Commentary

Chipping away at stem cells

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Until recently, conventional wisdom has held that the lineage potential of stem cells was restricted to the tissue of origin. That is, stem cells derived from tissue A could only give rise to differentiated cells of tissue A and not to differentiated cells of tissues B, C, or D. This notion has been challenged since by a plethora of reports suggesting that stem cells have an inherent plasticity that allows them to respond to extrinsic signals present in the transplanted environment (1–3). In these studies, trans-differentiation or reprogramming has been substantiated mostly by cell morphology and/or the expression of antigenic proteins specific to the transplanted tissue environment and not to the tissue of origin. Although more rigorous criteria of functional activity and sustainable multilineage engraftment should be applied to future reports of trans-differentiation (4), if true, the phenomenon of trans-differentiation suggests that most if not all stem cells share an intrinsic genetic program that is not present in nonstem cells. Put another way, one hypothesis is that stem cells originating from different tissues may share a common genetic program responsible for maintaining them in an undifferentiated proliferative state. Once placed in a novel environment, the plastic nature of stem cells would allow them to respond to local cues and activate the appropriate differentiation pathway. Whether stem-specific genes exist or not is at present difficult to test given that the existence of stem cells itself is difficult to prove in many organ systems. In the case of neural stem cells (NSCs) of the murine central nervous system (CNS), the lack of known surface markers has hindered the prospective identification of these cells (i.e., for direct isolation of NSCs from fresh tissues). Currently, NSCs of the CNS are identified retrospectively. The existence of stem cells is inferred by the analysis of their differentiated progeny, using a complex series of cloning and differentiation assays. If there is indeed a universal stem cell gene-expression profile, the process of maintaining the "stem" state is in all likelihood highly complex, requiring the interactions and contributions of many different cellular genes in a spatial and temporal order. Thus, unraveling this gene puzzle is no easy task.

In this issue of PNAS, Terskikh *et al.* (5) address the question of stem-specific genes by building on two previous studies that have examined separately the gene-expression profiles of hematopoietic stem cells (HSCs; ref. 6) and NSCs (7). In these earlier studies, cDNA derived from cells with minimal to no functional "stem activity" was subtracted from cDNA derived from cells enriched for stem activity. This approach was necessary to remove normal housekeeping genes along with transcripts unrelated to stem cell biology. The results are two cDNA libraries that are enriched selectively for transcripts believed to be specific to fetal HSCs (6) and postnatal day 0 (P-0) NSCs (7). Subjected to high-throughput sequencing, biochemical analysis, and verification [i.e., quantitative reverse transcription (RT)-PCR, Northern and *in situ* hybridization], the subtracted cDNA libraries eventually were used to produce fetal HSC (6) and NSC (7) cDNA arrays. By using an extension of this strategy, Terskikh *et al.* ask whether there are adult HSC-enriched transcripts that are also expressed in mouse NSCs (5).

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In all three studies, cDNA chips were used to examine gene-expression profiles of HSC and/or NSC (5–7). To interpret more accurately the differences or similarities between two expression profiles, it is best to start with purified or homogeneous populations of all of the specific cell types under study. For HSCs and NSCs, obtaining a homogeneous population is especially critical, as they are present at very low frequencies. It has been estimated that in the fetal liver (the site where fully functional HSCs are first found) and the bone marrow (the site where HSCs are located throughout adulthood), HSCs are present at a frequency of 1 in 10^4 or 10^5 cells (8). Similarly, NSCs are estimated to comprise only 3–4% of neurospheres (murine CNS cells tend to proliferate as neurospheres or balls of cells in culture; ref. 9). The rarity of these cells and the possibility that stem-specific transcripts may be present at low abundance signify that in the absence of homogeneity, the majority of the gene expression data would be from nonstem cells.

The problem of cell and tissue heterogeneity is thus one of the most formidable issues confronting stem cell biologists. In the hematopoietic system, the problem has been largely solved as stem cells are identified and purified prospectively through a combination of negative and positive selections. In day 14 fetal liver and bone marrow, HSCs with the phenotype $AA4^{+}Sca^{+}Kit^{+}Lin^{neg/low}$ and $Sca^{+}Kit^{+}Thy1.1^{low}Lin^{neg/low}$, respectively, are isolated by using fluorescence-activated cell sorting (FACS; refs. 5, 6, and 10). Earlier studies have shown that when these two respective hematopoietic cell populations are transplanted back into a lethally irradiated host whose endogenous hematopoietic system has been disabled, the cells are able to reconstitute the normal hematopoietic system functionally (10). Thus, *in vivo* transplantation studies have established these phenotypic cell populations as HSCs (i.e., they are able to self-renew and give rise to mature blood cells). In the present study by Terskikh *et al.* (5), bone marrow cells are sorted into two cell populations: the $Sca^{+}Kit^{+}Thy1.1^{low}Lin^{neg/low}$ population (HSC) and the non-HSC remaining fraction of the bone marrow (BM). cDNA prepared from the BM cell population was subtracted from HSC cDNA, thus selectively enriching for transcripts specific to adult HSCs (5). It is noteworthy that this same strategy was used earlier to generate a gene-expression profile of HSCs derived from fetal day-14 liver (6). Interestingly, a comparison of the expression profiles of fetal (available in the Princeton Stem Cell

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Database at http://stemcell.princeton.edu) and adult HSCs by Terskikh *et al.* (5) revealed "overlapping but not identical expression profiles," as might be expected from two distinct developmental stages.

Although apparently less of an issue in the hematopoietic system, cell heterogeneity is a defining theme in the study of CNS NSCs. As previously mentioned, the lack of specific cell surface markers prevents prospective isolation of a cell population with functional NSC activity. As a result, the original cell mixture is heterogeneous, containing not only the NSCs of interest, but also cells at various stages of specialization. True multipotent stem cells cannot at present be separated physically from lineage-restricted progenitors.

In a recent report of a gene-expression profile of neural progenitor cells (7), the issue of cell heterogeneity was addressed through the use of subtractive hybridization. To obtain putative NSCs, the authors isolated the cortex from P-0 mice, dissociated the cortical cells, and propagated them in medium containing fibroblast growth factor (FGF-2). Subsequently, the cell population was split into two groups: the "neural progenitors" population that was maintained in FGF-2 and the "differentiated" cell population in which FGF-2 was withdrawn for 24 h. cDNA derived from the differentiated population was subtracted from cDNA of the neural progenitor cell population, and clones from the resulting subtracted library were spotted subsequently to produce a cDNA array containing neural progenitor-specific transcripts. It is this same cDNA array that also was used by Terskikh *et al.* to cohybridize amplified cDNA derived from the adult HSCs and bone marrow to arrive at a set of transcripts common to adult HSCs and P-0 NSCs.

There are, however, several caveats to this study. (*i*) It is not certain in the case of NSCs that Geschwind *et al.* (7) have solved the cell heterogeneity problem. Although they have approached the problem by using subtractive hybridization, the subtracted library was derived from a mixed population. Additionally, it is not clear that this approach has enriched the low-abundant stem-specific transcripts. To establish homogeneity, the putative NSC population should display functional characteristics of self-renewal and multipotency; that is, on preferential differentiation, the majority of the cells should express lineage-specific markers of one specific differentiated cell type. Ideally, to ensure that the culture conditions have not selected for lineage-specific progenitors, preferential differentiation into each of the three CNS cell types (astrocytes, oligodendrocytes, and neurons) should be demonstrated also. (*ii*) Because the neural cell population is derived from P-0 mouse cortex and has been cultured for at least 2 weeks, it is possible that glial progenitors constitute a significant proportion of the original neural cell culture. In the mouse cortex, neurogenesis initiates around embryonic day 12 (E12), peaks at E15, and terminates at birth (11). The generation of glial cells occurs after neurogenesis, primarily during the first month after birth (11). Time-lapse studies also have shown that with increasing time in culture, cortical stem cells have a greater tendency to produce glial cells (11). Therefore, it may be possible that the putative NSC-specific transcripts found preferentially expressed in the germinal neuroepithelium by *in situ* hybridization actually may be transcripts involved in glial cell proliferation and/or maintenance.

With these caveats in mind, the study by Terskikh *et al.* (5) may be tantalizing evidence of a universal stem cell profile. What emerges from the report is a streamlined collection of candidate genes that is believed to be specific to HSCs and NSCs and not to their differentiated cell types. Validation of the candidate genes, using quantitative RT-PCR, Northern hybridization, and more importantly *in situ* hybridization, of the developing murine brain ranging from E13 to the adult indicates that most of the transcripts are expressed in germinal zones (areas of proliferation where stem cells are likely to reside; ref. 5). Remarkably, one of the transcripts commonly expressed in adult HSCs and P-0-NSCs is a putative seven-transmembrane receptor, Cyt 28 (5). Cyt 28 is expressed also in the fetal HSC-enriched cDNA library. If indeed a universal stem cell profile does exist, one would predict genes comprising this profile to be independent of the site of stem origin and also of developmental stages. Cyt 28 fulfils both these expectations. Cyt 28 also could serve potentially as a cell-surface marker for the prospective isolation of NSCs.

COMMENTARY

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