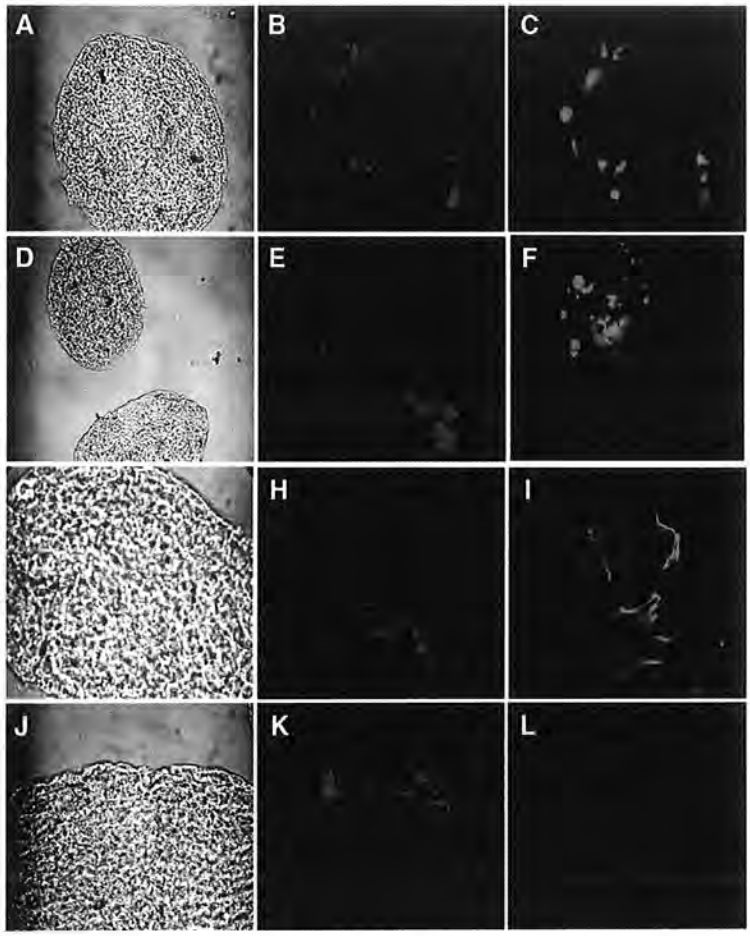

Fig. 13.5 Examples of phenotype detection using cell type-specific antibodies. Cultispher G gelatin microcarriers (Percell Biolytica) were seeded with mESCs and subjected to combinatorial cell culture to obtain neuronal lineages. Left panels show phase micrographs of the beads (under various magnifications) while the middle and right panels respectively show staining with DAPI (showing cell nuclei) and various neuron-specific primary antibodies. Panel C shows staining of dopaminergic neurons (using anti-tyrosine hydroxylase antibody); Panel F shows staining of GABA-ergic cells (anti-GABA antibody); Panel I shows astrocytes (anti-GFAP antibody). Panel L shows the secondary antibody control (*See Color Plates*)

of a cell type-specific promoter, to follow differentiation without the need to stain the cell units (Fig. 13.6). Additional advantages of integrated real time reporters are that they allow continuous monitoring of cell differentiation and unlike phenotypic screens, which are limited by the availability of a specific antibody, genetic markers can be applied to any differentially expressed gene. Reporter genes have been integrated into hESCs using phiC31 phage integrase to target genomic hotspots that

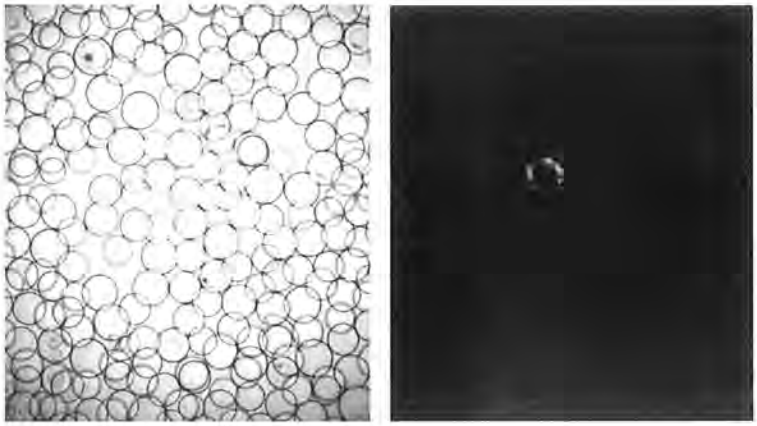

Fig. 13.6 Example of a cell unit screen to isolate a 'positive' bead. A population of Cytodex 3 microcarriers (GE Healthcare) viewed using phase (left) and epifluorescence (right) microscopy reveals a unit bearing cells that express GFP. The 'positive' bead can be isolated manually using a pipette, or using an automated bead sorter (see text) (*See Color Plates*)

support gene expression before and after cell differentiation [94] and it is likely that hESC lines modified with such reporters will become commonplace. Finally, it is possible to carry out functional screening of cell units, for instance to detect a characteristic enzyme activity (e.g. cytochrome P450 activity in hepatocytes) or other cell-specific function (e.g. phagocytosis by macrophages).

Cell units displaying large numbers of correctly differentiated cells can be isolated by a variety of techniques, but most straightforwardly by manual sorting of the cell units (under a microscope) using a pipette. Automated sorting is also possible, for instance by techniques similar to fluorescence activated bead sorting but conventional fluorescence activated cell sorting (FACS) instruments are not normally suitable owing to the large size of microcarriers. The COPAS™ instrument (Union Biometrica), devised for sorting live samples (typically embryos or small model organisms) that are too large for conventional cytometers, is capable of sorting objects including beads up to 1 mm in size based on size and fluorescence parameters.

13.5.2.5 Determining Cell Culture History of Cell Units

The absolute number of cell units to be processed for analysis depends firstly on the frequency of 'hits' and also on the level of background that can result in a certain proportion of false positives (i.e. beads which score positive in the screen but the cells have not acquired the phenotype as a result of directed differentiation). Besides false positives which occur as a result of screening artifacts, false positives may arise as a result of the interaction (signaling) of two separate cell units during

any of the experimental steps, or as a result of spontaneous differentiation of cell units that pass through a series of culture conditions that are permissive for differentiation but do not necessarily reliably direct differentiation. Both these types of differentiation are difficult to reproduce. Such false positives occur more frequently when: (i) the target cell type lies in an 'easy-to-obtain' lineage; (ii) the experimental matrix is comprised predominantly of conditions which result in differentiation towards the lineage of interest; or (iii) when the media contain a general inducer of differentiation such as serum. The implication is that Combinatorial Cell Culture is practically more useful for determining conditions that lead to extremely rare events. In these cases there will presumably be a small number of 'hits' and many fewer if any false positives.

The precise method of determining the cell culture history of a cell unit depends largely on the type of tags used in the Combinatorial Cell Culture experiment, for instance whether the tags are read during or after the experiment. Our lab generally uses either microsphere or optically-encoded, nanomaterial tags, both of which are processed at the end of an experiment in a rather similar manner. Both tags attach to the microcarrier substrate generally within the pores and so must be recovered by total digestion of the cell unit (Fig. 13.3). Following digestion, tags become arrayed on a glass surface and are imaged *in situ* using an epifluorescence microscope equipped with appropriate filter sets (Fig. 13.4) or alternatively using FACS instruments. It is often not necessary to image and categorise all tags from a cell unit in order to be able to deduce the cell culture history, however it can be the case (depending on the efficiency of tagging) that certain tagging data are scarce or even absent, or that there are excess flavours of tags present (indicating tag 'hopping' between units) and in this case fuller analysis of tagging may be required. An automated image acquisition system is desirable and image recognition software is almost certainly absolutely necessary to classify the different tags.

13.5.2.6 Validation of Pathway and Cell Phenotype

Following tag analysis and determination of the cell culture history it is necessary to repeat and validate the putative differentiation protocols in order to quantify and rank the efficiency of each and eliminate irreproducible 'false positives'. Individual protocols are typically assayed by exposing a few thousand cell units to a single series of media – these conditions are not exactly equivalent to those which originally gave rise to the 'hit' since cell units in the two different experiments will almost certainly secrete different types and quantities of growth factors which condition the culture media and therefore influence cell growth and differentiation. Nevertheless this type of experiment, followed by detailed phenotypic analysis of the target cell type, will indicate whether the protocol is reproducible, providing a starting point for further development and optimisation.

Finally, an advantage of using microcarriers in Combinatorial Cell Culture is that the protocol may be adapted for the production of large quantities of differentiated tissue through straightforward scale up of the microculture.

Acknowledgements I would like to gratefully acknowledge John Girdlestone, Nadire Ali, Fraser Hornby, Ying Chen, Marina Tarunina, Bettina Huhse and Chris Johnson who at various times (in the order in which they are listed) contributed to the development of Combinatorial Cell Culture methods at Plasticell Ltd., and (in alphabetical order) Chris Adam, Nick Allen, Hugh Cochrane, Martin Evans, Danny Green, Andrew Griffiths and Aaron Klug for their help and support in this project. I am thankful to N Allen, BH, TM and CJ for their comments and suggestions of improvements to the text and to CJ for help in preparing the manuscript.

References

1. Scrip's Complete Guide to Transplantation (1998) PJB Publications, New York, USA
2. McNeish J (2004) Embryonic stem cells in drug discovery. Nat Rev Drug Discov 3:70–80
3. Pouton CW, Haynes JM (2007) Embryonic stem cells as a source of models for drug discovery. Nat Rev Drug Discov 6:605–616
4. Takahashi K, Tanabe K, Ohnuki M, et al. (2007) Induction of pluripotent stem cells from adult human fibroblasts by defined factors. Cell 131:861–872
5. Evans MJ, Kaufman MH (1981) Establishment in culture of pluripotential cells from mouse embryos. Nature 292:154–156
6. Martin GR (1981) Isolation of a pluripotent cell line from early mouse embryos cultured in medium conditioned by teratocarcinoma stem cells. Proc Natl Acad Sci U S A 78:7634–7638
7. Thomson JA, Itskovitz-Eldor J, Shapiro SS, et al. (1998) Embryonic stem cell lines derived from human blastocysts. Science 282:1145–1147
8. Guhr A, Kurtz A, Friedgen K, et al. (2006) Current state of human embryonic stem cell research: an overview of cell lines and their use in experimental work. Stem Cells 24:2187–2191
9. Adewumi O, Aflatoonian B, Ahrlund-Richter L, et al. (2007) Characterization of human embryonic stem cell lines by the International Stem Cell Initiative. Nat Biotechnol 25:803–816
10. Thomson H (2007) Bioprocessing of embryonic stem cells for drug discovery. Trends Biotechnol 25:224–230
11. Keller G (2005) Embryonic stem cell differentiation: emergence of a new era in biology and medicine. Genes Dev 19:1129–1155
12. Hewitt ZA, Amps KJ, Moore HD (2007) Derivation of GMP raw materials for use in regenerative medicine: hESC-based therapies, progress toward clinical application. Clin Pharmacol Ther 82(4):448–452
13. Fletcher JM, Ferrier PM, Gardner JO, et al. (2006) Variations in humanized and defined culture conditions supporting derivation of new human embryonic stem cell lines. Cloning Stem Cells 8:319–334
14. Mallon BS, Park KY, Chen KG, et al. (2006) Toward xeno-free culture of human embryonic stem cells. Int J Biochem Cell Biol 38:1063–1075
15. Terstegge S, Laufenberg I, Pochert J, et al. (2007) Automated maintenance of embryonic stem cell cultures. Biotechnol Bioeng 96:195–201
16. Thomson H (2007) Bioprocessing of embryonic stem cells for drug discovery. Trends Biotechnol 25:224–230
17. Xu C, Inokuma MS, Denham J, et al. (2001) Feeder-free growth of undifferentiated human embryonic stem cells. Nat Biotechnol 19:971–974
18. Stacey GN, Cobo F, Nieto A, et al. (2006) The development of 'feeder' cells for the preparation of clinical grade hESC lines: challenges and solutions. J Biotechnol 125:583–588
19. Mallon BS, Park K-Y, Chen KG, et al. (2006) Toward xeno-free culture of human embryonic stem cells. Int J Biochem Cell Biol 38:1063–1075
20. Joannides A, Fiore-Heriche C, Westmore K, et al. (2006) Automated mechanical passaging: a novel and efficient method for human embryonic stem cell expansion. Stem Cells 24:230–235

21. Ellerstrom C, Strehl R, Noaksson K, et al. (2007) Facilitated expansion of human embryonic stem cells by single-cell enzymatic dissociation. Stem Cells 25:1690–1696
22. Watanabe K, Ueno M, Kamiya D, et al. (2007) A ROCK inhibitor permits survival of dissociated human embryonic stem cells. Nat Biotechnol 25:681–686
23. Terstegge S, Pochert J, Brustle O (2004) Hamilton's new cellhost system for full automation of embryonic stem cell cultures. Nat Meth 1:271–272
24. Hewitt ZA, Amps KJ, Moore HD (2007) Derivation of GMP raw materials for use in regenerative medicine: hESC-based therapies, progress toward clinical application. Clin Pharmacol Ther 82:448–452
25. Rao M (2008) Scalable human ES culture for therapeutic use: propagation, differentiation, genetic modification and regulatory issues. Gene Ther 15(2):82–88
26. Kurosawa H (2007) Methods for inducing embryoid body formation: *in vitro* differentiation system of embryonic stem cells. J Biosci Bioeng 103:389–398
27. Rideout WM, 3rd, Eggan K, Jaenisch R (2001) Nuclear cloning and epigenetic reprogramming of the genome. Science 293:1093–1098
28. Blyszczuk P, Czyz J, Kania G, et al. (2003) Expression of Pax4 in embryonic stem cells promotes differentiation of nestin-positive progenitor and insulin-producing cells. Proc Natl Acad Sci U S A 100:998–1003
29. Miyazaki S, Yamato E, Miyazaki J-i (2004) Regulated expression of pdx-1 promotes *in vitro* differentiation of insulin-producing cells from embryonic stem cells. Diabetes 53:1030–1037
30. Kim J-H, Auerbach JM, Rodriguez-Gomez JA, et al. (2002) Dopamine neurons derived from embryonic stem cells function in an animal model of Parkinson's disease. Nature 418:50–56
31. Martinat C, Bacci J-J, Leete T, et al. (2006) Cooperative transcription activation by Nurr1 and Pitx3 induces embryonic stem cell maturation to the midbrain dopamine neuron phenotype. Proc Natl Acad Sci U S A 103:2874–2879
32. Woods NB, Bottero V, Schmidt M, et al. (2006) Gene therapy: therapeutic gene causing lymphoma. Nature 440:1123
33. de-Leon SB-T, Davidson EH (2007) Gene regulation: gene control network in development. Annu Rev Biophys and Biomol Struct 36:191–212
34. Keller GM (1995) *In vitro* differentiation of embryonic stem cells. Curr Opin Cell Biol 7:862–869
35. Trounson A (2006) The production and directed differentiation of human embryonic stem cells. Endocr Rev 27:208–219
36. D'Amour KA, Agulnick AD, Eliazer S, et al. (2005) Efficient differentiation of human embryonic stem cells to definitive endoderm. Nat Biotechnol 23:1534–1541
37. Ma F, Wang D, Hanada S, et al. (2007) Novel method for efficient production of multipotential hematopoietic progenitors from human embryonic stem cells. Int J Hematol 85:371–379
38. Koay EJ, Hoben GM, Athanasiou KA (2007) Tissue engineering with chondrogenically differentiated human embryonic stem cells. Stem Cells 25:2183–2190
39. Barberi T, Willis LM, Socci ND, et al. (2005) Derivation of multipotent mesenchymal precursors from human embryonic stem cells. PLoS Med 2:e161
40. Lamba DA, Karl MO, Ware CB, et al. (2006) Efficient generation of retinal progenitor cells from human embryonic stem cells. Proc Natl Acad Sci U S A 103:12769–12774
41. Xu C, He JQ, Kamp TJ, et al. (2006) Human embryonic stem cell-derived cardiomyocytes can be maintained in defined medium without serum. Stem Cells Dev 15:931–941
42. Xu C, Police S, Rao N, et al. (2002) Characterization and enrichment of cardiomyocytes derived from human embryonic stem cells. Circ Res 91:501–508
43. Laflamme MA, Chen KY, Naumova AV, et al. (2007) Cardiomyocytes derived from human embryonic stem cells in pro-survival factors enhance function of infarcted rat hearts. Nat Biotechnol 25:1015–1024
44. Leor J, Gerecht S, Cohen S, et al. (2007) Human embryonic stem cell transplantation to repair the infarcted myocardium. Heart 93:1278–1284
45. Barberi T, Bradbury M, Dincer Z, et al. (2007) Derivation of engraftable skeletal myoblasts from human embryonic stem cells. Nat Med 13:642–648

46. Flaim CJ, Chien S, Bhatia SN (2005) An extracellular matrix microarray for probing cellular differentiation. Nat Methods 2:119–125
47. Anderson DG, Levenberg S, Langer R (2004) Nanoliter-scale synthesis of arrayed biomaterials and application to human embryonic stem cells. Nat Biotechnol 22:863–866
48. Mei Y, Goldberg M, Anderson D (2007) The development of high-throughput screening approaches for stem cell engineering. Curr Opin Chem Biol 11:388–393
49. Wichterle H, Lieberam I, Porter JA, et al. (2002) Directed differentiation of embryonic stem cells into motor neurons. Cell 110:385–397
50. Reubinoff BE, Itsykson P, Turetsky T, et al. (2001) Neural progenitors from human embryonic stem cells. Nat Biotechnol 19:1134–1140
51. Zhang SC, Wernig M, Duncan ID, et al. (2001) *In vitro* differentiation of transplantable neural precursors from human embryonic stem cells. Nat Biotechnol 19:1129–1133
52. Li XJ, Du ZW, Zarnowska ED, et al. (2005) Specification of motoneurons from human embryonic stem cells. Nat Biotechnol 23:215–221
53. Perrier AL, Tabar V, Barberi T, et al. (2004) From the Cover: derivation of midbrain dopamine neurons from human embryonic stem cells. Proc Natl Acad Sci U S A 101:12543–12548
54. Yan Y, Yang D, Zarnowska ED, et al. (2005) Directed differentiation of dopaminergic neuronal subtypes from human embryonic stem cells. Stem Cells 23:781–790
55. Roy NS, Cleren C, Singh SK, et al. (2006) Functional engraftment of human ESC-derived dopaminergic neurons enriched by coculture with telomerase-immortalized midbrain astrocytes. Nat Med 12:1259–1268
56. Ohyama K, Ellis P, Kimura S, et al. (2005) Directed differentiation of neural cells to hypothalamic dopaminergic neurons. Development 132:5185–5197
57. Salero E, Hatten ME (2007) Differentiation of ESCs into cerebellar neurons. Proc Natl Acad Sci U S A 104:2997–3002
58. Mizuseki K, Sakamoto T, Watanabe K, et al. (2003) Generation of neural crest-derived peripheral neurons and floor plate cells from mouse and primate embryonic stem cells. Proc Natl Acad Sci U S A 100:5828–5833
59. Rathjen J, Haines BP, Hudson KM, et al. (2002) Directed differentiation of pluripotent cells to neural lineages: homogeneous formation and differentiation of a neurectoderm population. Development 129:2649–2661
60. Motohashi T, Aoki H, Chiba K, et al. (2007) Multipotent cell fate of neural crest-like cells derived from embryonic stem cells. Stem Cells 25:402–410
61. Keirstead HS, Nistor G, Bernal G, et al. (2005) Human embryonic stem cell-derived oligodendrocyte progenitor cell transplants remyelinate and restore locomotion after spinal cord injury. J Neurosci 25:4694–4705
62. Brustle O, Jones KN, Learish RD, et al. (1999) Embryonic stem cell-derived glial precursors: a source of myelinating transplants. Science 285:754–756
63. Aubert J, Stavridis MP, Tweedie S, et al. (2003) Screening for mammalian neural genes via fluorescence-activated cell sorter purification of neural precursors from Sox1-gfp knock-in mice. Proc Natl Acad Sci U S A 100:11836–11841
64. Klug MG, Soonpaa MH, Koh GY, et al. (1996) Genetically selected cardiomyocytes from differentiating embronic stem cells form stable intracardiac grafts. J Clin Invest 98:216–224
65. Li M, Pevny L, Lovell-Badge R, et al. (1998) Generation of purified neural precursors from embryonic stem cells by lineage selection. Curr Biol 8:971–974
66. Laflamme MA, Chen KY, Naumova AV, et al. (2007) Cardiomyocytes derived from human embryonic stem cells in pro-survival factors enhance function of infarcted rat hearts. Nat Biotechnol 25:1015–1024
67. Joannides AJ, Fiore-Heriche C, Battersby AA, et al. (2007) A scaleable and defined system for generating neural stem cells from human embryonic stem cells. Stem Cells 25:731–737
68. Bouhon IA, Kato H, Chandran S, et al. (2005) Neural differentiation of mouse embryonic stem cells in chemically defined medium. Brain Res Bull 68:62–75
69. Watanabe K, Kamiya D, Nishiyama A, et al. (2005) Directed differentiation of telencephalic precursors from embryonic stem cells. Nat Neurosci 8:288–296

70. Conti L, Pollard SM, Gorba T, et al. (2005) Niche-independent symmetrical self-renewal of a mammalian tissue stem cell. PLoS Biol 3:e283
71. Yasunaga M, Tada S, Torikai-Nishikawa S, et al. (2005) Induction and monitoring of definitive and visceral endoderm differentiation of mouse ESCs. Nat Biotechnol 23:1542–1550
72. D'Amour KA, Agulnick AD, Eliazer S, et al. (2005) Efficient differentiation of human embryonic stem cells to definitive endoderm. Nat Biotechnol 23:1534–1541
73. Itskovitz-Eldor J, Schuldiner M, Karsenti D, et al. (2000) Differentiation of human embryonic stem cells into embryoid bodies comprising the three embryonic germ layers. Mol Med 6:88–95
74. Chen S, Hilcove S, Ding S (2006) Exploring stem cell biology with small molecules. Mol Biosyst 2:18–24
75. Takahashi T, Lord B, Schulze PC, et al. (2003) Ascorbic acid enhances differentiation of embryonic stem cells into cardiac myocytes. Circulation 107:1912–1916
76. Wu X, Ding S, Ding Q, et al. (2004) Small molecules that induce cardiomyogenesis in embryonic stem cells. J Am Chem Soc 126:1590–1591
77. Chen S, Do JT, Zhang Q, et al. (2006) Self-renewal of embryonic stem cells by a small molecule. Proc Natl Acad Sci 103:17266–17271
78. Karen H. Chang, Peter W. Zandstra (2004) Quantitative screening of embryonic stem cell differentiation: endoderm formation as a model. Biotechnol Bioeng 88:287–298
79. Arnold FH (1998) Design by directed evolution. Acc Chem Res 31:125–131
80. Smith GP (1985) Filamentous fusion phage: novel expression vectors that display cloned antigens on the virion surface. Science 228:1315–1317
81. Tawfik DS, Griffiths AD (1998) Man-made cell-like compartments for molecular evolution. Nat Biotechnol 16:652–656
82. Choo Y (2002) Cell Culture. GB0222846.8, 3 Oct 2002
83. GE (http://www6.gelifesciences.com/aptrix/upp00919.nsf/Content/WD:Microcarrier + ce(148896581-B345)) Microcarrier cell culture - principles and methods.
84. Nicolaou KCE, Hanko RE, Hartwig WE (2002) Handbook of Combinatorial Chemistry: Drugs, Catalysts, Materials. Wiley-VCH, Weinheim, Germany
85. Terrett NK (1998) Combinatorial Chemistry (Oxford Chemistry Masters). Oxford University Press
86. Braeckmans K, De Smedt SC, Leblans M, et al. (2002) Encoding microcarriers: present and future technologies. Nat Rev Drug Discov 1:447–456
87. Walton CA (1983) Portable radio frequency emitting identifier. US Patent 4,384,288, 17 May 1983
88. Cardullo MW, Parks WL (1973) Transponder apparatus and system. US Patent 3,713,148, 23 Jan 1973
89. Finkel NH, Lou XH, Wang CY, et al. (2004) Barcoding the microworld. Anal Chem 76:353A–359A
90. Nicewarner-Pena SR, Freeman RG, Reiss BD, et al. (2001) Submicrometer metallic barcodes. Science 294:137–141
91. Gudiksen MS, Lauhon LJ, Wang J, et al. (2002) Growth of nanowire superlattice structures for nanoscale photonics and electronics. Nature 415:617–620
92. Matthias S, Schilling J, Nielsch K, et al. (2002) Monodisperse diameter-modulated gold microwires. Adv Mater 14:1618–1621
93. Probst MCO, Rothe G, Schmitz G (2003) Bead-based multiplex analysis. Bead-basierte multiplexanalyse. Laboratoriums Medizin 27:182–187
94. Thyagarajan B, Liu Y, Shin S, et al. (2008) Creation of engineered human embryonic stem cell lines using phiC31 integrase. Stem Cells 26:119–126