Chapter 2
The Significance of Culture Adaptation of Embryonic Stem Cells for Regenerative Medicine

Neil J. Harrison, Duncan Baker and Peter W Andrews

Abstract The promise that human embryonic stem (ES) cells hold for regenerative medicine has generated much excitement since their initial derivation. However, before the potential of these cells can be realised, efficient differentiation protocols must be devised, and the cells should be shown to pose no safety risk. Despite initial reports suggesting that human ES cells are karyotypically stable, during the last decade it has become apparent that they do acquire genetic and/or epigenetic changes during culture, reflecting an adaptation to life in vitro. This culture adaptation can affect ES cell growth and differentiation, but of particular concern is the potential link between adaptation and cancer, which would become an issue if the cells are to be used for transplantation. In this chapter we discuss the issues surrounding culture adaptation of ES cells, and the potential impacts, both positive and negative, it may have on the use of these cells for regenerative medicine.

2.1 The Principles of Culture Adaptation

> ..natural selection is daily and hourly scrutinising, throughout the world, the slightest variations; rejecting those that are bad, preserving and adding up all that are good; silently and insensibly working, whenever and wherever opportunity offers, at the improvement of each organic being in relation to its organic and inorganic conditions of life—Charles Darwin, *The Origin of Species* (1870).

N. J. Harrison · P. W. Andrews (✉)
Centre for Stem Cell Biology, Dept Biomedical Science,
The University of Sheffield, Sheffield, UK
e-mail: p.w.andrews@sheffield.ac.uk

N. J. Harrison
e-mail: N.J.Harrison@sheffield.ac.uk

D. Baker
Sheffield Diagnostic Genetic Services, Sheffield Children's NHS Trust, Sheffield, UK

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The derivation of embryonic stem (ES) cells was achieved almost a century after Darwin's death, yet the evolutionary principles he described are inescapable in their maintenance. The ability to self-renew is one of the defining characteristics of ES cells, yet they are not resistant to mutation, and as such evolutionary pressures during culture will invariably lead to the generation of variant cells with improved growth capacity. The selection of cells is evident from the initial derivation of an ES cell line, since generation of an immortal cell line from the transient population of inner cell mass (ICM) cells in the blastocyst stage embryo requires some adaptation, albeit that, at least, initially such changes can be reversed to allow, in the laboratory mouse, the generation of germline chimaeras if ES cells are returned to a developing blastocyst. However, ES cells can continue to 'evolve' during their time in vitro, a process termed culture adaptation.

The culture adaptation of ES cells must then involve two, potentially independent, phenomena: mutation and selection. Without selection, and in the absence of a population bottleneck, such as cloning, a mutation would never reach sufficient levels in the population to be detected, and without mutation, no variants would exist. The selection pressure for advantageous mutations must be quite high since, assuming a routine passage ratio of 1:3 every 3–4 days, and a cell cycle time of 16 h, it is likely that upward of 90% of cells must be lost between passages. One study has used a Monte Carlo simulation to assess the relationships of these are key factors in ES cell growth [1]. In this study, the selective advantage of the variant cells that commonly appear in ES cultures was assessed by spiking cultures with cytogenetically abnormal cells and then comparing the rate at which they overgrew the culture with predictions from the simulation. Indeed the selective advantage of the variant cells was high and variant cells typically predominant in cultures within 10–15 passages, sometimes less. From this it was estimated that human ES cells acquire beneficial mutations at a rate of approximately 1×10^{-6}. This rate is similar to that reported for other cell types [2], though the short cell cycle time for ES cells perhaps makes them more prone to the acquisition of such mutations. Selection is more difficult to quantify, though it has the potential to be quite significant. With a large opportunity for selection, and seemingly frequent mutation, human ES cells are obviously strongly subject to adaptation.

2.2 Culture Adaptation and Cancer

The culture adaptation of ES cells increases their growth capacity in culture, which has indeed been observed during the prolonged passage of human ES cells [3]. For a stem cell, this increase in growth capacity must result from an increased propensity for self-renewal, which would increase the stem cell pool, over differentiation and/or death, which would decrease the stem cell pool. A similar shift in cell fate would benefit cancer cells, particularly cancer stem cells, since the evolution of a phenotype strongly biased towards self-renewal in a cancer stem cell would result in a highly aggressive tumour. With this in mind, it is notable that the

genetic changes most frequently reported in culture adapted ES cells are also commonly observed in embryonal carcinoma (EC) cells, their malignant counterparts and the stem cells of teratocarcinomas [4, 5]. Teratocarcinomas are germ cell tumours (GCT) that occur most commonly as a testicular cancers (TGCT), and are perhaps the first examples of a stem cell-based cancer. Here, Kleinsmith and Pierce [6] showed that a single EC cell from a murine teratocarcinoma could recapitulate the original tumour when transplanted to a syngeneic recipient mouse. It should be noted that when culture adapted human ES cells are allowed to form teratomas in immunecompromised mice, the tumours tend to behave more like teratocarcinomas, retaining a stem cell component that can be excised and re-cultured [7, 8].

The karyotypic changes most frequently seen in ES and EC cells are the gains of material from chromosomes 12 and 17 (Fig. 2.1), particularly the p arm of chromosome 12 and the q arm of chromosome 17 [4]. The gain of material from chromosome 12p is also common in TGCT, so much so that it is considered diagnostic for this malignancy. In TGCT, the gain of material from chromosome 12p is mainly from 2 amplicons, 12p11.2-12 [9, 10] and 12p13 [11], and the specific amplification of 12p13 has also been seen in a culture adapted iPS cell line [12]. The potential candidate genes in the 12p13 band include GDF3, DPPA3, CCND2 and NANOG. NANOG and GDF3 were shown to be overexpressed in the culture adapted iPS cells with 12p13 amplification, and forced overexpression of NANOG has previously been shown to maintain human ES cells in an undifferentiated state [13]. However, not all culture adapted cells show an increase in NANOG [14, 15], including those which are trisomic for chromosome 12 (Alagaratnam et al., in preparation), suggesting that there may be a number of ways in which ES cells can adapt to culture.

The amplicons on chromosome 17 in TGCT were originally identified as 17q11-21 and 17q24qter [16], though recent work [17] suggested the region 17p11.2-q21.32 is most commonly amplified in EC cells. These data are in general agreement with the amplicon suggested for culture adapted human ES cells, approximated as 17q21-qter [4]. In particular, the terminal end of 17q (q25-ter) has been implicated in human ES cell culture adaptation, with reduced expression of Survivin (BIRCS) inducing apoptosis [18]. However, a small amplicon at 17p11.2 has also been identified in human ES cells. Amplification of this region has been reported in two independent human ES cell lines, and in one case as a homogeneous staining region (HSR), a cytogenetic representation of genetic amplification that is almost unique to cancer cells. The amplification of this region has also been reported in breast cancer [19] retinoblastoma [20], and frequently in osteosarcoma [21], suggesting this region may contain gene(s) which can impact on cell fate.

Aside from the changes on chromosomes 12 and 17, gains of material from chromosomes 1, 20 and X have also been seen with relative regularity. On chromosome 20, an amplicon at 20q11.21 has been identified through array-CGH by a number of groups [22–24] and may hold most promise for identifying specific genes involved in adaptation. The use of array-CGH technology has helped to identify and refine the common regions of genetic change in human ES cells, yet confirms the earlier karyological reports that chromosomal gain is far more

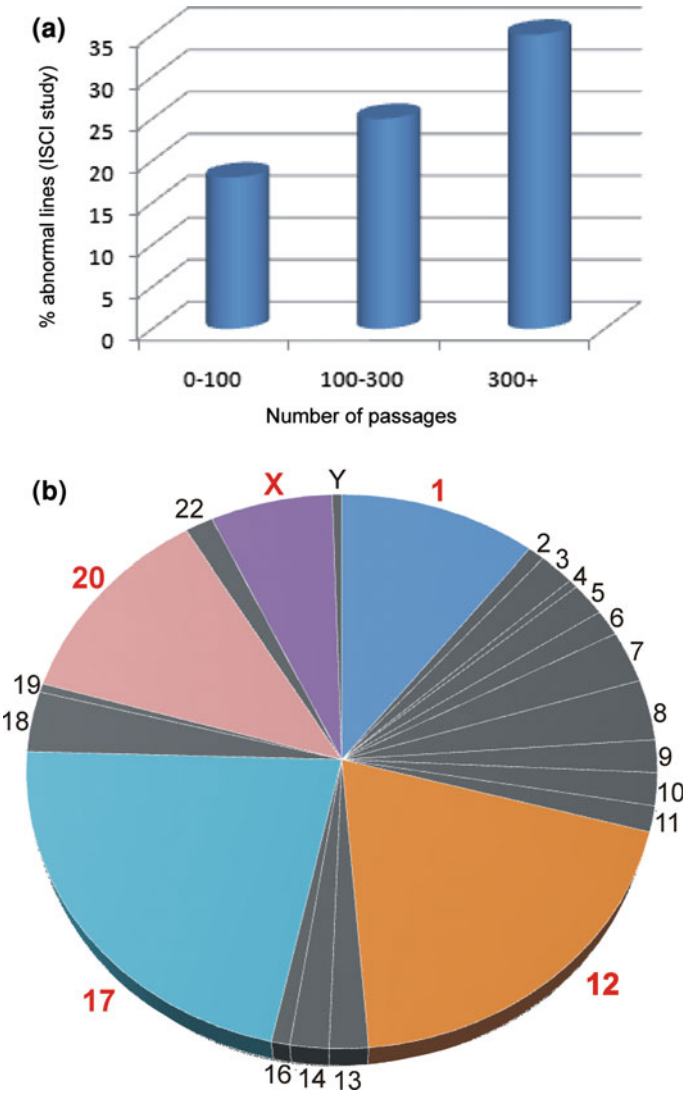

Fig. 2.1 Karyotypic abnormalities in hES cells. **a** The percentage of karyotypically abnormal cell lines (mosaic cultures are considered abnormal) compared against passage number. As passage number increases, so does the percentage of abnormal lines. **b** The percentage of abnormalities, per chromosome, reported for hES cells. The most common abnormalities are highlighted, and are associated with chromosomes 12, 17, 20 and X. Data shown is from the International Stem Cell Initiative (ISCI) study [39]

frequent than chromosomal loss in these cells. This may reflect a quirk in the DNA repair machinery of human ES cells, or suggest that increased gene expression may be more readily achieved through DNA amplification than epigenetic mechanisms in these cells.

2.3 Culture Adaptation and Regenerative Medicine

The use of stem cells in regenerative medicine will require their efficient differentiation into the cell type(s) of interest. Culture adaptation may change fate propensities in ES cells, biasing them not only against differentiation per se, but also potentially against differentiation along certain lineages. Human ES cells are not islands, these cells will be influenced by their neighbours, and the signals that they provide. As such, to survive as a stem cell, it would be beneficial to be surrounded by cells that provide signals that facilitate self-renewal, as opposed to those that encourage death or differentiation. Here, it is of interest that some culture adapted cells seem to be biased against endoderm differentiation [25], since endoderm cells are known to produce bone morphogenetic proteins (BMPs) which can cause human ES cell differentiation [26]. This offers an obvious mechanism by which variant cells could acquire a selective advantage, and would prove an obstacle to those trying to differentiate the cells to, e.g. pancreas or liver.

2.4 Identification of Culture Adapted ES Cells

The concerns associated with using culture adapted cells in regenerative medicine mean that methodologies to detect these cells, and/or mimimise the likelihood of their appearance, have become increasingly important. In this regard, defining what exactly constitutes ‘culture adapted’ is a fundamental question, yet one which is still without a definitive answer.

If one assumes that for a mutation to reach detectable levels it must bestow a growth advantage on the variant cell, then the identification of culture adapted ES cells could be achieved based on mutation. With karyotyping services readily available and array-CGH becoming more accessible, any change in DNA content can be observed. In addition, the advent of sequencing technologies may also allow identification of point mutations, or any novel splice variants. However, to demonstrate that a mutant has spread through a population requires regular monitoring, and the procedures to detect mutations are destructive to the cells. The use of CD30 as a marker for karyotypically abnormal cells was suggested [27], since this can be detected on live cells, yet subsequent results have shown that CD30 is not always associated with aneuploidy [28, 29]. In any case, altered gene expression patterns can also be acquired by epigenetic changes in the absence of detectable genetic changes. Although imprinting patterns tend to be fairly stable in human ES cells [30, 31] marked changes in DNA methylation patterns have been reported [32, 33]. A further example is the reported loss of X-chromosome inactivation upon adaptation leading to the functional overexpression of X-linked genes in the absence of any identifiable karyotypic change affecting that chromosome [14].

Functional tests likely present the best way to show that a cell has adapted, yet the assay(s) of choice have still to be decided. Across a number of studies, culture adapted cells have shown decreased apoptosis, increased cloning efficiency, lack of growth factor dependence, reduced differentiation and increased clonogenicity [14, 27, 34], yet sometimes there is discrepancy. For example, [29] showed no difference in apoptosis between a culture adapted subline and its karyotypically normal sister line. Perhaps a more simple way to show that a variant cell has a growth advantage is to mix it with an early passage sister line, and monitor its spread through the culture.

A further issue to be considered when assaying for adapted cells is their in vitro environment. Human ES cells are maintained in a number of different ways, with variation in substrate, media and passaging strategy, so that certain conditions are likely to promote/reduce the selection of certain mutants. Anecdotally, manually passaging human ES cells was assumed to guard against genetic change, and the work of [35] did suggest that when human ES cells are passaged manually they retain a normal karyotype, yet this study does not factor in population size. Manual passaging generally involves transfer of fewer cells on passage than bulk dissagregation techniques, so that the chances of propagating a relatively rare mutation are much decreased. Indeed, [1] showed that population size alone can have a major impact on the likelihood of acquiring a culture adapted cell line. Interestingly, this study also revealed that the maintenance of human ES cells in small cultures as opposed to large cultures reduced the probability of abnormal cell appearance. However, one must also remember that the most common changes observed in culture adapted ES cells (i.e. gain of material from chromosomes 12 and 17) are also seen in TGCT, which are maintained in very different conditions. Thus these particular mutations seem to provide stem cells with an intrinsic growth advantage, and may be very difficult to completely avoid. On the other hand, altered culture conditions might also affect the mutation rate.

2.5 Can Culture Adapted Cells be Utilised in Regenerative Medicine?

It is unlikely that mutation in ES cells will ever be completely prevented, and so eventual culture adaptation after a period in culture is perhaps unavoidable. Given this, it is pertinent to ask how hazardous these cells may be, and whether, in fact, culture adaptation actually presents any opportunities for research and regenerative medicine.

Inevitably, the initial derivation of immortal human ES cell lines must involve a degree of adaptation, although this might be expected to be initially reversible given the experience of using mouse ES cells to produce germ line chimeras and genetically altered mice. Thus, at no point is any ES cell line identical to those cells which exist in the ICM of the embryo. Further, although culture adapted cells

may show tumourigenic behaviour when undifferentiated, there is no guarantee that the growth advantage which afforded their selection will persist when the cells are differentiated. In other words, if those pathways affected by adaptation are intrinsic to the stem cell state, then they will no longer be active when the cells differentiate, and as such these cells will likely behave as normal somatic cells. Since it is unlikely that undifferentiated cells would themselves be used in therapy, then these cells may not pose a safety risk, provided that there are adequate methods for ensuring that no undifferentiated cells persist in preparations of their differentiated derivatives for transplantation. However, since many of the genetic changes that underlie adaptation involve large chromosomal fragments, it may be that genes involved in driving stem cell adaptation might have other consequences for differentiated cells. It will therefore be essential that assays that assess the growth potential of differentiated culture adapted cells, and also the tissue stem cells formed from variant ES cells, are performed.

Apart from their applications in regenerative medicine, a more immediate use for differentiated cells derived from ES cells, and iPS cells, is in toxicology and drug screening in the pharmaceutical industry. Since culture adaptation has the effect of increasing the propensity of stem cells for self-renewal, it may then offer the practical advantage of making the cells easier to maintain. Toxicology screens using ES cells are likely to require large-scale productions of homogeneous cell populations, and these may be more easily achieved using culture adapted cells. Of course it would be essential to confirm experimentally that the adaptive changes in the stem cells do not significantly affect the behaviour of their differentiated derivatives in the particular application for which they are to be used.

2.6 Culture Adaptation as a Tool to Study Stem Cell Fate

To maximise the potential of ES cells, it is imperative that their basic biology is fully understood. Culture adaptation must change the fate of potential ES cells, and as such this process may provide an insight into pathways that affect cell growth. Further, considering the similarity between culture adapted ES cells and EC cells of TGCT, it seems plausible that this in vitro process may act as a model to study this cancer. The use of culture adapted cells may be particularly useful here, since these cells show only a small number of genetic changes compared to the highly aneuploid TGCT. Speculatively, bearing in mind the alleged transcriptional similarity between ES cells and cancer cells [36], it is possible that culture adaptation may also act as a paradigm for other cancers, particularly those with a stem cell component.

At present, there is little information relating to the molecular pathways affected by culture adaptation. From the common amplicons it is possible to pick potential candidate genes, but testing these genes, and their up- and down-stream targets, is not a trivial matter in human ES cells. Genetic manipulation will exert pressures on ES cells, which could promote culture adaptation, making results

difficult to interpret. The issue is further complicated by the fact that adaptation could occur in many ways. A number of mechanisms appear to exist through which pluripotency pathways and/or survival pathways can be regulated, any of which could be the target of an adaptive change. This highlights the earlier point that culture adapted cells may have a range of phenotypes, making their identification challenging.

2.7 Summary

The culture adaptation of human ES cells is an inescapable truth. These cells are maintained in sub-optimal conditions, and over time variant cells will inevitably arise which show an increased growth capacity, and will overtake their normal neighbours. Certain passaging strategies, and of course reduced culture time, are likely to reduce the chances of generating abnormal cells, yet since it appears that at least some adaptive advantages are intrinsic to stem cell behaviour, such methods will only act to stem the tide for so long. Based on their similarity to human ES cells, the same principles will likely apply for iPS cells, and indeed a number of groups have reported mutations in these cells [37, 38]. It is then important to understand the potential problems culture adaptation may cause for regenerative medicine, and also to grasp the opportunities this process may provide.

One of the most concerning issues is the relationship between culture adaptation and oncogenesis. Culture adapted cells have been shown to acquire the same changes as those observed in EC cells, their malignant counterparts, and a number of their behaviours (e.g. increased clonogenicity, reduced apoptosis) are associated with transformation. However, at present, it is not known whether the growth changes observed in culture adapted cells will still manifest when the cells are fully differentiated. Since it is unlikely that undifferentiated cells would be used in therapy, it is possible that the adaptive changes active in the stem cells would have no impact on somatic cells. If this were true, then culture adapted cells may still be suited to therapy, particularly since they are much easier to maintain and expand. However, this would need to be assessed on a case-by-case basis. There are concerns regarding the differentiation potential of the adapted cells, since evidence suggests they may be biased towards, or against certain lineages. Although this may be the case, Melton and colleagues have already reported differing differentiation propensities between different ‘normal’ lines, making adapted lines no more problematic than those already available.

The culture adaptation of ES cells must bias their fate towards self-renewal, and away from differentiation or death. As such, adaptation provides an insight into the fundamental biology of these cells, and also potentially cancer stem cells. The relatively small number of changes observed in these cells should help hone the search for amplicons, and genes of interest, in TCGT in particular. The variety of manners through which adaptation can occur could in fact shed light on a

number of pathways which could impact on stem cell fate, influencing both regenerative medicine and oncological stem cell research.

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