

Editorial

Restoring balance to liver stem cell research

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This month's report from Saji et al., entitled 'Basic fibroblast growth factor promotes the trans-differentiation of mouse bone marrow cells into hepatic lineage cells via multiple liver-enriched transcription factors' [1], is one of a handful of recent papers helping to restore balance to the contentious field of adult stem cell research in general and, more specifically, to investigations of liver stem/progenitor cells and the roles played by different mechanisms of cell plasticity in hepatic responses to injury.

Since the field first exploded with publications indicative of unexpected adult stem cell plasticity in 1999, several challenges to the findings have arisen, forcing ever more careful demonstrations and analyses of the phenomena [2]. These, in turn, have led to more nuanced understandings of cell differentiative potential, both *in vivo* and *ex vivo*.

The first challenge to the field, of course, was reproducibility. Though some negative results papers have been published, the negative findings have often proven to be based on differences in study design rather than a true lack of reproducibility of the underlying mechanisms (for an example see Wagers et al. and Technical Comments on that paper [3,4]). Virtually all of the *in vivo* studies have involved transplantation experiments, either of bone marrow or of the target organ of interest. Many variables in study design may contribute to differences in outcomes, but these are rarely discussed in the experimental reports or in reviews and commentaries. Some of these variables are uncontrolled physiological aspects of the study designs, including the respective ages of the donors and recipients, differences between strains and between species, and differences in the selection criteria and pre-conditioning of the donor cells (selection methods themselves being initial steps of pre-conditioning), and the varied models of injury employed to heighten the possibility of plasticity. The different techniques used to detect and quantify engraftment and plasticity

phenomena may also be an important source of variability in studies which, again, often goes unremarked. This aspect will be discussed further on in this editorial.

The next challenge involved whether the plasticity phenomena could be demonstrated as clonal events, actually deriving from plasticity of a single cell, or rather as representing direct differentiation from heretofore unrecognized, marrow-derived, organ progenitors which were not in fact plastic. In other words, might there be liver specific progenitors in the circulation and marrow that can only give rise to liver? Clonal studies, however, soon followed, indicating that there was indeed true plasticity of adult cells [5,6]. But it has also been argued that clonality is not actually an appropriate criterion as experiments which abstract a single cell out of its normal context are not the equivalent of the systems level interactions of multitudes of interacting cells that guide regeneration [7].

Additional questions were raised about the usually 'low' frequency of the findings, with the frequencies of a few percent or a fraction of a percent being considered unimportant. Subsequent studies, though, have demonstrated more robust engraftment, the degree of which depends on the degree and type of injury affecting the organ in which plasticity is being studied. Engraftments greater than 10% have now been reported in liver [8], lung [9], skin [10], and gastrointestinal tract [11]. Furthermore, we have suggested that the low level engraftment, far from being trivial and non-physiological, may actually be evidence of the stochasticity necessary for complex systems of interacting cells to give rise emergently to tissues, organs, and bodies [12,13].

In March 2004, two studies were published in *Nature* that represented the most serious challenge to the adult stem cell plasticity field [14,15]. These studies showed that hematopoietic and neural stem cells, respectively, could spontaneously fuse with co-cultured embryonic stem cells giving rise to tetraploid fusion products with the functional properties of embryonic stem cells. Thus, it was suggested

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that the markers used to detect progenitor cell engraftment into unexpected organs—such as the Y chromosome in gender mismatch transplant experiments or transgenes placed into donor cell populations (for example, green fluorescent protein [GFP] or beta-galactosidase) could be explained not by direct differentiation, but rather by cell–cell fusion events, perhaps followed by nuclear–nuclear fusion, resulting in a tetraploid cell. This challenge to the field was so strong that Nature questioned ‘Adult stem cells: Not so flexible after all?’ and Science editorialized that ‘Studies cast doubt on plasticity of adult cells’ [16,17].

Soon, work from Markus Grompe’s laboratory demonstrated extensive fusion [18] as being responsible for the plasticity events that they had reported earlier in the fumaryl acetoacetate hydrolase (FAH)-null mouse, a model for human tyrosinemia, type I. Several papers concerning different organ systems followed, with *in vivo* and *in vitro* evidence mounting that cell–cell fusion, perhaps even followed by reductive division back into two functioning diploid cell populations (‘unstable fusion’), made some contribution to the plasticity phenomena. Most of these papers however also contained abundant evidence of simultaneous non-fusion, direct differentiation events. For example, in the first FAH-null mouse report of fusion, in which they claim ‘that hepatocytes derived from bone marrow arise from cell fusion and not by differentiation of hematopoietic stem cells’, the authors do not adequately acknowledge that the data also show a small percentage of cells that may or may not represent non-fusion events. Overstatements that ‘in liver only fusion matters’ [19] probably say more about the politics of stem cell research than they do about the science underlying it [20].

A key report published from the laboratory of Darwin Prockop should have restored some balance to the field, though the general discourse did not respond to this paper as one might have expected [21]. In this study, investigators applied heat shock to co-cultured human mesenchymal stem cells (MSCs) and human lung epithelial cells. They watched the response to the injury with real-time videography and were able to see two processes: (1) direct change in phenotype of MSCs to that of lung epithelia; (2) rare fusion events where pseudopods extended from an MSC, making contact with an epithelial cell, leading to coalescence into a fused, binucleated cell. Thus, both processes occur.

So a broader, more accurate (and exciting) conclusion, borne out by the sum of the research published to date, is that we are truly speaking of genomic plasticity, rather than cell plasticity, *per se* [22,23], with transdifferentiation representing genomic plasticity in response to microenvironmental cues and fusion representing genomic plasticity in response to cytoplasmic cues. The latter turns out to be a precise physiological equivalent of the *ex vivo* heterokaryon experiments of Helen Blau and collaborators performed in the late 1970s which helped demonstrate that ‘differentiation requires continuous active control’ [24]. They were significantly ahead of the pack by many years. The cloning of Dolly and the abundant

confirmations of plasticity by many means now clearly validate her intuition.

Despite the clear evidence for both processes, however, the high level of attention paid to the fusion phenomenon has engendered a widespread misunderstanding that the plasticity bubble had burst. Most important, since *in vivo* fusion was first demonstrated in a liver disease model, restoration of balance to the field requires convincing demonstration of direct differentiation without cell fusion in the liver. The first such demonstration was in hepatic engraftment of human cord blood stem cells in NOD/SCID immunodeficient mice [25]. With no demonstration of cells containing mixtures (either two separate nuclei or one fused nucleus) of human and mouse chromosomes, clearly human cord blood cells had become hepatocytes without a prior fusion event. Similar work from Esmail Zanjani’s laboratory has now been published with human hematopoietic stem cells (HSCs)—from bone marrow, cord blood, and peripherally mobilized cells—transplanted into fetal sheep, also with no demonstration of fusion [26]. Some critics however are unconvinced by the morphologic and immunophenotypic characteristics of the images in these studies and doubted whether they were truly hepatocytes. More rigorous data was required. Three studies now provide such data.

The first is from the laboratory of my collaborator Saul Sharkis, in which a bone marrow derived subpopulation that had been enriched for HSCs was co-cultured with damaged liver tissue with barriers to prevent direct cell–cell contact [27]. Using this approach, in which only substances secreted into the culture medium from the minced liver tissue would stimulate hepatocytic differentiation in the marrow-derived cells, they confirmed direct differentiation of bone marrow cells into hepatocytes. Not only did they rigorously exclude fusion events with this approach, proven by genotypic analysis, they demonstrated functionality by secondary transplantation of the hepatocytes into recipient mice with CCl₄-induced injury. Direct differentiation as a pathway of marrow-to-liver plasticity was confirmed.

Then the laboratory of another of my collaborators, Diane Krause, demonstrated that cell–cell fusion was not required for the differentiation of bone marrow derived cells into epithelial cells *in vivo*, using a sophisticated cre-lox transgenic model [28]. In this model, fusion would be signaled by GFP expression and direct differentiation would be demonstrated by diploid Y-positive, GFP negative cells with or without beta-galactosidase expression. No GFP expression could be found even with RT-PCR for GFP mRNA. Another paper, by Alvarez-Dolado et al., used a similar approach, with GFP expression marking direct differentiation and beta-galactosidase marking fusion [29], they found the reverse: only fusion, but no direct differentiation. This study may have missed some GFP+ cells, however, by an insensitive approach to detection of GFP, relying solely on fluorescence rather than immunohistochemical or PCR detection of GFP expression, the gold standards

of such investigations. That the negative transdifferentiation result arises from insensitive detection methods is suggested by the fact that no engraftment was found in tissues where it has already been well documented, such as skin, lung, kidney, biliary tree, and the gastrointestinal tract. In this study, engraftment by fusion was only detected in cells which are commonly multiploid and known to have fusogenic potential: hepatocytes, cardiac myocytes, and Purkinje cells. A greater and more global problem in methodology, however, is suggested: might GFP itself interfere with plasticity phenomena? In all the studies using cre-lox approaches to the problem, including those from my own laboratory [30], whichever phenomenon is to be detected by GFP expression is the one not found. This clearly requires further investigation.

Now, we have a third paper showing transdifferentiation of HSCs toward hepatocyte gene expression without the possibility of fusion. Saji et al. cultured mouse bone marrow cells with and without a variety of growth factors [1]. They found that basic fibroblast growth factor (bFGF) was most effective for inducing albumin production in these cultured cells. Indeed, within days of adding bFGF to the culture medium, not only could cells expressing hepatocyte markers cytokeratin 18 and albumin be found, but they could also demonstrate production of mRNA for HNF1a, HNF3a, HNF3b, HNF4a, GATA4, and GATA6, i.e. induction of transcription factors which yield hepatocyte differentiation. The mechanisms by which bFGF results in these gene expression changes remain unclear. While it has not yet been shown that these cells can function as hepatocytes *in vivo*, the finding may well be important in defining the extracellular microenvironmental conditions that can promulgate hepatocyte differentiation in adult bone marrow cells for therapeutic purposes.

In conclusion, it is safe to say that there is a ‘flexible arrangement’ regarding cell differentiation in the body [31]. At least four pathways to plasticity have now been established experimentally: (1) stem or progenitor cells can differentiate down different pathways depending on microenvironmental effects; (2) mature cells can undergo metaplasia to become cells of another mature lineage, perhaps through ‘de-differentiation’ to a progenitor cell intermediate; (3) transdifferentiation, i.e. genetic reprogramming of a differentiated cell under induction of microenvironmental signaling; (4) cell–cell fusion, with genetic reprogramming accomplished by exposure of a donor nucleus to cytoplasmic differentiative factors. However, we should not be content to assume that these four are the only pathways to plasticity. It is worth repeating a quote which I have often used in recent years. Barbara McClintock, Nobel laureate for her work in genetics in 1979, stated: ‘There’s no such thing as a central dogma into which everything will fit...any mechanism you can think of, you will find—even if it is the most bizarre kind of thinking. Anything... So if the material tells you, ‘It may be this,’ allow that. Do not turn it aside and call it an

exception, an aberration, a contaminant...’ [32]. We would do well to heed this sage advice.

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