Plastic adult stem cells: will they graduate from the school of hard knocks?

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Summary

Notwithstanding the fact that adult bone marrow cell engraftment to epithelial organs seems a somewhat uncommon event, there is no doubt it does occur, and under appropriate conditions of a strong and positive selection pressure these cells will expand clonally and make a significant contribution to tissue replacement. Likewise, bone-marrow-derived cells can be amplified in vitro and differentiated into a multitude of tissues. These in essence are the goals of regenerative medicine using any source of stem cells, be it embryonic or adult. Despite such irrefutable evidence of what is possible, a veritable chorus of detractors of adult stem cell plasticity has emerged, some doubting its very existence, motivated perhaps by more than a little self-interest. The issues that have led to this state of affairs have included the inability to reproduce certain widely quoted data, one case where the apparent transdifferentiation was due to contamination of the donor tissue with haematopoietic cells and, most notoriously, extrapolating from the behaviour of embryonic stem cells to suggest that adult bone marrow cells simply fuse with other cells and adopt their phenotype. While these issues need resolving, slamming this whole new field because not everything is crystal clear is not good science. The fact that a phenomenon is quite rare in no way mitigates against its very existence: asteroid collisions with the Earth are rare, but try telling the dinosaurs they do not occur! When such events do occur (transdifferentiation or collision), they certainly can make an impact.

Key words: Stem cells, Bone marrow, Transdifferentiation, Cell fusion

Introduction

Work on murine embryonic stem (ES) cells over many years has established the amazing flexibility of embryonic stem cells, which essentially can generate almost all cells that arise from the three germ layers. However, since the publication of two papers in 1998 describing the growth in vitro of human ES cells derived either from the inner cell mass (ICM) of the early blastocyst (Thomson et al., 1998) or the primitive gonadal regions of early aborted foetuses (Shamblott et al., 1998), the field of stem cell research has gone into overdrive. There is now a genuine belief that stem cell research will deliver a revolution in terms of how we treat cardiovascular disease, neurodegenerative disease, cancer, diabetes and the like. Regenerative medicine has been grabbing many headlines in both the biomedical and the popular press over this period – not just concerning ethical issues but also regarding the therapeutic potential of ES cells versus adult stem cells, although we suspect that sometimes a view on the latter may be clouded by one's moral standpoint.

Within the past few months doubt has been cast upon claims that certain adult stem cells, particularly from bone marrow and the central nervous system, when removed from their familiar niches, can jump lineage boundaries to generate completely new cell types. This has led to a stream of banner headlines along the lines of 'Cell fusion leads to confusion' (Wurmser and Gage, 2002), 'Biologists question adult stem-cell versatility' (DeWitt and Knight, 2002), 'Plasticity: time for a reappraisal?' (Holden and Vogel, 2002), 'Is transdifferentiation in trouble? (Wells, 2002) and 'Are somatic stem cells pluripotent or lineage-restricted? (D'Amour and Gage, 2002). Here, we examine the key issues.

The case for adult stem cells

The evidence for so-called adult stem cell plasticity often relies on the appearance of Y-chromosome-positive cells in a female recipient of a bone marrow transplant from a male donor. Alternatively, markers such as LacZ or GFP have been used, and these techniques are usually combined with lineage markers in attempts to decide whether there has been a switch in the fate (transdifferentiation) of the transplanted cells. Claims have been made for adult bone marrow stem cells differentiating into all manner of tissues, including skeletal muscle, cardiomyocytes and endothelia, neurones, hepatocytes and bile duct epithelia, renal epithelia and podocytes, and gut mucosal cells and associated myofibroblasts (reviewed by Poulsom et al., 2002).

Two recent publications in *Nature*, however, have suggested that these phenomena could be due to the fusion of bone marrow cells with the differentiated cells in the new organ – for example, the liver. When genetically altered bone marrow from GFP transgenic mice was mixed with ES cells, a very

small proportion of the bone marrow cells fused with the ES cells (2-11 hybrid clones per 10^6 marrow cells), and these cells could subsequently adopt many of the phenotypes typical of ES cell differentiation (Terada et al., 2002). A similarly low frequency of fusion (one event per 10^5 CNS cells) has also been reported when mouse CNS cells (also genetically altered) were mixed with ES cells, and in this case the derived hybrid cells were able to show multilineage potential when injected into blastocysts, most prominently into liver (Ying et al., 2002).

Before one rushes headlong to the conclusion that all apparently transdifferentiated cells are heterokaryons, and although one should look at the genotype of cells claimed to have been generated from tissue of another type, several observations nevertheless strongly indicate that the phenomenon of adult stem cell plasticity is not dead in the water – far from it. It is interesting to look at epithelial tissue from mothers of male offspring. Post-partum exacerbation of thyroiditis is sometimes observed and could be due to transplacentally acquired foetal cells that cause an alloimmune disease previously regarded as an autoimmune disease (Srivatsa et al., 2001). Particularly noteworthy was one female patient with clusters of fully differentiated thyroid follicular cells bearing one X and one Y chromosome; of course the source of the transdifferentiated cells was the foetus rather than a deliberate transplant, but nevertheless no follicular cells were XXXY, which suggests cell fusion was not responsible for the phenomenon, even when foetal cells were the source. In a similar vein, an investigation by fluorescence in situ hybridisation (FISH) of the karyotype of male donor peripheral blood stem cells that had apparently transdifferentiated into epidermal, hepatic and gastric mucosal cells in human female recipients clearly demonstrated the presence of only one X and one Y chromosome (Korbling et al., 2002). Likewise, Okamoto et al. (Okamoto et al., 2002) have found no evidence for cell fusion being responsible for the apparent engraftment and differentiation of bone marrow cells into mucosal epithelial cells throughout the gastrointestinal tract of human female recipients of male bone marrow. They noted sustained engraftment over many months/years, up to 13% (much higher than the reported fusion rates with ES cells) of colonocytes being marrow-derived shortly after the development of GVHD (graft versus host disease), but more significantly Y-chromosome-positive epithelia did not stain more intensely than other epithelial cells with 4,6-diaminidino-2 phenylindole (DAPI): these observations argue against cell fusion being the mechanism. Moreover, male recipients of male marrow had only one Y chromosome. Of course, the detection of polyploidy by in situ hybridisation is not without its problems, not least of which is the fact that not all chromosomes can be visualised in a tissue section of finite thickness. Perhaps a better approach is that of Kleeberger et al. (Kleeberger et al., 2002), who examined liver chimerism in liver allografts by PCR analysis of a highly polymorphic tetranucleotide repeat marker at the human β-actin-related pseudogene. Using laser-assisted microdissection of small areas of pure hepatocytes and bile duct cells, they did indeed find that almost all samples displayed the genotype of both donor and recipient, thus suggesting engraftment from an extrahepatic source. However, cell fusion could not be excluded since the samples contained more than one cell, but if single cells could be captured this approach could settle the cell fusion issue.

Although not directly disproving that cell fusion happens in vivo, a number of in vitro observations also strongly point to the plasticity of adult cells. For example, Catherine Verfaillie and colleagues (Schwartz et al., 2002; Jiang et al., 2002a; Jiang et al., 2002b) have isolated so-called multipotent adult progenitor cells (MAPCs) from mesenchymal cell cultures obtained from human and rodent bone marrow. These MAPCs are capable of in excess of a hundred population doublings and can be induced to differentiate not only into mesenchymal lineages but also into endothelia, neuroectoderm (neurons, astrocytes and oligodendrocytes) and endoderm (hepatocytes). Moreover, the group has provided evidence of function as well as phenotype, allaying the fears expressed by some commentators that many transdifferentiated cells merely take on the appearance rather than the function of their new creation.

In the rat, a population of bone-marrow-derived hepatocyte stem cells (BDHSC) has been identified on the basis of being β_2-microglobulin negative and Thy-1 positive (β_2m^-/Thy-1^+) (Avital et al., 2001). These cells are more numerous in damaged liver and express albumin, even in the bone marrow. After these BDHSCs are co-cultured with cholestatic hepatocytes (separated by a semi-permeable membrane, so no fusion could occur) they differentiate into hepatocytes and are able to metabolise ammonia into urea as efficiently as existing hepatocytes; prior co-culture with healthy hepatocytes is not sufficient to achieve this. So here we have another situation where fusion could not be responsible for the transdifferentiation. Likewise, pancreatic cells can readily differentiate into their embryological cousins, the hepatocytes, both in vitro (Shen et al., 2000) and in vivo (Krakowski et al., 1999), and no fusion or heterokaryon formation has been described. Moreover, in the in vitro study, the induced transdifferentiation commonly occurred directly without cell cycle traverse and involved the vast majority of a pure population of exocrine pancreatic cells – which could not involve fusion with another cell type.

Cells derived from sorted bone marrow cells can also apparently differentiate into cardiomyocytes. In female mice, direct injection of *Lin*$^-$ *c-kit*$^+$ bone marrow cells (from male EGFP transgenic donors) into the contracting area bordering an experimental infarct results in more than half the infarcted area being colonised by donor cells within nine days (Orlic et al., 2001). Since cardiomyocytes are cells that have a minimal regenerative capability, it is extremely unlikely that cell fusion has occurred and bestowed the resultant hybrids with hitherto-unrecognised migratory/proliferative powers.

In a ‘proof of principle’ demonstration of the potential therapeutic use of bone marrow, mice with a metabolic liver disease have been cured (Lagasse et al., 2000). Female mice lacking the enzyme fumarylacetoacetate hydrolase (FAH$^{-/-}$, a model of fatal hereditary tyrosinaemia type 1), a key component of the tyrosine catabolic pathway, can be rescued biochemically by 10^6 unfractionated bone marrow cells that are wildtype for FAH. Moreover, only purified HSCs (c-kit^{hi}Thy^{lo}Lin$^-$Sca-1^+) are capable of this functional repopulation, as few as 50 of these cells being capable of hepatic engraftment when haematopoiesis is supported by 2×10^5 FAH$^{-/-}$ congenic adult female bone marrow cells. The salient point to arise from this powerful demonstration of the therapeutic potential of bone marrow cells was that, although

the initial engraftment was low (approximately one bone marrow cell for every million indigenous hepatocytes), the strong selection pressure exerted thereafter on the engrafted bone marrow cells resulted in their clonal expansion to occupy almost half the liver. This positive selection was achieved by withdrawal of 2-(2-nitro-4-trifluoro-methylbenzoyl)-1,3 cyclohexanedione (NTBC), a compound that blocks the breakdown of tyrosine to fumarylacetoacetate (FAA) in the FAH-deficient mice. In the absence of NTBC, FAA accumulates and destroys the hepatocytes; thus the ensuing regenerative stimulus promotes the growth of the engrafted cells. Furthermore, in the absence of NTBC, no engraftment is seen (Wang et al., 2002). Likewise, bone-marrow-derived hepatocytes can be selectively expanded if they are engineered to overexpress Bcl-2, and then the indigenous cells are targeted for destruction by an anti-Fas antibody (Mallet et al., 2002). One could also add that, if fusion were responsible for all these observations made in the liver, then clearly these hybrids would have a selective growth advantage, turning unhealthy hepatocytes into metabolically competent hepatocytes and would not negate the therapeutic potential of bone marrow cells in the liver. Expressing a similar sentiment, Blau has suggested that if cell fusion is responsible for the apparent reprogramming of certain adult cells then there is something 'exciting' about rescuing damaged cells through fusion, with, for example, bone-marrow-derived cells providing a healthy and entire genetic complement, even one that has been manipulated for gene therapy (Blau, 2002).

The case against adult stem cell plasticity

Apart from cell fusion, the only other major issue has centred on the reproducibility of certain high profile observations. For example, Bjornson et al. demonstrated that single $LacZ^+$ neural stem cells form large colonies (neurospheres) in vitro that have all three neural lineages present and that such neurosphere cells also have haematopoietic potential when transplanted into sublethally irradiated mice (Bjornson et al., 1999). An in vitro clonogenic assay of the bone marrow from the transplanted mice showed that in these studies most (~95%) of the colonies were positive for β-galactosidase, which suggested they were of neural stem cell origin. Significantly, cultured neural stem cells neither proliferated nor formed haematopoietic progeny in the same clonogenic assays without prior injection into irradiated host mice – this indicated that an appropriate microenvironment is necessary for transdifferentiation (Watt and Hogan, 2000). Likewise, clonally derived human neurosphere cells derived from foetal tissue and expanded in vitro by EGF and/or FGF-2 show no haematopoietic potential in culture but can establish long-term haematopoiesis in human bone fragments in SCID-hu mice (Shih et al., 2001).

A recent study using a similar protocol to that emplyed by Bjornson and colleagues rigorously tested the haematopoietic potential of murine neurosphere cells and was unable to find any evidence of haematopoietic differentiation in a large group (108) of sublethally irradiated mice each transplanted with 10^6 neurosphere cells, which suggested that haematopoietic potential was not a general property of neural stem cells (Morshead et al., 2002). However, Lako et al. have found that cells surrounding the murine vibrissa (whisker) follicle (dermal sheath cells) and also dermal papilla cells can exhibit both in vitro haematopoietic potential and reconstitute the bone marrow of lethally irradiated mice and may be passaged into secondary recipients (Lako et al., 2002) . Moreover, the authors considered that contamination of the dermal-derived cells by haematopoietic cells was not responsible for their haematopoietic potential, unlike another recent report that found that the haematopoietic potential of muscle cells was due to haematopoietic cell contamination of the donor muscle cells (McKinney-Freeman et al., 2002). From a similar tissue source, and supporting the concept of adult stem cell plasticity, multipotential cells have been isolated from rodent and human skin, specifically from the dermis, and named skin-derived precursors (SKPs) (Toma et al., 2001). These cells can undergo multiple rounds of cell division in vitro and can be directed to undergo differentiation along neuroectodermal lines (neurons and glial cells) or mesodermal lines (adipocytes and smooth muscle) – so no cell fusion here! These cells are distinguishable in their behaviour from plastic-adherent bone marrow mesenchymal cells, and apparently clonally derived spheres of these cells could generate all the above lineages – so perhaps they are equivalent?

Another controversial issue centres on the claim that just one single cell from a male mouse bone marrow population (lineage-depleted and enriched for $CD34^+$ and $Sca\text{-}1^+$ by in vivo homing to the bone marrow) can, when injected into female recipients along with 2×10^4 female supportive haematopoietic progenitor cells, give rise to a spectrum of epithelial cells: at 11 months a surprisingly high proportion of type II pneumocytes were Y chromosome positive, although fewer Y-chromosome-positive cells were seen in other tissues – for example, 2% were cytokeratin-positive in the skin (Krause et al., 2001). The high level of lung engraftment was attributed to lung damage caused by either the irradiation to eradicate endogenous bone marrow to facilitate bone marrow transplantation or viral infection in the temporarily immunosuppressed animals. Although the experiments are not directly comparable, the observations of Wagers at al. led the authors to speculate that 'transdifferentiation of circulating HSCs is an extremely rare event if it occurs at all' (Wagers et al., 2002)! In one approach, they transplanted single GFP-marked HSCs into lethally irradiated nontransgenic recipients and, although GFP^+ HSCs colonised the bone marrow, no significant contribution was made by these cells to epithelia. The other approach involved the long-term study of parabiotic pairs between GFP^+ mice and wild-type mice, and once again significant chimerism was observed in the bone marrow but not in other organs.

A third area where apparently conflicting observations have been made concerns the ability of bone marrow to contribute to neural tissue. For example, Mezey and colleagues, studying homozygous PU.1 mutant female mice (PU.1 is a transcription factor required for the histogenesis of six of the haematopoietic lineages) rescued these mice with a life-saving bone marrow transplant from male wild-type donors and found that up to 4.6% of cells in the CNS were Y chromosome positive and that up to 2.3% of Y-positive cells possessed the neuronal markers NeuN and neuron-specific enolase (NSE) (Mezey et al., 2000). By contrast, Goodell and colleagues (Castro et al., 2002) found no neuronal differentiation in eight lethally irradiated recipients of 2×10^3 SP cells from ROSA26 donors or in twelve recipients of 2×10^6 whole bone marrow cells, even though

some of the recipients in both groups had a neuronal injury. What are we to make of these discrepancies? It is difficult to say, but these are isolated examples, and in each case the experimental conditions were not identical.

Conclusions

So what does all the criticism add up to? Regarding cell fusion, we propose that, until experiments showing heterokaryon formation when adult stem cells 'transdifferentiate' in vivo are carried out, then extrapolations from rare events involving cultured ES cells are premature. The field of adult stem cell plasticity is still very much in its infancy, and although one or two quite startling observations have not been confirmed, this is no reason to damn the whole field. Remember that apoptosis was almost laughed to ridicule in the early seventies, one of its original proponents (Andrew Wyllie) being unable to secure appropriate funding for further research: 7 October 2002 saw the Nobel Prize for Physiology and Medicine awarded in recognition of the discovery of cell death controlling genes in *Caenorhabditis elegans*!

Of course, it is one thing for a circulating cell to engraft in another organ and assume some or all of the phenotypic traits of that organ (transdifferentiation – the acquisition of a new phenotype), but it is quite another for one to claim that the engrafted cell has become a local stem cell in its new niche. So while a scattering of engrafted, apparently transdifferentiated cells in no way establishes that these cells are capable of robust clonal expansion in their new environment, and indeed this is what is observed in the majority of recently published papers, it should not be taken as a criticism. As we have noted, significant clonal expansion requires the presence of a persistent selection pressure strongly favouring the engrafted cells (Lagasse et al., 2000; Wang et al., 2002).

The demonstration of engrafted cells becoming stem cells within their new location would, ideally, require the isolation and transplantation of single cells that self-renew and produce a family of descendents that eventually become fully functional. However, some commentators have added that this phenomenon should be shown to occur 'naturally' (without intervening culture) in organs not forced to undergo organ degeneration before accepting that stem cells jump a lineage boundary (Anderson et al., 2001). Clearly, it is difficult to track cells without intervention, and most of the studies to date involve damage consequent upon ablation of bone marrow by irradiation or chemical means, or the traumas of surgery and rejection, where organs have been transplanted and then studied some time later. A counter argument is that a degree of organ damage is essential to allow transdifferentiation or stem cell plasticity to take place at recognisable levels. It may be that migration of bone marrow stem cells throughout the body acts essentially as a back-up system that can *in extremis* augment an organ's intrinsic regenerative capacity. As Jonas Frisen has eloquently reiterated (Holden and Vogel, 2002), the 'no alteration in culture' postulate is only relevant if the goal is to study normal physiology, but if you are studying what is possible then it is absolutely OK to culture.

Finally, stem cell plasticity is encountered in other situations: disturbances in the local stem cell microenvironment occur commonly in vivo, resulting in major switches in tissue phenotype – metaplasias (Tosh and Slack, 2002) – and even more extremes of stem cell plasticity must occur when animals are reproductively cloned by somatic cell nuclear transfer. Of course, nothing is really new; scholars of Icelandic literature (The Prose Edda, written in 1220) will be aware that Thor knew that he could regenerate his slaughtered goats by placing their intact bones (with the bone marrow) on their skins (Thor's Journey to Utgard).

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