STEM CELLS — OPINION

Embryonic stem-cell culture as a tool for developmental cell biology

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Abstract | The cell biology of the early processes of mammalian embryogenesis, such as germ-layer formation, has been technically challenging to study owing to the size and accessibility of mammalian embryos. Embryonic stem cells, which can generate the three germ layers *in vitro*, are useful for studying embryogenesis at the cellular level. So, how can the study of embryonic stem cells and their differentiation provide a deeper understanding of the cell biology of early development?

All developmental biologists are well aware that embryogenesis is the integrated sum of the changes that occur in individual cells of the embryo. These changes can be examined through the development of various technologies that allow the *in vivo* and *ex vivo* imaging of the behaviour of cells during mammalian embryogenesis[1]. However, there are at least two basic problems that are difficult to address using *in vivo* imaging. First, although it is possible to observe cell behaviour *in vivo* for some model organisms, visualization of individual cells during mammalian embryogenesis has been difficult because of the problem of accessibility. Short-term tracking of cells in post-implantation embryos has been reported[2], but it is still difficult to obtain live imaging of cells during mammalian embryogenesis in whole embryo culture. The second problem is theoretical. Multiple molecules that are localized in the embryonic microenvironment regulate different cell fates. Therefore, although it is possible to define the function of individual molecules, it is not easy to determine the 'sufficient' conditions for a given process by *in vivo* analysis.

Embryonic stem (ES) cells are derived from the inner cell mass (ICM) of blastocysts (embryos that are ~3.5 days post coitum)[3,4]. The ICM of blastocysts contains all of the cells that will give rise to the embryo itself and the primitive endoderm (see Glossary). In the embryo, ICM cells partially proliferate, but all of them differentiate and lose their pluripotency within a short period of time. However, in specific culture conditions, ICM cells give rise to ES cells, which can be maintained indefinitely in an undifferentiated state without losing their pluripotency (FIG. 1). Although ES cells are clearly not identical to ICM cells, they maintain the ability to undergo differentiation to all cell lineages, and when injected into blastocysts they behave in the same manner as the host ICM cells and participate in embryonic development.

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Because ES cells maintain the ability to undergo differentiation to all cell lineages, they can provide a universal cell source for the study of the cell biology of embryogenesis. This is particularly important for the study of human development[5] because the availability of normal cells from human embryos is extremely limited. The potential to produce unlimited numbers of cells in culture is another advantage of ES cells. Finally, ES cells can easily be genetically modified, allowing the creation of lines that bear specific markers and providing a means of interfering with genetic functions.

The aim of this article is to highlight current and future strategies for the exploitation of ES cells in developmental cell biology, and to discuss the unique set of opportunities and problems presented by *in vitro* differentiation of ES cells.

ES cells and pluripotency

ES-cell culture has shown its great potential in cell biology studies of pluripotency and self-renewal[6,7] — the two characteristic features of ES cells. It is perhaps surprising that ES-cell lines were established despite the fact that self-renewal of ICM cells is limited *in vivo*. Nonetheless, our understanding of the molecular mechanisms that control the choice between self-renewal and differentiation have been greatly advanced by recent studies of ES cells.

Transcriptional and epigenetic control of pluripotency. Three transcription factors — OCT3/4, NANOG and SOX2 — were suggested to form a core network that controls the maintenance of pluripotency of mouse ES cells[8–11], albeit in distinct pathways. The evidence indicates that one role of these factors is to counteract molecules that are involved in the differentiation programme. In fact, OCT3/4 and SOX2 form a complex that suppresses the expression of *Cdx2*, which directs differentiation to the primitive endoderm[7,12]. NANOG can inhibit the differentiation of primitive endoderm[9] and mesoderm[13] by influencing the expression of *Gata6* and brachyury, respectively. By some unknown mechanisms, NANOG can also inhibit differentiation to the neural lineage[14]. So, suppression of differentiation programmes is an essential requirement for maintenance of ES-cell pluripotency.

Although the molecular basis of pluripotency has been studied mostly using mouse ES cells, it seems likely that these three core transcription factors are also responsible for pluripotency in human ES cells. In fact, recent high-throughput studies of target genes to which OCT3/4 and NANOG bind in both mouse and human ES cells suggest that many of the important

downstream genes are regulated by common mechanisms in the two species[15,16].

Recent data also suggest the existence of an ES-specific epigenetic state that might be important for the maintenance of pluripotency[17–19]. This state was described as a bivalent state because both active (methylated Lys4 of histone H3) and suppressive (methylated Lys27 of histone H3) chromatin modifications appear to coexist in genes that are required for lineage-specific differentiation. This bivalent state is thought to resolve to either an active or a suppressive state during differentiation. A set of Polycomb molecules have also been implicated as global gene silencers that repress the differentiation programme to maintain pluripotency [20,21].

Proliferation of ES cells. Sustained expression of several proto-oncogenes has been implicated in the proliferation of ES cells. This is interesting because teratoma formation is an important property of ES cells. Yamanaka's group demonstrated that a *Ras* family gene, ES-cell-expressed *Ras* (*ERas*), is involved in self-renewal and teratoma formation of ES cells[22], probably through the activation of the phosphatidylinositol 3-kinase (PI3K) pathway[23]. Cartwright *et al.* reported that the proto-oncogene *c-Myc* is downstream of the leukaemia inhibitory factor (LIF)–signal transducer and activator of transcription-3 (STAT3) pathway, which is required for ES-cell maintenance[24]. Finally, *b-Myb* is another oncogene that has been implicated in the self-renewal of ES cells[25]. How the expression of these proto-oncogenes is sustained in ES cells, to what extent they are involved in self-renewal of ES cells, and how ES cells are protected from senescence are by no means fully understood yet. The answers to these questions will contribute towards understanding the derivation of ES cells.

Cell biology of ES-cell differentiation

As described above, ES cells have enormous potential for the study of embryogenesis at the cell level. Differentiation of ES cells into the three germ layers is attained through the use of specific culture conditions: embryoid-body (EB) formation[26]; cultures on feeder cells[27]; and cultures on matrices[28]. Currently, the most popular protocol is to make an embryo-like structure known as the EB, in which most cell lineages differentiate spontaneously. The basic idea behind the EB method is to mimic the process of embryonic development. By contrast, cultures on feeder cells or matrices aim to induce guided differentiation to a limited number of cell types (FIG. 2).

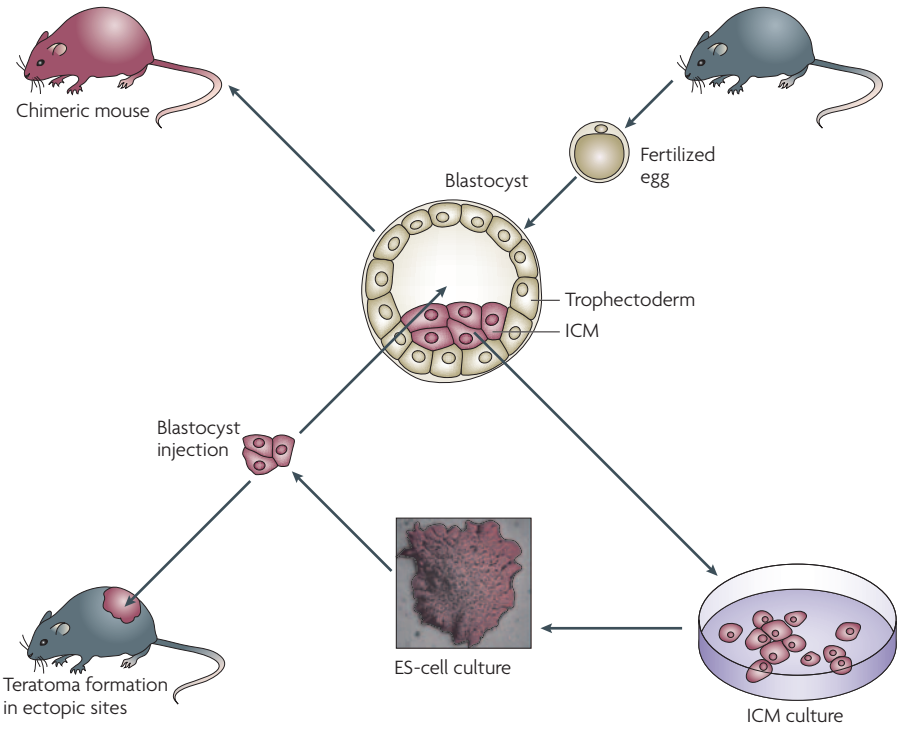


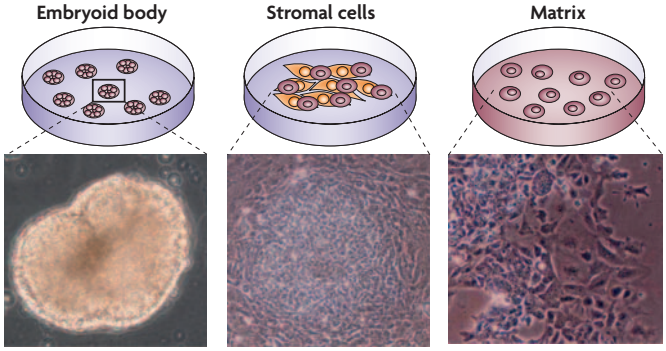
Figure 1 | **Derivation of embryonic stem cells.** After several rounds of divisions, the fertilized egg develops into the blastocyst. The blastocyst consists of trophectoderm and the inner cell mass (ICM), which give rise to extra-embryonic tissues such as the placenta and the cells of the embryo, respectively. When ICM cells are cultured under appropriate conditions — for example, in a medium that contains the cytokine leukaemia inhibitory factor (LIF; mouse only) or on mouse embryonic fibroblasts (both human and mouse) — they become able to proliferate continuously; these cell lines are designated as embryonic stem (ES) cells. In the mouse, ES pluripotency has been shown by injecting ES cells into blastocysts. These cells can be integrated into the embryo and give rise to all cell lineages in the body, including germ cells (chimeric mouse). For obvious reasons, this method has not been applied to human ES cells. Instead, the pluripotency of human ES cells is usually tested by their ability to give rise to teratomas.

Triggering differentiation. Compared with the mechanisms that are important for the maintenance of pluripotency, little is known about the molecular mechanisms that govern ES-cell differentiation. Although withdrawal of LIF is common to all protocols that are used to induce the differentiation of mouse ES cells, it is not a requisite. The current consensus is that the factors that promote pluripotency and differentiation are finely balanced in ES cells. Any small perturbations to this balance in favour of differentiation will result in the loss of pluripotency and commitment to a specific lineage.

In fact, disturbance of the balance between NANOG and GATA6 in favour of GATA6 (through a decrease of NANOG or an increase of GATA6 levels) leads to the differentiation of primitive endoderm[9,29]. The same is also the case for the balance between OCT3/4 and CDX2 for the trophectoderm-fate determination[12]. It is noteworthy that GATA6 or CDX2 overexpression results in highly selective differentiation to primitive endoderm or trophectoderm, respectively. In contrast to the differentiation of these two lineages, the molecular processes that are involved in triggering differentiation to other lineages such as mesoderm remain obscure. For example, the transcriptional regulators that are sufficient to mediate ES differentiation to mesoderm are not yet known, although it is clear that downregulation of NANOG is essential for differentiation.

Embryoid-body culture. The EB was first used to induce the differentiation of embryonic carcinoma cells[30]. The main concept of EB culture is to mimic the embryonic process of germ-layer formation from the ICM. It is indeed striking that this process can be studied in both embryonic carcinoma and ES cells[30,26].

On withdrawal of LIF, aggregated ES cells consistently develop into a structure that has visceral endoderm in the outer layer and other lineages derived from



Protocols:	Embryoid body	Stromal cells	Matrix
General features:			
Purpose	Mimicry of embryonic process	Selective differentiation	Selective differentiation
Serum-free culture	Possible	Possible	Possible
Determination of culture condition	Easy	Easy	Laborious
Technical difficulty	Easy, time-consuming for hanging drop	Requires stromal-cell maintenance	Easy (if condition is established)
Guided differentiation	Difficult	Theoretically difficult	Possible
Monitoring of cell behaviour	Difficult (owing to three-dimensional structure)	Possible	Easy
Morphogenesis	Possible	Difficult	Difficult
Cost	Low	Medium	High
Utility for preparing mature cells:			
Inducible cell lineage	Multiple, permissive	Moderately selective	Selective
Cell purification	Absolutely required	Required	Often not necessary
Limiting factors	Cell purification	Cell purification	Establishment of culture conditions

Figure 2 | **Comparison of three protocols for ES-cell differentiation culture.** To induce differentiation of embryonic stem (ES) cells into the three germ layers, three protocols are currently available. The protocol using embryoid body initiates the culture from aggregates of ES cells that develop to an embryo-like architecture with primitive endoderm outside and other lineages inside (left image). The protocol using feeder cell layers places ES cells on a monolayer of feeder cells (OP9 stromal cells; central image). The third protocol is a simple culture (right image), in which ES cells are placed on a defined matrix such as collagen IV (REF. 44). Each method has specific merits and drawbacks, several of which are enumerated in the figure.

primitive ectoderm inside the structure. However, the structure of the EB is far less organized than the actual embryo. EBs also recapitulate the formation of the proamniotic cavity and this allowed the study of the mechanism that underlies egg-cylinder formation[31]. Most cell lineages, including the neuronal and mesoderm lineages, have been shown to be generated within the EB. Moreover, recent studies demonstrate that the precursors of germ cells are also induced in EBs[32–34]. Given the lack of spatial organization in EBs, it is notable that the time course of expression of several important molecules in EBs is the same as the time course observed in embryos. Because of the convenience of EB culture, this method has become the standard method for inducing ES-cell differentiation in mouse and human ES cells.

Feeder-cell-dependent culture. Monolayers of freshly isolated cells or cell lines are often used as feeder cells to support ES-cell activities. Before the discovery of LIF, cell lines such as the STO cell line were used as a tool to maintain proliferation and pluripotency of ES cells in culture[35]. The maintenance of human ES cells still requires feeder cells[5] because these cells do not respond to LIF.

Many feeder cell lines used for inducing specific differentiation were originally stromal cell lines that were established to support *ex vivo* haematopoiesis; however, most feeder cell lines support differentiation of ES cells to multiple lineages. For example, the OP9 stromal cell line supports differentiation of ES cells to most haematopoietic cell lineages, including T and B lymphocytes[36], but also to other mesoderm lineages such as paraxial mesoderm, which can give rise to somites[28,37].

Under certain circumstances, a highly selective microenvironment is provided by feeder cells. The most remarkable example is that of the PA6 stromal cell line, which induces selective differentiation of ES cells to the neuronal lineage. The model proposed by Kawasaki *et al.* suggested that the activity that promotes differentiation to the neuronal cell lineage is present in the matrix formed by PA6 (REF. 38). This matrix component (the nature of which remains to be elucidated) can overcome the activity of several other secreted factors that are expressed by PA6 cells that would normally be expected to induce mesodermal differentiation[38]. This activity was designated as SDIA (stromal-derived inducing activity) and also applies to ES cells derived from other species, including primates and humans[39,40]. Recently, immortalized midbrain astrocytes have also been used as feeder cells to induce the differentiation of ES cells to dopaminergic neurons[41]. In conclusion, differentiation on feeder cell lines is worthwhile considering because they sometimes enable the induction of selective differentiation.

Towards fully defined conditions. One goal of ES-cell differentiation is to mimic the process of cell specification under fully defined conditions and, in the process, generate a specific type of mature cells at a high purity. Although some progress has been made, this is still difficult to achieve for most lineages; the main obstacle is the laborious screenings that are required to determine optimal conditions for each lineage. Furthermore, it is inherently difficult to control culture conditions fully. For example, although the culture may be initiated under fully defined conditions, undefined factors might be introduced by subsequent changes of the cultured cells. Factors such as cell diversification, an increase in cell density and the formation of new cellular interactions are difficult to control. Furthermore, all cellular interactions — both with the underlying matrix and with other cells — will introduce a range of different physical forces such as tension and shearing forces, which can have profound effects on the differentiation and survival of cells[42].

Despite this intractable problem, continuous attempts have been made to induce ES-cell differentiation under serum-free and chemically defined conditions in the absence of EB formation. For ES-cell differentiation in monolayer cultures under chemically defined conditions, only a few successes have been reported. Ying *et al.* showed that ES cells cultured on gelatin-coated dishes

with a conventional serum-free medium differentiate selectively to *Sox1*$^+$ neuronal cells[43]. We also reported two serum-free conditions for definitive endoderm and visceral endoderm that are based on the SFO3 serum-free medium that contains insulin, transferrin and bovine serum albumin[44,45]. Under these conditions, the purity of cells that are generated in culture often exceeds 90%, as assessed by the expression of molecular markers. Therefore, monolayer culture under serum-free conditions must be the ultimate direction to be pursued for other cell lineages.

Promising areas for ES studies

The current protocols for ES-cell differentiation range from EB cultures that recapitulate actual embryogenesis to a significant extent, to monolayer cultures under fully defined conditions that are selective for specific lineages. Although EB culture is regarded as a more embryology-orientated protocol than monolayer culture, morphogenetic events such as axis formation cannot be analysed. In this section, we present areas to which ES-cell differentiation culture is expected to provide useful insights.

The cell biology of embryogenesis. There are at least two areas in which the analysis of ES-cell differentiation can contribute to the understanding of the cell biology of embryogenesis. The first area is the preparation of specific types of embryonic cell for cell biological analysis. The EB method that easily supports differentiation of multiple cell lineages might be the most convenient method to provide many different cell types. Although unwanted cell types are generated in EBs, these can be removed using lineage-specific markers to purify the desired cell types. A series of surface markers are available for the purification of distinct lineages and stages using specific antibodies[28,46,47]. Therefore, the EB method provides a convenient tool for preparing several cell types, which can be used for cell biological analysis after the purification of the desired cell types.

The other area to which ES-cell differentiation studies are expected to contribute substantially is the cell biology of the early processes of embryonic differentiation; particularly, the early specification of the germ layers, which remains largely uninvestigated because of the difficulty in obtaining sufficient numbers of cells. For example, an embryonic day 3.5 (E3.5) embryo consists of *Cdx2*$^+$ primitive endoderm and ICM in which *Gata6*$^+$ and *Nanog*$^+$ cells are mixed in a 'salt-and-pepper' manner[48]. How this early

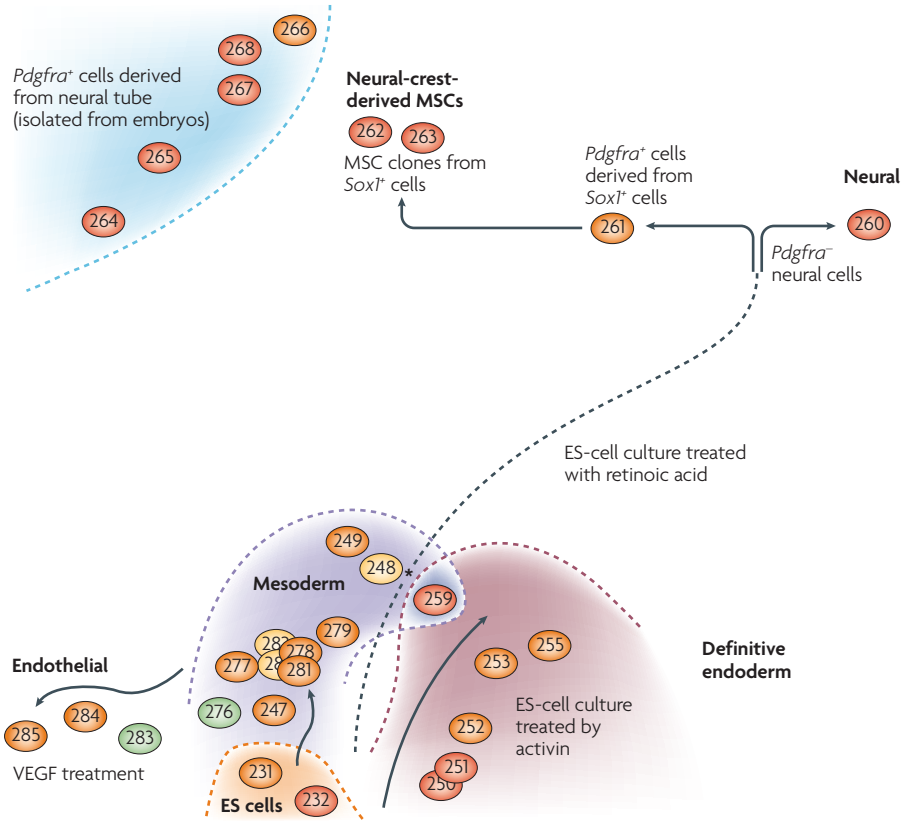


Figure 3 | **Relationships between a set of ES-derived cells and embryonically derived cells.** The diagram indicates distances between different cell populations during the differentiation of embryonic stem (ES) cells, as calculated from the signals of 724 Affymetrix probe sets selected by their probe pair covariance and variance across the different samples[58]. Each circle indicates an individual sample. All cell populations except samples 264–268 (in the blue area) were derived from ES cells (samples 231 and 232; orange area). Samples 262 and 263 are clones with mesenchymal stem-cell (MSC) properties that were derived from ES cells. These clones are most similar to several cell populations that were derived from embryonic day 14.5 (E14.5) neural-crest derivatives that have MSC properties (samples 264–268). Samples in the magenta area were derived by culturing ES cells in the presence of activin, with samples taken from day 1 (sample 250) to day 5 after culture (samples 255 and 259). Sample 259 consisted of E-cadherin-negative cells derived from the same culture, indicating that it is equivalent to mesendoderm-derived mesoderm. Samples in the purple area are ES-derived mesoderm and are all positive for vascular-endothelial growth factor receptor-2 (VEGFR2; also known as FLK1) and/or platelet-derived growth factor receptor-α (PDGFRα). All mesoderm samples except sample 259 were derived by differentiation in the presence of serum. Note that samples 248 and 259 are positive for both VEGFR2 and PDGFRα, which indicates a close correlation between surface phenotype and global gene expression even for samples that were derived by different methods. Samples 283–285 are vascular endothelial (VE)-cadherin$^+$ populations that were derived from *Vegfr2*$^+$ ES-derived precursors.

divergence is regulated would be a good subject for ES-cell culture because of the ease of monitoring and manipulating the intermediate processes in culture. Although study in this direction will require enormous effort to determine the culture conditions, it should be a fruitful area of research.

Evaluation of extrinsic signals. Concentration gradients have been implicated in specifying cell types in a position-specific manner. However, determination of the actual concentration that specifies a given cell fate can be assessed only in simple culture systems that avoid the involvement of other extrinsic factors. Under such conditions, only specific lineages might be permitted to differentiate.

We have shown that the concentration of activin, a molecule that induces differentiation to mesoderm in various species, correlates with the proportion of *Gsc*$^+$ mesendoderm cells derived from ES cells cultured under serum-free conditions[44]. Obviously, the expression level of one marker alone is not sufficient for prospective prediction of the actual cell fate. Therefore, detailed fate analyses of the

Glossary

Embryonic carcinoma
A type of testicular cancer that maintains the potential to give rise to mature tissues.

Mesoderm
The middle germ layer of the developing embryo that occupies an intermediate position between the ectoderm and the endoderm. It gives rise to the skeleton, muscles and connective tissue.

Pluripotent
Embryonic stem cells that are able to form all of the cell lineages of the body, including germ cells, and some or even all extra-embryonic cell types.

Polycomb
A class of proteins — originally described in *Drosophila melanogaster* — that maintains the stable and heritable repression of several genes.

Primitive endoderm
The extra-embryonic tissue that gives rise to the visceral and parietal endoderm; it diverges directly from the inner cell mass to form the outer layer of the embryo.

Teratoma
Tumours that contain various differentiated cells from all three primary germ layers.

Trophectoderm
The outer layer of the blastocyst.

cells induced at a given condition should be pursued in parallel with the development of other markers that correlate with specific fates. This type of analysis should enable elucidation of the desired positional information in cultures; this knowledge will then contribute to the development of new methods for guided differentiation of ES cells. So far, only a limited set of molecules such as activin, bone morphogenetic proteins (BMPs) and Wnt signals have been investigated[44,49–51], but precise analyses of the dose response of these molecules and their combinations in serum-free monolayer cultures are necessary. Such studies are also essential for determining the optimum conditions to steer ES-cell differentiation to a particular lineage.

Genes to systems. The ability to obtain large numbers of cells from ES-cell differentiation cultures easily is important for introducing high-throughput technologies for gene-expression profiling (for example, DNA microarray). This type of analysis helps the discovery of genes that are involved in specific embryonic processes, and allows a map of gene-expression patterns during the developmental process to be drawn. Indeed, it is possible to convert microarray data into a map that displays the relationship among various intermediates isolated from ES-cell differentiation cultures (as well as embryos) in terms of expression profiles of many genes (FIG. 3; for neuronal differentiation see REF. 52). Although such analyses have been unable to prove that ES-cell differentiation is equivalent to embryonic differentiation (largely owing to the lack of embryonic data), our data (FIG. 3) and a recent report[52] suggest that meaningful comparisons can be made between different ES derivatives and that their paths of derivation are consistent with our understanding of the process. In addition, we have found that there is a good correlation between surface phenotype, function and global gene-expression profile (FIG. 3). However, it should be emphasized that this does not constitute proof of such equivalence, merely that high-throughput analyses are broadly in line with this being the case.

Microarray analysis is also expected to be useful for identifying novel genes involved in the earliest stage of germ-layer formation because it is usually difficult to prepare a sufficient number of cells from early embryos. Although this advantage has been fully exploited in the investigation of pluripotency, its use for early differentiation processes has been limited.

One often-overlooked advantage of microarray data is that the analysis is based on highly standardized materials and methods. Standardized methods, in combination with the large amount of data that any single hybridization provides, make it easy to compare measurements directly from a large number of different samples. We are currently using this feature of microarray data to establish a database of various intermediates purified from ES-cell differentiation culture or directly from mouse embryos. We expect that such a database has the potential to meet the need for gene-expression analysis of embryonic cells repeatedly without the cost associated with carrying out the analyses on embryonic tissues.

Conclusions and future perspectives

In this article, we have reviewed how ES cells are used in the cell biology of embryogenesis. Despite many remaining problems, we emphasize the necessity of developing defined culture conditions for ES-cell differentiation without using EB or stromal cell co-culture. Although establishing defined culture conditions for distinct cell types requires an enormous amount of work, we expect that recent progress in high-throughput technologies will facilitate this process. Indeed, a high-throughput screen has been applied to identify small molecules that can support the maintenance of pluripotency and self-renewal of ES cells[53].

It should be emphasized that recent high-throughput technologies are only compatible with simple monolayer culture protocols owing to their requirement for an automated assay system that requires *in vitro* imaging of cellular responses. Indeed, several ES-cell lines are already available in which the signal status of the cell is reflected by fluorescent markers. Once optimum culture conditions are determined by such screens, other technologies become applicable to ES-cell differentiation culture. These technologies include gene-transfection arrays[54], RNAi-transfection arrays[55,56] and extracellular matrix arrays[57]. When combined, these technologies may also allow us to draw a detailed landscape of how the transcriptome changes during the cell-specification process. Such technologies acting in concert may enable the generation of diversity to be visualized during early differentiation of ES cells under defined conditions. This progress will, in turn, enable us to prepare and manipulate normal human cells of desired lineages and stages. As such, the list of future possibilities is endless, but future analyses of normal processes will include many that are based on ES-cell culture and differentiation.

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Competing interests statement
The authors declare no competing financial interests.

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