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# Stem cells and regenerative medicine: principles, prospects and problems

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## Abstract

Stem cells have been used routinely for more than three decades to repair tissues and organs damaged by injury or disease, most notably haematopoietic stem cells taken from bone marrow, umbilical cord or, increasingly, from peripheral blood. Other examples, such as grafts of skin to treat severe burns, entail transplantation of stem cells within organized tissue rather than following isolation. The prospect of exploiting stem cells more widely in regenerative medicine was encouraged both by the development of human assisted conception and growing evidence that various adult cells retained greater versatility than had been suspected hitherto. The aim is to employ stem cells as a source of appropriately differentiated cells to replace those lost through physical, chemical or ischaemic injury, or as a result of degenerative disease. This may entail transplantation of just a single type of cell or, more challengingly, require a complex of several different types of cells possessing a defined architecture. Cardiomyocytes, hepatocytes or neuronal cells producing specific transmitters offer promising examples of the former, although how transplanted healthy cells will function in a perturbed tissue environment remains to be established. Recent success in repairing urinary bladder defects with grafts of urothelial and muscle cells seeded on a biodegradable collagen scaffold is an encouraging step towards assembling organs in vitro. Nevertheless, this is still far removed from the level of sophistication required to counter the ever increasing shortfall in supply of kidneys for transplantation. Various problems must be addressed if recent advances in the laboratory are to be translated into clinical practice. In many cases, it has yet to be established that cells derived from adults that retain plasticity are actually stem cells. There is also a pressing need for appropriate assays to ensure that, regardless of source, stem cells maintained in vitro are safe to transplant. Assays currently available for human ES cells are far from ideal. It is, in addition, important to ensure that differentiated cultures are pure and, depending on whether cell renewal is required or to be avoided, retain or lack appropriate stem cells. Neither autografts nor those obtained by so-called ‘therapeutic cloning’ are options for treating condition with an obvious genetic basis. Moreover, claims that some stem cells are more likely than others to yield successful allografts have yet to be confirmed and explained. **To cite this article: R.L. Gardner, C. R. Biologies 330 (2007).**

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## 1. Introduction

Regenerative medicine has a much longer history than present excitement about the potential of stem cells

would lead many to believe. Grafting of skin from the face or buttocks to restore noses amputated for various crimes was carried out in India several thousand years ago [1,2]. In modern times, routine grafting was first performed with the cornea, initial success in the allografting of which was achieved by Edward Zirm in

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Vienna in 1905. Nowadays, autografting is practised for a wide range of purposes, including restoring burnt skin, repairing damaged bone, and by-passing occluded coronary arteries. The first enriched stem cells to be grafted were those of the haematopoietic system, and these remain the best characterized of all stem cells. While haematopoietic stem cells (HSCs) were originally obtained from bone marrow, mobilizing them with cytokines so that adequate numbers can be harvested from peripheral blood has gained support as a less invasive procedure [3]. There is also much interest in using cord blood as a hitherto neglected source of HSCs. However, until these cells can be made to proliferate substantially before frozen storage, without pooling they suffice for only a single graft [4].

The prospect of using stem cells more widely for repair of damaged tissues and organs emerged with the development of human assisted conception *in vitro*, a set of procedures that typically entail producing considerably more early conceptuses than are needed for treating infertility. The suggestion that otherwise ‘spare’ conceptuses might be used for regenerative medicine seems first to have been made by Robert Edwards who, together with the late Patrick Steptoe, pioneered the development of human *in vitro* fertilization [5]. One possibility considered by Edwards was to grow such ‘spares’ *in vitro* to provide organ rudiments for transplantation, an option that was closed in the UK with the passing of the Human Fertilization and Embryology Act in 1990. Recognizing the primitive streak as an indicator of the onset of development of the embryo proper, this legislation set a limit to the period for which human conceptuses could be cultured *in vitro* to 14 days or the appearance of this structure. The other possibility raised by Edwards was to use spare blastocysts to derive embryonic stem (ES) cell lines, as had then been achieved very recently in the mouse [6]. It is perhaps rather surprising that it took substantially more than a further decade for this to be adopted. It is also intriguing that derivation of the first putative human ES cell lines closely corresponded in time with the emergence of claims that cells from adult sources retained greater plasticity than had been generally supposed hitherto.

A defining property of stem cells is, of course, their ability to self-renew as well as to produce more differentiated derivatives. Additional attributes seem to relate principally to minimizing the risk of sustained cycling, namely accumulation of alterations in the genome through errors in DNA replication. Transit amplification is one way of reducing the number of times a stem cell must divide to an extent that depends on how many divisions intervene before differentiation. One view is that

commitment to differentiate rather than renew may not occur until after amplification is under way so that cells may be able to regain the status of stem cells if the latter suffer depletion [7].

Among further strategies proposed to guard against the deleterious effects of DNA damage are very efficient repair mechanisms or exquisite susceptibility to apoptosis in response to such damage. Yet another interesting possibility, originally suggested by Cairns [8] is selective segregation of the products of DNA replication so that stem cells retain template strands. Intriguing findings that are consonant with such a possibility have emerged from recent experiments involving double labelling of stem cells in crypts of the rodent small intestine [9]. Yet another attribute associated with an increasing variety of stem cells is active elimination of Hoechst 33342 and certain other dyes, so that they form a relatively non-fluorescent ‘side-population’ on sorting by FACS machine [10–13]. In view of the various other features they share stem cell might be expected to express certain genes in common whose better characterization may aid enrichment for them [14]. It is interesting in this regard that murine multipotent adult progenitor cells (mMAPCs) express both Oct-4 and Rex-1 [15], albeit at low level compared to murine ES cells, and pluripotential cells in human amniotic fluid are also positive for Oct-4 [16].

## 2. Principles

The essential aim of regenerative medicine is to obtain a sufficient mass of whatever specific type of cell, or more organized entity, such as tissue or organ, is needed to restore the normal physiology of a part of the body damaged by physical, chemical or ischaemic insult, or as a consequence of infectious or genetic disease. This is necessitated by the fact that, particularly postnatally, the intrinsic regenerative capacity of most tissues and organs is very limited in mammals compared to many lower vertebrates. Even where damage does induce significant cellular proliferation, the newly formed cells often fail to differentiate appropriately to replace those that have been lost. Thus, in the nervous system glia tends to be produced at the expense of neurones and, in chronically damaged liver, fibroblastic cells in place of hepatocytes. Better understanding of the conditions required to direct stem cells along one lineage rather than another is clearly needed in order to circumvent such problems. This can be achieved more readily by studying the growth and differentiation of stem cells *in vitro* than *in vivo* and, as well as providing optimal cells for transplantation, could suggest ways in which

endogenous regenerative potential might be enhanced (e.g., [17]).

The ideal is obviously to be able to take cells for transplantation, either directly or following a period of growth and differentiation *in vitro*, from the individual requiring treatment, thereby ensuring graft compatibility. In practice, however, the scope for this is likely to be rather limited, principally because it depends on obtaining appropriate cells from elsewhere in the body in sufficient number to establish a viable graft without further prejudicing the health of the patient. Thus far, the most widespread and successful use of this approach has been with keratinocytic cells for restoring epidermis or repairing damage to the cornea. This is because sufficiently rapid and sustained proliferation can be achieved *in vitro* with stem cells from both epidermis and the limbal region of the cornea to allow very modest biopsies to be taken [18–21]. Claims regarding the facility with which stem cells from bone marrow can be re-programmed are particularly interesting because this represents a rich source of such cells. Of course, allo- rather than auto-grafts are the only option where tissue damage has a clear hereditary basis, unless the cells used for grafting are modified genetically so as to correct the defect. However, even allografts where obvious histocompatibility differences exist and immunosuppression is limited to topical application of corticosteroids enjoy unusually high success rates in the case of the cornea. This does not seem to be attributable simply to this tissue's lack of vascularisation [22].

That evidence is emerging for the persistence of stem or progenitor cells in an increasing number of adult organs, which, unlike epidermis and intestine, do not undergo rapid turnover raises the possibility of using growth factor or cytokines for activating or enhancing endogenous repair. Here, the concern would be to ensure appropriate localization of an optimal combination of such molecules for the requisite period of time. Whether this might best be achieved by means of implanted genetically-modified cells or in other ways remains to be seen. This approach is likely to be informed by closer study of regeneration in vertebrates in which the phenomenon is much better developed.

### 3. Prospects

While further technical improvements will doubtless be made, the use of stem cells to restore the haematopoietic system, and repair wounds, particularly to the skin and cornea, is now an increasingly routine part of clinical practice. Extending the scope of this approach is perhaps most straightforward where there is a need for

grafts of single types of cells such as specific neurones, cardiomyocytes or hepatocytes. Greater challenges are posed where grafts composed of cells of more than one type organized in a specific architecture are required. Encouraging progress here is exemplified by recent success in using grafts established from biopsies of patients' urothelial and smooth muscle cells grown on opposite sides of a collagen matrix *in vitro* to rectify defective urinary bladders [23]. Nevertheless, this is a relatively modest challenge in terms of the complexity of organogenesis required compared to a structure such as the kidney.

The incidence of chronic renal failure is rising inexorably, largely as a consequence of an increase in obesity-induced type-II diabetes. Progress of this very serious condition can often be so insidious that patients remain asymptomatic until kidney function is irreversibly impaired. Moreover, the shortage of suitable cadavers is so acute that many clinicians are very actively promoting live donation. The basic functional units of the kidney are the nephrons and, in mammals, the total, species-specific complement of these sophisticated structures is established either before or shortly after birth. The production of new nephrons has not been observed thereafter, regeneration being limited to repair of damage within individual nephrons or their enlargement. Embryologically, the nephron is of dual origin, arising through a complex sequence of inductive interactions between the ureteric bud and metanephric mesenchyme, with the bud forming the collecting duct and the mesenchyme the remainder of its structure. In the human, development of the metanephric kidney begins by 5 weeks and the full complement of nephrons is formed before 32 weeks. Obviously, producing structures as complicated as nephrons *in vitro* poses very formidable technical difficulties. However, some progress has been made in developing more simple tissue arrangement from stem cells within nephrons, in conjunction with mechanical dialysis equipment [24]. Given that the genesis of nephrons occurs between 10 and 15 weeks of gestation in man, the possibility of using metanephric rudiments from abortuses as starting material clearly warrants consideration, especially since success in grafting embryonic metanephroi to adults had been accomplished in other mammals [25,26]. Also of interest in this context is recent work involving seeding the nephrogenic region of rodent fetuses during its morphogenesis with mesenchymal stem cells from human bone-marrow [27,28]. The human bone marrow derived cells are evidently directed thus to differentiate into various types of tubular and other metanephric cells.

## 4. Problems

### 4.1. *Assessing the developmental status and physiological normality of stem cells and their derivatives*

The only vertebrate from which pluripotential stem cells from early embryos that can form germ as well as all somatic cell types have been obtained is the mouse. Moreover, even in this species success has been achieved only with certain strains [29]. Cell lines derived from morulae or blastocysts of various other mammals have given somatic but not germline chimaerism following blastocyst injection or morula aggregation. Those from yet other species have so far altogether failed to give any discernible chimaerism [29]. This obvious heterogeneity in properties and potential of cells derived at similar stages in early development from different species raises questions about the appropriateness of assigning them the common status of ES cells. In the case of the mouse, it is clear that what may be termed ‘true’ ES cells originate from the epiblast, the progenitor tissue of both the foetus and various extra-embryonic structures [30]. Murine epiblast is permissive for ES cell derivation only during an extremely restricted interval, which seems to correspond rather precisely with the period during which its cells can colonize the blastocyst [31]. The basis of this restriction is not clear, especially since embryonal carcinoma cells obtained from ectopic grafts of more advanced epiblast can yield chimaeras following blastocyst injection [32]. Most intriguingly, even cells from adults can sometimes give generalized chimaerism by this route [15].

Although ES lines can be obtained efficiently from cleavage stages and morulae in the mouse, these do not differ discernibly in properties and developmental potential from those of blastocyst origin [33]. It seems, therefore, that regardless of the stage at which pre-implantation conceptuses are explanted, cells may have to attain the status of nascent epiblast in order for their continued division to be uncoupled from further differentiation. The generally low success rate in deriving human ES cell lines may relate, at least in part, to the unavoidable dependence as starting material on conceptuses developing in vitro. In vitro developed blastocysts are inferior to in vivo in yielding ES cells the mouse, [34], though the adverse effect of culture can be partially mitigated by sequential use of different media [35]. The latter may also aid the derivation of human ES cells [36].

How well do stem cells fare during prolonged passage in vitro? This is less easy to answer than is often

supposed even for ES cells, although it is commonly asserted that they can be propagated indefinitely in culture without loss of potency. This assertion is made especially with regard to human ES cells that, largely on the basis of retention of a normal karyotype, are often held to withstand prolonged passage in vitro better than their murine counterparts do. Failure of murine ES cells to give chimaerism by blastocyst injection following prolonged passage has been attributed to acquisition of epigenetic changes that cannot be reversed by re-programming their nuclei via what has come to be known as ‘therapeutic’ cloning [37]. However, in this, as in many other studies, the cells were assumed to have retained euploidy on the basis of chromosome counts rather than detailed karyological analysis. According to our own recent experience with mouse ES cells, even lack of karyotypic change may not be a reliable indicator of normality. Thus, although strictly monoclonally derived as opposed to presumptively polyclonal mouse lines can remain entirely euploid for up to 40 or more passages, and show no discernible genomic rearrangements, they do not necessarily behave normally when assessed by blastocyst injection. Notably, a euploid monoclonal line derived from a blastocyst which, at low passage, yielded respectable chimaerism on blastocyst injection, not only failed to give chimaerism at high passage but disrupted the development of host embryos (F.A. Brook, T.J. Davies, E.P. Evans and R.L. Gardner, unpublished observations). Curiously, a few weak postnatal chimaeras were obtained if the number of cells injected into each blastocyst was reduced from the usual 10–15 to just 3. Since, this monoclonal line showed no genomic changes when analysed by representational oligonucleotide microarray analysis at a resolution of ca. 30 000 base pairs (Ira Hall, personal communication of unpublished observations) a likely, but as yet unproven, explanation for its altered behaviour is that it has indeed undergone stable deleterious epigenetic change [38]. Since this is not seen with all monoclonal lines at high passage, we are at present attempting to ascertain whether such adverse change correlates with restriction in the capacity of the cells to differentiate in vitro. This could help inform studies concerned with critical evaluation of human ES lines that are destined for clinical use in regenerative medicine for which, unless done interspecifically, testing by blastocyst injection is not an option. So far, however, the results of injecting human ES cells into mouse blastocysts have not proved very informative [39]. Moreover, enduring intra-specific chimaerism with ES cells in non-human primates has yet to be demonstrated [40].

The haematopoietic stem cell from adult bone marrow is still the most thoroughly characterized stem cell. Putative stem cells from various other adult tissues and organs need to be assessed against this yardstick before contemplating their use for regenerative purposes. This is because it is conceivable that cells that would never have cycled again if left in situ may be induced to engage in extensive proliferation following isolation and exposure to a cocktail of growth factors they would not have encountered in vivo. Consequently, sustained growth in vitro in the absence of overt differentiation may not be unique to stem cells. Nonetheless, the case has been made for having identified stem cells from a variety of tissues, and this has been aided by finding an increasing number of molecular markers that are shared between such cells.

A further important issue, regardless of whether the stem cells are of embryonic, foetal or adult stem origin, is establishing that cells into which they are induced to differentiate are normal. While it may be convenient to assess differentiation in terms of expression of specific marker molecules, this is no substitute for devising rigorous physiological assays. A particular compelling example of such an assay is the grafting of foetal hypothalamic tissue into the brains of adult mice that fail to mature sexually through homozygosity for deficiency of gonadotrophin releasing hormone. Such grafts prompted rapid sexual maturation and reversal of infertility [41,42]. Obviously, for human cells the scope is limited to assays based on in vitro culture versus xenografts, the latter posing the problem of choice of appropriate graft site [43,44], as well as host species.

#### 4.2. Selection of appropriately differentiated cells

As well as ascertaining that differentiated cells are physiologically normal, there is the further question of whether it is important to ensure that they are homogeneous – i.e. that only cells of the desired type are included in grafts. The need to eliminate residual stem cells from differentiated cultures of ES origin is generally accepted because of their exceptional tumorigenicity at low inoculum size [43,45]. While injection of undifferentiated ES cells into articular cartilage defects produced repair without any tumours being detectable eight weeks thereafter [46], presumed contaminating ES cells yielded tumours after a similar interval following sub-retinal grafting of ES-derived neural precursor cells [47]. It is possible that interspecific grafts used in the cartilage study are less susceptible to tumorigenesis than interspecific grafts used in the retinal one. This may also be true of allogeneic as opposed to syn-

genic grafts. Murine ES cells transplanted locally into the brains of host mice showed down-regulation of expression of stem cell markers following multiplication and migration towards sites of experimentally-induced injury [48]. However, no evidence was provided that cells had differentiated appropriately, and the interval between transplantation and analysis was too short to exclude their participation in tumorigenesis.

Whether inclusion of some inappropriately differentiated cells as opposed to persisting stem cells in grafts really matters is not clear. It is interesting, in this context, that no prior differentiation or selection of cells was undertaken in the successful use of bulge stem cells from hair follicles to re-myelinate damaged sciatic nerve in rats [49]. If purity of cell populations for grafting is indeed necessary, it is questionable in view of public mistrust of genetic modification whether engineering cell type-specific expression of an antibiotic resistance gene will prove an acceptable approach for achieving this in a clinical situation. Use of flow cytometric methods offers an alternative providing, of course, that a suitable specific marker is available [50]. Yet another strategy is to exploit the differential sensitivity of ES cells relative to their differentiated derivatives to various agents such as ceramide analogues [45].

A further important issue is whether grafts are intended to make good loss of a population of cells that are terminally differentiated and thus entirely post-mitotic or are required to self-renew. In the latter case, appropriate stage stem cells need both to be present in the graft and to persist thereafter. The notion of dependence of stem cell on specific niches for their maintenance is now familiar [51], particularly as a result of elegant genetic analysis of germline stem cells of both sexes in *Drosophila* [52,53]. At present, rather less is known about conditions required for the maintenance of stem cells of somatic tissues in mammals. It is, however, encouraging that relatively simple conditions have been found to support sustained in vitro propagation of various types of stem cells in addition to ES cells. These include neural stem cells, and especially those of epidermis, hair follicles, and cornea [18,20,54]. In the case of the latter three types of stem cells, conditions of culture that are used presently for regenerative medical purposes are essentially as defined many years ago by Howard Green and his colleagues [55]. Interestingly, these include use of gamma-irradiated murine 3T3 feeder cells and bovine serum [18], components that many argue need to be avoided in culturing human ES cells lines intended for clinical use. Moves to the use of same species feeders for human ES cells have already been made (e.g., [56]).



### 4.3. Avoiding graft rejection

As noted earlier, wherever practicable, autografts are the obvious choice, although there are at present rather few situations where a suitable source of stem cells is available from the patient. Obviously, if efficient reprogramming of stem cells from skin or bone marrow directly rather than via nuclear transplantation proved feasible, the scope for autografting would be enormously enhanced. There is, however, evidence relating to skeletal myoblasts to suggest that cells may be altered by culture so as to render them susceptible to destruction by NK cells even when grafted syngeneically [57].

A further possible way of avoiding allografting, which has been much discussed, is to resort to what has rather prematurely been termed ‘therapeutic cloning’. This technique will unquestionably aid the study of human genetic diseases *in vitro* in the types of differentiated cells in which they are normally manifest (e.g., [58]). However, there is division of opinion within the biomedical community as to whether this is a realistic option clinically. Aside from the low efficiency of obtaining cloned blastocysts for deriving patient-specific ES cell lines and the consequent heavy demand for oocytes, questions remain regarding the likely normality of the resulting cells with respect to genomic imprinting. Hence, for the foreseeable future, the continued widespread use of allografting seems almost inevitable. Therefore, one pertinent consideration is whether tissues from some types of stem cells are likely to fare better as allografts than those from others. Another is the prospect of achieving graft-specific states of immunological tolerance.

A recent study in mice provided no support for the notion that the differentiated derivatives of ES are less susceptible to rejection than other types of grafts, even where there were only minor as opposed to major histocompatibility differences between donor and host (N. Robertson, F. Brook, R.L. Gardner, H. Waldmann and P. Fairchild, unpublished observations). However, the situation may be different for mesenchymal stem cells and, as noted earlier, also for limbal stem cells of the cornea. A growing number of studies attest to the possibility that mesenchymal stem cells enjoy a special status immunologically. This is suggested most cogently by the production of widespread enduring chimaerism in sheep following introduction of human mesenchymal cells into immunologically competent foetuses [59]. However, while numerous *in vitro* studies argue for active interference of these cells with the immune response rather than their failure to express histocompatibility antigens, there is still no general consen-

sus on how this is achieved [60–64]. More significantly, contradictory results have been obtained regarding the lack of immunogenicity of these cells in *in-vivo* studies [65–67]. Most interestingly, successful allografting of developing metanephroi has been achieved where corresponding grafts of adult kidneys failed [25].

Further areas of active exploration to avoid recourse to immuno-suppression, are induction of tolerance to grafts or their protection from humoral and cell-mediated immune attack, either by their artificial encapsulation or by their location in immunologically privileged sites. Inducing tolerance may possibly prove easier with tissues derived from ES cells than from other sources (N. Robertson, F. Brook, R. L. Gardner, H. Waldmann and P. Fairchild, unpublished observations).

### 4.4. Location of grafts

Grafts that are designed to restore specific endocrine functions can presumably be placed other than at the site of damage since, with few exceptions, their location is unimportant providing they become sufficiently well vascularised to work effectively. Introduction of dissociated cells directly into the vascular system may be sufficient to achieve the desired outcome where appropriate homing of transplanted cells can be effected by this route. Where grafts need to be placed specifically at the site of damage, e.g., in countering neurodegenerative diseases involving extracellular deposition of insoluble protein aggregates, there is the question of whether they will function any better than the native cells they are designed to replace unless a more normal environment is restored. Indications from several recent studies are encouraging with regard to the central nervous system in suggesting that transplanted stem cells may not only survive at sites of damage, but also actively migrate towards them [48]. The situation is less clear for damaged myocardium where it may be more pressing to obtain rapid neovascularization if grafts are to survive.

### 4.5. Structure of grafts

In some cases, the extracellular matrix of damaged tissue may remain sufficiently well ordered to provide the cues necessary to allow transplanted cells to become organized appropriately to restore normal function. Otherwise, as touched on earlier, it may be necessary to attempt to obtain organogenesis *in vitro*, which means achieving defined *in vitro* arrangements of cells in three dimensions rather than just two. This obviously depends on use of scaffolds for which a wide variety of

materials of both biological and non-biological origin is currently being evaluated. Many challenges confront tissue engineering, a rapidly developing field that has encouraged very productive interdisciplinary collaborations between the physical and biological sciences.

## 5. Conclusions

The burgeoning interest in stem cells will undoubtedly serve to enlarge understanding of their properties and potential in ways that hold great promise for extending the scope of regenerative medicine. Nevertheless, translating advances in the laboratory into sound clinical practice presents a series of formidable conceptual and technical challenges. As well as exploring the relevant attributes of stem cells from all sources, attention should be focused on ways of stimulating endogenous repair as an alternative to grafting.

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