Adult stem cells – perspectives in treatment of metabolic diseases

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Abstract

There is currently great excitement and expectation concerning the differential potential of adult stem cells or adult cells with capacity of differentiation. As the body of work concerning transdifferentiation of somatic stem cells and bone marrow derived stem cells grows, the number of critics increases steadily questioning the reliability of reported findings. So scientists are now challenged more and more to prove that resulting differentiated somatic cells originated from somatic adult stem cell through a transdifferentiation process. Phenomenons such as fusion of cells have to be ruled out and the origin of the differentiated cell has to be determined by specific techniques i.e. in situ hybridisation. Cellular mimicry through uptake of specific factors out of the medium is questioned to be the reason for cells staining positive for Insulin. Some multipotent adult stem cells can cross lineage boundaries and differentiate into somatic cells of other lineages after being relocated. Bone marrow cells have been described to have the greatest plasticity among adult stem cells regenerating damaged liver or myocardium. It has been proposed that the differentiation of bone marrow derived adult stem cells occurs naturally even in healthy organs as a physiologic process of tissue-regeneration. Others believe that organ damage is essential to induce transdifferentiation by release of organ specific microenvironmental factors. We here try to constitute necessary data which should be demonstrated to give substantial evidence for transdifferentiation of newly characterized cells including exclusion of fusion, phagocytosis or DNA uptake, description of the outset cell, differentiation into all three germ layers and functional parameters.

Introduction

There is currently great excitement and expectation concerning the differential potential of adult stem cells or adult cells with capacity of differentiation. As the body of work concerning transdifferentiation of somatic stem cells and bone marrow derived stem cells grows, the number of critics increases steadily questioning the reliability of reported findings (1). So scientists are now challenged more and more to prove that resulting differentiated somatic cells originated from somatic adult stem cell through a transdifferentiation process. Phenomenons such as fusion of cells have to be ruled out and the origin of the differentiated cell has to be determined by specific techniques i.e. in situ hybridisation.

Cellular mimicry through uptake of specific factors out of the medium is questioned to be the reason for cells staining positive for Insulin (2).

Some multipotent adult stem cells can cross lineage boundaries and differentiate into somatic cells of other lineages after being relocated. Bone marrow cells have been described to have the greatest plasticity among adult stem cells regenerating damaged liver or myocardium. It has been proposed that the differentiation of bone marrow derived adult stem cells occurs naturally even in healthy organs as a physiologic process of tissue-regeneration. Others believe that organ damage is essential to induce transdifferentiation by release of organ specific microenvironmental factors (3).

The fate of bone marrow derived stem cells was first demonstrated by identifying differentiated male cells in female recipients after bone marrow transplantation via in situ hybridisation. These cells were found in muscle (4), liver, kidney and heart (5,6,7).

Others reported the direct isolation of cells with stem cell characteristic out of peripheral blood (8,9). The group around Catherine Verfaille (8) reported of a cell described as Multipotent adult progenitor cell (MAPC) which they isolated out of mice, rats and humans and which is capable of differentiation into cells with endodermal, ectodermal and neurodermal characteristics (8) which then could even further be differentiated in vitro into hepatocyte-like cells (10). Huberman and colleagues (9) isolated a cell of monocytic origin and programmed this cell into cells of various somatic lines in vitro. Both groups however only yield a small amount of cells so the goal of clinical application seems limited by the accessibility and lacking methods of expansion for these cells to be used therapeutically.

We here try to constitute necessary data which should be demonstrated to give substantial evidence for transdifferentiation of newly characterized cells including exclusion of fusion, phagocytosis or DNA uptake (11), description of the outset cell, differentiation into all three germ layers and functional parameters.

Ruling out cellular mimicry

Ying *et al.* (12) co-cultured mouse neurospheres with pluripotent adult stem cells. Both cell strains were genetically altered for future identification. Neurospheres expressed a puromycin resistance gene and embryonic stem cells expressed EGFP and HSVTK making cells sensitive to ganciclovir. After co-culture brain derived cells were isolated via puromycin treatment and revealed GFP expression and characteristics of

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undifferentiated cells implicating that the embryonic stem cells underwent spontaneous fusion with the co-cultivated neuronal cells. This hypothesis was underlined by chromosomal analysis of these cells showing that they have a tetraploid chromosomal compliment.
Even for bone marrow cells this phenomenon was described by co-culture with embryonic stem cells showing fusion (13) although Il-3 treatment was necessary.

If co-culture methods are used, indirect co-culture systems should be used to exclude cell fusion by avoiding cell contact. In addition chromosomal analysis can be exemplarily included. The most superior method for induction of differentiation would be a treatment with cell conditioned media : This however has to be sterile filtered and cultured parallel to prove that there are no contaminating cells after filtration.

Uptake of specific growth factors or medium components can lead to a positive staining of cells not resembling gene expression and protein production. Rajogopal *et al.* (2) described treatment of ES cells with insulin containing media and the consecutive positive staining of the cells. After depriving the media from insulin cells did not stain positive any longer implicating that they did not produce insulin.

It should therefore be recommended to show that any differentiated cell also transcribes the specific mRNA and if possible other methods should underline the differentiation process i.e. for Insulin the production of C-Peptide or the lack of mouse insulin in diabetic mice treated with insulin producing cells from other species.

Evidence for transdifferentiation

Although fusion has been described as a possibility of mimicked transdifferentiation in *in vitro* co-culture systems, there are numerous contrary *in vivo* and *in vitro* findings. Sriwatsa (14) described a clinical case of postpartum thyroiditis caused by transplacentally acquired foetal cells causing an alloimmune disease. The differentiated follicular cells of the female patient had one X and one Y chromosome and were from the male foetus and none of the cells were XXXY, suggesting that cell fusion was not responsible for this phenomenon. Lagasse (15) reported the metabolic rescue of FAH-/- mice (a model of fatal hereditary tyrosinaemia type 1) by the injection of 10^6 unfractioned bone marrow cells that were wild type for FAH. The liver of these animals showed multifocal liver colonization by marrow-derived hepatocytes. This makes cell fusion almost impossible to be the underlying mechanism, since fusion was always described as a rarely occurring phenomenon. As a matter of fact it has never been shown that adult stem cells or cells with stem cell character develop a heterokaryon phenotype when transdifferentiation occurs *in vivo*.

In vitro there are two publications by the group around Catherine Verfaille (8,10) reporting the isolation of Multipotent Adult Progenitor Cells (MAPC's) out of bone marrow which had the capacity to differentiate *in vitro*, not only into cells of all three germ layers but into cells resembling hepatocytes. *In vitro* MAPC divide up to 120 times. For differentiation studies cells were allowed to differentiate after 35-50 population doublings. Since this group never used co-culture systems to induce differentiation but treated their cells with various growth factor cocktails in order to differentiate MAPC into hepatocyte-like cells, endothelium, neuroectoderm and endoderm fusion cannot be the underlying mechanism. Our own results (16) (Ruhnke *et al.*) showed that the same differentiation cocktail used by Schwartz *et al*. was able to induce hepatic differentiation in rat embryonic stem cells without co-culture. In the same experiment (unpublished data) we treated the rat embryonic stem cells with hepatocyte conditioned media, which was sterile filtered and had comparable results. Taken together these findings support little evidence for the fusion theory.

Huberman (9) presented a peripheral blood derived monocytic cell which has the capacity to differentiate *in vitro.* All differentiation studies were carried out by treatment with growth factors. These cells transdifferentiated into macrophages, T-Lymphocytes, epithelial cells, neuronal cells, endothelial cells and hepatocyte-like cells.

Since these findings clearly demonstrate that transdifferentiation does take place and there are numerous other publications which could be mentioned herein, the phenomenon of cell-fusion might take place in direct co-culture experiments but is certainly not the base of all transdifferentiation processes.

Functionality

Many authors describe transdifferentiation of adult stem cells or "programmable cells" into cells of all three germ layers or even specialized somatic cells. Predominantly however these cells are only characterized by their immunohistochemically detected expression of specific antigens.

Describing transcription of specific genes via amplification of mRNA by polymerase chain reaction demonstrates that there is an expression of the amplified gene mRNA and rules out the uptake of protein out of the medium. Despite this fact even PCR has to be judged very critically. Small amounts of mRNA are almost present in every cell so unless controls are convincing, the expression is quantified via real time PCR or at least semi-quantitative methods, the pure detection of specific mRNA is not satisfactory to describe a cells phenotype. In addition to this even real time-PCR cannot quantify the number of cells within a population that produce the transcript.

Nevertheless if one claims that a transdifferentiation takes place specific functional data have to be demonstrated.

For the differentiation of insulin-producing cells Rajogapal *et al*. (2) clearly define functional parameters which are essential to describe a beta cell. These para-

meters are c-peptide staining, electron microscopy, northern analysis, metabolic labelling, demonstration of biphasic, glucose dependant insulin secretion and a transplantation assay for ß-cell function that demonstrate rescue of the diabetic phenotype for more than a month.

Transdifferentiation of adult cells into ß-cells has been described in vitro for pancreatic duct cells (17) (Ramiya *et al.*). This group even demonstrated the reversal of insulin-dependant diabetes by the duct-derived *in vitro* generated ß-cells. *In vitro* they demonstrated transcription of mRNA and glucose dependant insulin secretion.

Susan Bonner-Weil and colleagues of Harvard Medical School (18) isolated human ductal pancreatic tissue and cultured the ductal cells as monolayers on Matrigel with keratinocyte growth factor and nicotinamid. After this treatment the monolayer of epithelial cells formed three-dimensional structures from which islet-like clusters budded. They could show a 10-15-fold increase of the insulin concentration in the medium and cells were identified as ß-cells by positive detection of Insulin by RIA, PCR for Insulin mRNA. Insulin-secretion was glucose dependant. It is not clear whether these cells would as well function *in vivo* although functional *in vitro* data are satisfactory.

Other authors describe the secretion of insulin in embryonic stem cells. Here functional data are mostly fragmentary (19, 20, 21) with exception of for example the group around Anna Wobus. Blyszczuk *et al*. (22) expressed the ß-cell specific Pax-4 gene in mouse embryonic stem cells and could show the promoted differentiation of nestin-positive pancreatic precursor cells and insulin producing cells out of embryonic stem cells. They demonstrated protein expression, mRNA transcription, Insulin secretion electron microscopy and *in vivo* function after transplantation over a period of 14 days compared to controls. Nevertheless since the phenomenon of transdifferentiation of adult cells has been described the usage of embryonic stem cells in terms of therapeutic options becomes more and more redundant. Another somatic cell type we would like to emphasize on is hepatocytes. Parameters describing a functional hepatocyte should include expression of specific membrane bound antigens via immunohistochemistry and PCR, albumin secretion, indocyanine green uptake, LDL-binding, and specific enzyme activities. In more detail these enzymes are divided into the cytochrome p450 isoenzymes, epoxy hydroxylase activity and activity of enzymes belonging to the phase 2 metabolism of hepatocytes (i.e. UDP-glucoronyl-transferase). For optimization enzyme induction via inductors such as rifampicin and testosterone should be demonstrated. Since these are the criteria defined by pharmaceutical companies for *in vitro* cultured human hepatocytes for drug metabolism studies one has to accomplish comparable data if one claims to have differentiated a true hepatocyte *in vitro.*

Of course these requirements are difficult to fulfil and if one does not routinely work with human hepatocytes many experimental procedures are not easily at hand, so that in currently published literature functional data concerning cells differentiating into the hepatic phenotype are very basic. Cells that express such function in vitro could be easily envisioned to be generated autologously or from different individual and serve to cure metabolic diseases after intraportal or intraperitoneal injection.

Zhao *et al*. (9) for example only showed positive staining for albumin, AFP, Cytokeratin 7 without showing any functional data nor PCR analysis. In contrast to this group Catherine Verfaille (8.10) initially only demonstrated that their MAP cells could differentiate into the hepatic lineage, but the following publication did reveal satisfactory functional data. Verfailles group showed that human, mouse and rat MAP cells secreted albumin, metabolized urea, were able to take up a-LDL and even showed broad cytochrome P450 activity via phenobarbital stimulated cells with the pentoxyresorufin assay.

Altogether the demonstration of functional data is indispensable with regard to possible therapeutic option of human transdifferentiated adult cells.

Origin of cells and genomic changes during differentiation

Demonstrating the direct origin of the transdifferentiated cell is often very hazardous. Especially, when cells are dividing very slowly or are dependant of intercellular interaction to expand. In these cases it is not possible to perform a single cell culture and alternatives have to be found. The group of Catherine Verfaille (8) demonstrated the origin of their MAPC by retrovirally transfecting ten cells with GFP showing that each cell of the resulting population after 100 population doublings expressed GFP and only had a single retroviral insert of GFP. This result underlines that progeny of only a single GFP transduced MAPC gave rise to continuous growing populations.

Huberman (9) did show that their cells differentiated from a single CD14 positive cell via single cell culture. If multipotent cells are isolated from bone-marrow or peripheral blood it should be ruled out that the small population of CD34+ haematopoietic cells are the source of the differentiated cells. Therefore a single cell culture should if possible be provided. Otherwise one could think about eliminating CD34+ cells before the beginning of the culture period.

A rigorous demonstration of true transdifferentiation of haematopoietic stem cells would require clonal analysis of a single CD45+ stem cell could demonstrably give rise to blood, muscle and hepatocytes (15) (Lagasse 2001). Progeny of the cell could in some cases be demonstrated by double staining of the cell revealing a co-expression of a lineage marker such as CD45 and a specific antigen of the differentiated cell as shown by Lagasse *et al.* 2000 (22).

If transdifferentiation takes place changes in differential gene expression should be presented, by proteomics or affimetrix analysis. Proteomics for example has been described in detail for dendritic cell maturation and differentiation by Richards *et al.* 2002. This additional information would reveal posttranscriptional changes taking place in the process of transdifferentiation which cannot be uncovered by PCR or northern blot. With this method the importance of yet unknown proteins could be revealed.

Since DNA methylation is a major epigenetic modification of the genome that regulates crucial aspects of its function, it would be of great interest to find out whether the transdifferentiation process goes along with changes in DNA methylation. Genomic methylation patterns in somatic differentiated cells are generally stable and heritable. However in mammals (24) there are at least two developmental periods, in germ cells and in preimplantation embryos-in which methylation patterns are reprogrammed genome-wide, generating cells with a broad developmental potential. Lineage specific methylation for example has best been described for the c-fms gene during the maturation of macrophages (25).

Analogous experiments with differentiating cells could promote and uncover the understanding of transdifferentiation substantially.

Accessibility and therapeutic options

As the insight in the differentiation process advances, so the interest of potential tissue that could be generated grows. It has been shown that epithelium such as skin or cornea, skeletal tissue and metabolising cells such as hormone-secreting ß-cells and hepatocytes can be differentiated out of adult autologous cells which theoretically could be isolated from every individual. Tissue transplants could be used to treat a wide variety of pathologies in order to replace perished cardiomyocytes, ß-cells, dopaminergic neurons or hepatocytes and if generated from the individual himself these tissue transplants could be autotransplanted without any need of immunosuppression.

Unanswered however remains the question if differentiated cells would really be needed to reconstitute function of a destroyed organ or if it would be enough to inject undifferentiated cells into the organ or even intravenously and these programmable cells would find the site of damage on their own and differentiate into the needed phenotype.

An insurmountable problem however is accessibility of the appropriate amount of cells in order to sufficiently replace organ function. Citing the most promising publications again, Catherine Verfaille for example isolated a minimum of $5x10^3$ cells from human bone marrow and showed that these cells could undergo up to 120 population doublings. This although was achieved over a period of 400 days. This means that in order to yield 6×10^5 programmable cells it would take over a year. Verfaille and co-workers to not state how many MAPC's can be isolated from a healthy human beings bone marrow or whether these cells could even be drawn from blood. They did however show that injection of the undifferentiated MAPC allows the cells to integrate into various tissues without being programmed *in vitro.*

Eliezer Humberman in contrast isolated monocytes from peripheral blood they reported of a cell division every 3 days. After 10 days the cell number did not increase any further. Since only 30-50% of isolated monocytes did actually differentiate into the programmable f-Mo the total number of programmable cells seems not enough. If one imagines that an autologous leukapheresis product would yield approximately 1×10^9 monocytes i.e. approximately 5×10^8 f-Mo. If these then divide up to four times the total amount of 2×10^9 transplantable cells.

Not wanting to go in further detail it seems obvious that there or a number of hurdles which have to be overcome before even autologous adult cells which now even seem to be easily accessible can successfully be transferred into a clinical protocol.

Conclusion

Although there has been great progress in research of adult stem cells and transdifferentiation of adult cells, the number of unanswered question is growing as research progresses. It will take further effort to convince the critics that certain adult cells are capable of transdifferentiation. Scientists will have to prove unequivocally that the newly generated cell is clonally derived from one homologous cell population and that it takes on the function of cells of its new phenotype. The origin of the cell and epigenetic changes has to be thoroughly characterized. Rarely occurring phenomenon such as cell fusion, DNA uptake or other cellular mimicry have to been ruled out.

Before going into clinical settings it is indispensable to learn more about inter-and intracellular mechanisms and interactions. Carcinogenic potential of the envisaged cell has to be ruled out securely.

In order to make cells applicable for clinical settings appropriate methods of clonal cell expansion or easier access to the source have to be developed.

If all these suppositions are fulfilled autologous cell therapy will open a new field of medicine with up to now unimaginable scope.

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