The future for stem cell research

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Stem cells have offered much hope by promising to greatly extend the numbers and range of patients who could benefit from transplants, and to provide cell replacement therapy to treat debilitating diseases such as diabetes, Parkinson's and Huntington's disease. The issue of stem cell research is politically charged, prompting biologists to begin engaging in ethical debates, and generating in the general public an unusually high level of interest in this aspect of biology. But excitement notwithstanding, there is a long way to go in basic research before new therapies will be established, and now the pressure is on for scientists and clinicians to deliver.

Scientists and clinicians have been beavering away in the background on stem cell research for years, but it took two breakthroughs — animal cloning by Ian Wilmut, Keith Campbell and colleagues[1] and the derivation of pluripotent human embryonic stem (ES) cells by Jamie Thomson and co-workers[2] — to really shake things up. The first showed that an adult cell nucleus can be reprogrammed to produce an entire animal, a dramatic demonstration of hidden potential, and the second provided a possible source of cells for cell-based therapies for many human diseases. Even more intriguing was the notion of combining the two in the process of 'therapeutic cloning', whereby ES cells could be (in theory anyway) derived from a patient's cell, thus avoiding problems of immune rejection, and then coaxed into forming large quantities of the cell types needed for a cure. The derivation of human embryonic germ (EG) cell lines from aborted fetuses, with a similar potential to ES cells, by John Gearhart's group[3], provided an alternative route to obtain cells for treating patients, avoiding the need to use early embryos. But then, more recently, adult stem cells have leapt into prominence with many claims that they are more plastic than previously thought, and can in some circumstances contribute to cell types very different from those in their tissue of origin. This has suggested that patient-specific stem cell therapy can be carried out without cloning.

As is evident from reading the articles comprising this Insight, stem cell research has opened for exploration a vast new terrain of basic biology. Fundamental questions remain, such as what defines a stem cell in molecular terms, what signalling events control stem cell differentiation, and what does it mean to reprogramme a cell? Scientists are now reconsidering the very definition of a stem cell, with the attendant notions of lineage restriction and the permanency of the differentiated cell state. Although this uncertainty is unnerving, in the end we will profit from a better

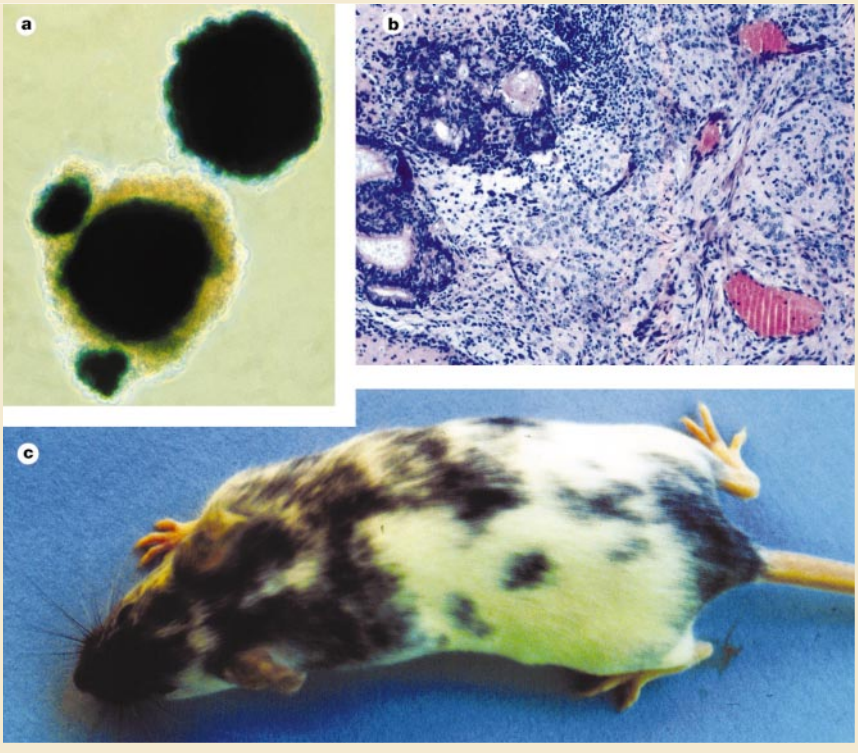

Figure 1 Pluripotency of mouse embryonic stem (ES) cells. **a**, Aggregates of mouse ES cells forming embryoid bodies. The dark staining shows expression of Sox2 in the less differentiated cells, whereas the rind of differentiated endoderm is unstained. (Image courtesy of A. Avilion). **b**, Histological section of a teratocarcinoma derived from mouse ES cells. Many different cell types are found, all formed from the ES cells, including representatives of all three germ layers. (Image courtesy of M. Parsons.) **c**, The ultimate test of pluripotentiality: a chimaera made by injecting ES cells into a blastocyst. The pigmented areas reveal the contribution of ES cell derivatives to the skin, but all tissues are composed of a mixture of ES and host embryo derivatives. Even a single ES cell can give a chimaera like this.

understanding of how stem cells work as well as how to identify, isolate and maintain them, and trigger their differentiation along useful directions.

We would love there to be common rules, but given the multitude of stem cell types, this may be asking too much. Some types of stem cells, such as bone marrow and skin, have already been used in therapies, and others are effectively being used in trials, including fetal midbrain cells for Parkinson's disease, and pancreatic duct cells for diabetes. But all have different properties reflecting the normal requirement for the tissue to which they belong. Some are easy to identify and to grow; others remain a mystery. Paradoxically, one of the easiest types to grow and expand in culture are ES cells, which are those with the greatest potential. This is one reason why it is so important to learn how to control their differentiation and to be able to select specific cell types to be used for therapies[4]. It is also crucial to understand the nature of pluripotentiality, one of the defining properties of ES cells (Fig. 1), discussed at length in the articles by Azim Surani (pages 122–128) and by Peter Donovan and John Gearhart (pages 92–97).

In political terms, the use of early embryos to derive ES cells has stimulated intense debate among professional ethicists, moral philosophers, religious leaders and scientists. Most ethicists believe that human embryo research raises no new ethical problems, at least none that were not already considered with respect to methods of assisted conception, such as *in vitro* fertilization. If ES cells turn out to be the best route to cure a particular disease, then many would argue that it would be morally wrong not to use embryos that would otherwise be discarded. Anne McLaren discusses the ethical and social considerations of stem cell research on pages 129–131.

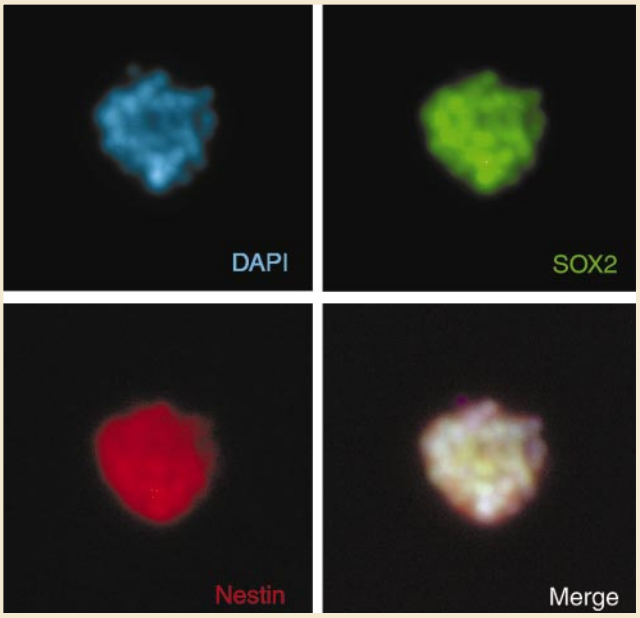

Figure 2 A small primary neurosphere, obtained from one or a few cells from the dorsal telencephalon of a 14.5-d.p.c. mouse embryo, which has been grown in culture for 21 days. This is stained with DAPI (blue) to reveal nuclei and with both SOX2 (green) and Nestin (red) to reveal neural progenitor cells. Many, if not all, of these cells have properties of neural stem cells. (Figure courtesy of E. Remboutsika.)

Realizing potential

The story of ES cells starts from the study of teratocarcinomas, first worked on in the mouse by Leroy Stevens, and discussed in this issue by Donovan and Gearhart. These are complex tumours containing a mix of differentiated cell types and a population of undifferentiated cells, termed embryonal carcinoma (EC) cells. The latter were shown to give rise to cell types from all three classical embryonic germ layers: ectoderm, mesoderm and endoderm[5] (Fig. 1b). The idea that such pluripotent EC cells might provide a source of cells for therapy was therefore around since at least the mid-1970s and encouraged much work on human EC cells. But they never seemed ideal because they were aneuploid and had come from tumours. It was known that teratocarcinomas could be made experimentally by transplanting early postimplantation mouse embryos to ectopic sites, so it seemed natural to try to avoid the tumour step by putting early embryos directly in culture. An understanding of the biology of EC cells and early embryos led Martin Evans and Matt Kaufman to put blastocysts that had been kept in diapause (or delayed implantation) into culture conditions that had been optimized for the best available EC cell lines. The successful derivation of ES cells that followed[6], and the demonstration that they had a normal karyotype and were truly pluripotential[7], has of course had ramifications far outside even the important area of stem cell biology.

ES cells are known to correspond roughly to cells of the early epiblast, but they are not identical to them. Mouse ES cells depend on LIF (leukaemia inhibitory factor) to remain undifferentiated, whereas embryos mutant in LIF or its receptors show normal development. Nethertheless, there is a requirement for LIF signalling by the inner-cell-mass cells of blastocysts in diapause[8]. Perhaps these cells are closer to ES cells, even though they are essentially non-dividing cells. On the other hand, it is known from many systems that stem cells are often quiescent. Perhaps this will also turn out to be a theme — stem cells maintained *in vitro* may rarely correspond to their counterparts *in vivo*.

Cells fitting the classic definition of stem cells also exist in the adult, where they function to replace cells lost from normal turnover or damage. Through asymmetric divisions, adult stem cells produce just the right number of differentiated cells, as well as daughter stem cells with properties identical to those from which they arose. This is true self-renewal. In contrast, in the embryo, although many cells undergo a similar asymmetric division with respect to daughter cell fate, this does not necessarily involve exact self-renewal. The daughter stem cells progressively become restricted in the variety of differentiated progeny they can produce, thereby losing developmental potential. This can occur rapidly, as in the transition between the inner cell mass and epiblast, or gradually, as in the developing central nervous system (CNS), as discussed by Sally Temple on pages 112–117. In considering the range of cell types to which a stem cell can give rise, Temple highlights the importance of the cell's environment and the increasing complexity of the embryo as it develops, as well as its history — cells isolated from different parts of the developing CNS remember from where they have come, even after many cell divisions *in vitro* (Fig. 2).

This notion of history versus environment has been discussed eloquently by David Anderson elsewhere[9]. As he points out, without history (or lineage) providing some hard-wiring, in combination with an organized body plan to put signals in the right place, it would be difficult to build an embryo, whereas environment alone must dictate what a stem cell does in the adult. But, seemingly to the surprise of many an embryologist, sometimes environment is able to overcome any amount of careful upbringing and historical propaganda. This is evident in recent data suggesting that adult stem cells isolated from one tissue can give rise to cell types characteristic of another. They can even cross over from one classic germ layer to another[10]. There are many dramatic examples of this, some mentioned in the following articles. But perhaps the most extreme example is that from Frisen and colleagues showing that adult CNS stem cells can contribute to cell types of all three primary germ layers after being introduced into blastocysts, where they behave something like ES cells[11].

Home alone

However, one important caveat with many of the recent experiments looking at the potential of stem cells is that they are rarely performed with single cells. Picking single EC or ES cells and showing that they

can give rise to a wide range of cell types *in vitro* or *in vivo* was the only real proof that they were truly pluripotent stem cells[5,7]. If, on the other hand, one begins with a population of cells, then that is all one is testing — many different cells, perhaps each with a unique potential. The possibility of contamination of one stem cell type with another has been stressed by Tannishtha Reya, Irving Weissman and colleagues (pages 105–111), who caution that haematopoietic stem cells (HSCs) could well be distributed throughout the body. The presence of differentiated cells among the stem cells might also affect results, but perhaps in the other direction. In Frisen's experiments, neurospheres were able to contribute to normal development to make chimaeric embryos in only 1% of attempts[11]. This could mean that the potential to do so was found only in rare neural stem cells. Alternatively, if differentiated cells tend to be present in the neurospheres, these could exert an inhibitory influence on the stem cells, in most cases preventing them from fulfilling their true potential. These are all problems that could impinge on the usefulness of stem cells in the clinic.

It is therefore of no surprise that the importance of testing the fate of single cells is stressed by several authors, notably by Alan Spradling and colleagues (pages 98–104) and Sally Temple (pages 112–117). Even if we set aside problems of contamination, it would be useful to know if some unusual potential, such as neural stem cells contributing to muscle, is a property of specific rare cells in the population or whether each cell has a low probability of differentiating in this direction. And even if the population of cells is clonally derived, there could still be variation, which could be explained if stochastic epigenetic changes affected the activity of certain critical genes. In fact, this could well be the explanation for the variable patterns of imprinted gene expression and phenotypes seen in cloned mice made using donor nuclei from ES cells[12]. But it is not always easy to test single cells. It has been attempted for bone-marrow stem cells, although a rather complex procedure had to be adopted to overcome the problem in identifying them in the first place[13]. We clearly need better markers for stem cells and efficient ways to test their potential both *in vitro* and *in vivo*.

Home truths

Spradling and colleagues highlight the importance of the stem cell niche — essentially the environment in which stem cells exist and which contributes to their self-renewal and differentiation. Cell–cell or cell–basement membrane contacts could provide a simple means by which asymmetric cell division can lead to these alternate fates. However, more diffuse signalling, such as by growth factors, could also be used to regulate the proportion of stem versus differentiated cells and rates of proliferation. This leads to the notion of two types of niche, lineage based and population based, but it is stressed that in many cases both may operate together.

They also suggest a rigorous way in which a stem cell niche can be identified, by showing that a single stem cell can repopulate an empty niche. But there is a caveat to this. The proper functioning of niches is likely to depend on the continued presence of stem cells and empty niches are likely to lose their abilities and degenerate. Testes devoid of germ cells eventually lose Sertoli cells (P. Burgoyne, personal communication). It is therefore not surprising if they also lose the ability to support newly transplanted spermatogonia (germ stem cells)[14]. This may be a problem that needs to be faced with stem cell-based therapies, that is, it may be necessary to rejuvenate or replace the niche as well as the stem cells. This area is covered by Paolo Bianco and Pamela Robey on pages 118–121, in an article which places stem cell biology in the context of tissue engineering, and highlights some of the many practical problems to be encountered in achieving cell-based therapies.

Plus ça change

Other types of cells, notably HSCs, seem to differentiate in the absence of a niche or the differentiating cell population, changing from long-term, self-renewing true stem cells to transient amplifying cells in a process that is apparently resistant to environmental influences, as discussed by Irving Weissman and colleagues. They also discuss the relationship between stem cells and cancer cells, and the evidence that mutations 'hijack' the self-renewing population of stem cells. They note that tumours are often a mix of cell types, with teratocarcinomas and Wilms' tumours being the obvious cases. Even when cell morphologies are similar, often only a small proportion of the cells are tumorigenic. But ES cells seem to break all the rules, as every undifferentiated cell is tumorigenic, without any mutations[15]. We clearly need to understand more about the relationship between cancer cells and stem cells, especially if we are to use the latter for therapies.

What are the molecular correlates of changes in stem cell potential, whether due to temporal, positional or lineage restrictions?

Box 1
A few definitions

Blastocyst. A mammalian embryo before implantation. Blastocysts consist of outer trophectoderm cells, which allow the embryo to implant, surrounding the inner cell mass, within which are found the pluripotent epiblast cells.
Cell fate. What a cell can do in either its natural location in the embryo or in an ectopic site. This is usually determined by marking the cell in as neutral a way as possible.
Committed. Used to describe cells whose fate is already determined along a particular path of differentiation — at least within the bounds of the experimental assay.
Epigenetic. Heritable but reversible changes in gene function without changes in DNA sequence. Usually involves chemical modifications of DNA or the binding of specific proteins to DNA sequences. Epigenesis is a related term used to describe formation of new structures during embryonic development.
Epigenetic asymmetry. Two genomes with identical DNA sequences, but with different forms of reversible but heritable modifications that regulate gene expression and repression.
Imprinted gene. A gene that is marked in the germ line; this denotes its maternal or paternal origin and influences its expression in the developing embryo.
Lineage. The natural progression from an immature cell type to one or more differentiated cell types.
Lineage restriction. The inability of one lineage to give cell types of another, that is, to cross lineage boundaries.
Molecular memory. The inheritance of specific pattern of gene expression, which persists in daughter cells from generation to generation.
Multipotent. Able to give rise to more than one differentiated cell type.
Plasticity. The ability to cross lineage boundaries.
Pluripotent. Able to give rise to all cell types found in the embryo and adult animal.
Progenitor cells. These can include both stem cells and transient amplifying cells, or even cells that are well on the way to becoming differentiated.
Stem cell. Generally used to describe a cell that is capable of both self-renewal and differentiation. However, the term has been used in many other contexts. (See ref. 27, pages 1–16 for more details, and the entire volume for many current ideas on stem cell biology.)
Therapeutic cloning. Reprogramming the nucleus of an adult cell through transfer into the cytoplasm of an enucleated oocyte. This is sometimes referred to as 'cell-nuclear replacement' or 'somatic cell nuclear transfer'.
Totipotent. Able to give rise to all cell types. In mammals, only the fertilized egg and early cleavage stage blastomeres are truly totipotent. Cells of the inner cell mass and ES cells are unable to differentiate into cells of the trophectoderm lineage.
Transient amplifying cells. Progeny of stem cells that undergo replication, but are not able to self-renew and eventually give only one or more differentiated cell types.

Some of these issues are raised by Azim Surani on pages 122–128. Presumably there are changes in the transcriptional activity of important genes within the stem cells. But are there any rules we can understand that might prove useful? Should we be looking for changes in the activation or repression of specific genes, or for changes in chromatin through the action of, for example, members of the polycomb and trithorax groups of factors, or for changes in histone modifications (acetylation and methylation) or methylation of DNA? No doubt it will be a combination of several of these processes acting on particularly critical genes. These genes may be of two types: those that confer stem cell properties, such as allowing self-renewal and maintaining potential, and those that give them a certain 'flavour', in other words, determining the range of differentiated progeny they can give rise to. If we can find the most critical of these genes then perhaps we could achieve the ambitious aim of reversing the process, turning differentiated cells directly into stem cells, which could then be used to cure disease or trauma. Both Spradling and colleagues and Temple mention the importance of identifying stem cell genes, but so far we know of very few: perhaps *piwi* as mentioned by the former, and *Sox2* and *Oct4* in early mouse development[4,16].

As mentioned above, it was a surprise to many that embryological rules seem to be broken by recent experiments on stem cells. One such rule is that of lineage restriction: cells derived from one of the three primary germ layers that arise at gastrulation — ectoderm, mesoderm and endoderm — should not be able to form cell types characteristic of another. The breaking of this rule has now been reported many times in experiments on stem cells[11,13,17]. But, in fact we have known natural examples of this for a long time. Neural crest is ectodermal in origin, but it gives rise to tissues, such as cartilage and muscle during normal development of the head, that are indistinguishable from those that have arisen from mesoderm formed via gastrulation[18]. There are also several examples of transdifferentiation, such as within the eye; these are situations where cellular reprogramming occurs naturally, and examples have been studied for many years[19].

Another rule of the embryo is the progression from an undifferentiated to more differentiated state. This rule can also be violated, as demonstrated recently by Kondo and Raff, who showed that O2A oligodendrocyte precursors can be induced by specific culture conditions to revert to neural stem cell-like cells[20]. But this rule was also broken before, at least 9 years ago. The articles by Surani and by Donovan and Gearhart discuss EG cells: pluripotent cells similar to ES cells that are derived from primordial germ cells (PGCs) within (or migrating towards) the genital ridges. Germ cells are very specialized cells, and become more so as they differentiate into sperm and oocytes. They usually have to go through meiosis to realize their totipotency, and sperm achieve this only after fertilization and reprogramming of their DNA by egg cytoplasm. Thus, it is thought that the culture conditions used to establish EG cells effectively regress the PGCs to a more primitive state. Germ cells undergo a complex history during their formation, but by changing their environment in culture they can be reverted all the way back to cells with properties similar to those of the epiblast from which they arose[21,22].

Back to the future

It may also be useful to take an evolutionary approach in studying stem cells. There are known to be species differences in the way embryos are made, perhaps even for the CNS[23], suggesting that if it is possible to get to the same cell types by different routes, then we should not be so surprised if certain stem cells are able to do things that contravene established thinking. It is also interesting to ask why regeneration occurs readily in some vertebrates, but not others. This includes the coordinated renewal of many cell types, such as with limb regeneration in newts[24], or cases where only a single cell type is involved. For example, cochlear hair cells of birds are renewed from adjacent cells with stem cell-like properties, whereas those of mammals are never replaced once lost[25,26]. Perhaps this is because song is so important to bird society, although it is little consolation for the pheasant being shot at, that the human wielding the gun will probably go deaf.

The next few years will no doubt bring great advances in understanding stem cells at the molecular level. New techniques will aid in identifying critical genes involved in controlling their self-renewal and differentiation. Perhaps these will allow us to manipulate stem cells *in vivo* in a useful way. Similarly, genes involved in reprogramming will be found. So far the only even remotely reliable way of reprogramming an adult cell type into another is by transferring its nucleus into the cytoplasm of an oocyte. This presumably reflects the normal ability of the egg to reprogramme the incoming sperm DNA to behave like its own. But is this due to one or many cytoplasmic factors? Identifying these and understanding how can they restore totipotentiality will be a substantial but very worthwhile challenge. □

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