The power of stem cells reconsidered?

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> We dance round in a ring and suppose,
> But the Secret sits in the middle and knows.
>
> Robert Frost, "The Secret Sits"
>
> Because something is happening here
> But you don't know what it is
> Do you Mr. Jones?
>
> Bob Dylan, "Ballad of a Thin Man"

The mammalian hematopoietic system is charged with the lifelong daily production of numerically large mature cell populations, which collectively represent a wide variety of peripheral blood cell lineages. In this sense, hematopoiesis is self-renewing and resembles other developmental systems such as the small intestine, the epidermis, and hair follicles of the skin, as well as the male germ line. Other tissue types such as the central nervous system, the liver, and muscle seem to replenish mature cell types much more slowly or in response to injury. Greater than 4 decades of elegant *in vivo* transplantation studies have defined the activities of a rare bone marrow stem cell that is both self-renewing and multipotential in its abilities to produce all blood cell types in engrafted hosts permanently and clonally (1–4). Eventually, a biological activity became a defined cellular entity with the development of numerous strategies designed to purify the hematopoietic stem cell physically from adult bone marrow and from other sources such as fetal liver (5–8). Individual hematopoietic stem cell purification schemes come in different flavors. There is general agreement that the degree of enrichment is quantitatively similar in most strategies and that the only reliable measure of stem cell activity in any physically purified population is by *in vivo* transplantation. One particular purification strategy that is relevant to the present discussion employs vital dye uptake and efflux properties together with flow cytometry to define a subset of bone marrow cells called the "Side Population" (SP) (9, 10). These cells are highly enriched in transplantable stem cell activity. The complexity of the whole-animal assay system does not permit accurate estimates of the absolute homogeneity of any purification protocol; that is, it is not possible to obtain a quantitatively rigorous one-to-one "mapping" of stem cell activity onto individual physically purified cells. Remarkably, however, in some cases, it has been possible to show that a single transplanted stem cell is both necessary and sufficient to transfer an intact, normal hematopoietic system to a recipient host (11–13). Although less extensive, other studies have physically identified candidate stem cells from a number of other tissues (14–18).

The traditional view, based on embryological considerations, the available assay systems, and also in part on intellectual preconceptions, holds that stem cells obtained from individual somatic tissues will be "dedicated" to the tissue in question. In other words, the undifferentiated stem cell "state" as defined by the ability of a stem cell to produce mature cell populations is limited to the range of cell types characteristic of each individual tissue. According to this notion, during development, distinct somatic stem cell populations are set aside or specified to be self-renewing at least to some degree but with a limited degree of lineage plasticity. This dogma also posits that any given somatic stem cell population is physically resident within its appropriate tissue.

Recently, several reports have appeared that warrant a fresh look at the prevailing concepts of somatic stem cell potentials. In particular, there are suggestions that the functional plasticity of somatic tissue-derived stem cells may be greater than expected. On page 14482 of this issue of PNAS, Jackson *et al.* (19) describe robust transplantable hematopoietic activity in cell populations originally obtained from muscle. A separate study by Gussoni *et al.* (20) published in *Nature (London)* also describes hematopoietic activity in muscle and, notably, muscle differentiation ability in highly purified bone marrow SP cells. Are these studies, either alone or together, sufficient reason to revise our comfortable views of stem cells? If not, exactly how do they contribute to stem cell biology? Both studies are conceptually grounded in the existence of a population of satellite cells in muscle, which can be considered a muscle stem cell compartment (21).

Jackson *et al.* (19) adopt a fairly straightforward and perhaps serendipitously fruitful approach to obtain transplantable material from the muscle. In brief, these investigators established short-term *in vitro* cultures from disaggregated muscle tissue. The culture conditions do not resemble those employed in any previous efforts to maintain or propagate hematopoietic stem cell activity. Hematopoietic activity in the cultured cell population was measured in the quantitatively rigorous competitive repopulation transplantation assay. In this assay, a given cell population is required to compete in the same recipient with a genetically distinguishable standard source of hematopoietic stem cell activity (22, 23). Remarkably, on a per cell basis, the muscle-derived material contains about 10 times more hematopoietic activity than whole bone marrow. Using diagnostic cell surface markers, these investigators conclusively show the reconstitution of lymphoid and myeloid cell lineages. In addition, the bone marrow of the transplanted animals contains an SP population that is derived from the donor cells of muscle origin, indicating that a hematopoietic stem cell compartment can arise from a muscle-derived source. Consistent with this interpretation, retransplantation of bone marrow from these mice into secondary recipients showed continued hematopoietic activity. Such retransplantable activity is considered to be a good indicator of a self-renewal process and is commonly used as a definitive measure of the "primitiveness" of a stem cell population. In short, by all accepted criteria, the cultured cell population functions like a true hematopoietic stem cell population.

Do these results indicate that a stem cell compartment for muscle tissue also possesses hematopoietic ability? The answer has to be no or, at least, not yet, because the identity of the hematopoietically competent cells in the cultures has not been directly established. Moreover, it has not been shown that these same cells possess muscle differentiation ability. Although the authors establish the existence of an SP subset in the cultured population, these cells were not transplanted by themselves.

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It is also not clear from these studies whether the SP phenotype can be extended to the muscle stem cell compartment. In addition, the authors did not attempt to measure the hematopoietic activity of any muscle-derived population before the culture period. Therefore, it is not yet possible to ask whether the cells that have hematopoietic activity acquire this ability as a consequence of mysterious culture events.

It is quite clear that the cultured cell population possesses striking hematopoietic activity; however, the biological significance of these data can be interpreted in a number of ways. The most interesting, of course, would be that muscle stem cells possess a broader range of differentiation potentials. Indeed, as mentioned by the authors, at least some candidate muscle stem cells do not express the myogenic transcription factors MyoD and Myf5 (24, 25). This lack of expression may suggest that such cells are not yet committed to a muscle fate. A rigorous proof of this suggestion would require clonal analysis where the progeny of a single cell could demonstrably yield muscle as well as hematopoietic progeny. Although this challenge is a difficult one, it is nonetheless necessary, given the long tradition of hematopoietic clonal studies that collectively provide a ''gold standard'' set of definitions for any stem cell population. An intriguing possibility is that some muscle-derived cells are being ''reprogrammed'' in some manner during the culture period to acquire a hematopoietic fate. In fact, flow cytometric analysis shows the expression of the panhematopoietic marker CD45 and ckit on a sizeable subset of the total cultured cell population. Further analysis of the SP population in the cultures reveals ckit expression on most of the cells and CD45 expression on less than 1% of the cells. Based on the previously estimated stem cell content in the bone marrow, it is possible that the small number of CD45-positive cultured cells could account for the hematopoietic activity observed by the competitive repopulation assay (23, 26). Clearly, it will be of paramount importance to address directly which subpopulation of the cultured material contains the transplantable blood-forming ability. It will also be important to measure the kinetics with which the cells acquire hematopoietic activity and cell surface markers during the culture period (assuming of course, that these are not present on day 0).

The studies of Jackson *et al.* (19) may be of greatest significance in a practical sense. Traditionally, it has been exceedingly difficult to maintain or expand robust levels of transplantable hematopoietic activity in any *in vitro* culture system. A general observation is that hematopoietic cultures may support the proliferation of stem cells but only in concert with differentiation events (27, 28). It would be surprising indeed if the conditions employed in the muscle cultures (which only include fetal calf serum and chick embryo extract) could support bona fide hematopoietic stem cells isolated from bone marrow. This possibility needs to be tested; ideally by including genetically distinguishable purified bone marrow stem cells in the cultures that also contain muscle cells. If the bone marrow-derived cells do not retain hematopoietic stem cells activity and the muscle-derived cells retain or acquire it, then these results would be strong evidence that the muscle-derived cells are different in some significant biological sense and in that something unique is happening in these cultures. It will also be important to ask whether the hematopoietically active cells obtained from these cultures are the products of active cell division. If, as seems likely, they are indeed the products of active cell division, it may provide an unprecedented avenue to explore the biology of stem cells by facilitating gene transfer as well as other manipulations. In short, this culture system and the precise definition of the events occurring in these cultures seem to hold great promise.

The paper by Gussoni *et al.* (20) describes studies in which either whole male bone marrow or small numbers of the SP population were intravenously injected into irradiated female mdx mice. (The mdx mouse is a model for Duchenne's muscular dystrophy.) A partial restoration of dystrophin expression in the muscle tissue was observed. The authors show the donor-derived origin of this restoration by showing that a high percentage of dystrophin-positive myofibers contain a Y chromosome-positive nucleus. Previous studies have shown that bone marrow contains myogenic precursors, and, at least in tissue culture experiments, the existence of a multipotential mesenchymal precursor in the bone marrow has been shown (29, 30). By physically defining the muscle-restoring activity in bone marrow to be contained in the SP fraction, the present studies have begun to build a link that may ultimately prove that hematopoietic and myogenic potentials are indeed present in the same cell. The transplanted dose of SP cells in these experiments was relatively small (only 10-fold greater than necessary for full hematopoietic reconstitution), and at least qualitatively, it seems that, on a per SP cell basis, the muscle-restoration activity is robust.

It is still premature to extrapolate these data in a way that challenges existing stem cell dogma. Clearly, what is also needed here is a clonal approach. In this regard, the observations that both blood-forming and myogenic activities reside in the same physically defined cell population and that the reconstitution of both tissues occurs simultaneously in the same irradiated mdx animals strongly suggest that such studies will be feasible in the near future. As a beginning, it might be interesting to use bone marrow cells purified according to other physical criteria; in particular, those criteria by which hematopoietic reconstitution can be accomplished with single cells (12, 13). It would also be interesting to introduce bone marrow SP cells directly into muscle as a way to ask whether these cells can differentiate into this tissue without obligatory events that may need to occur elsewhere. As in the case of the paper by Jackson *et al.* (19), the value of these experiments may be mostly practical. Specifically, it is most significant that the restoration of muscle can occur through systemic introduction of precursor cells via the circulation.

In complementary experiments, Gussoni *et al.* (20) identified an SP fraction in freshly isolated muscle. The cell surface marker profile of these cells is different from that of the bone marrow SP cells. Engraftment of these muscle SP cells into irradiated mdx mice yielded both reconstitution of the hematopoietic system and contribution to muscle tissue. The nature of the hematopoietic transplantation design in this case was radioprotective. This design is very different from the competitive repopulation strategy employed by Jackson *et al.* (19). Specifically, the muscle SP cells must rescue the recipient animal from radiation-induced blood system failure. Whereas considerably more muscle SP cells were required to accomplish this rescue than bone marrow SP cells, it is hard to argue with the fundamental conclusion that hematopoietic activity can indeed be directly isolated from a muscle source without extensive *in vitro* manipulation. Interestingly, in experiments in which a bone marrow SP fraction was competitively transplanted together with 30-fold more muscle-derived SP cells, all *in vivo* hematopoietic activity originated from the bone marrow. By the competitive repopulation criteria, it would seem that these muscle SP cells are not as ''adept'' in hematopoietic function as their bone marrow counterparts. Therefore, at least in their freshly isolated states, these two populations are functionally different. If, in fact, the muscle SP population in the studies by Jackson *et al.* (19) and by Gussoni *et al.* (20) is responsible for hematopoietic function, it would seem that the *in vitro* culture period is instrumental in explaining the competitive repopulation differences.

Whether all of the myogenic and hematopoietic activity in muscle tissue resides in the SP compartment has not yet been

addressed adequately. However, as suggested by Gussoni *et al.* (20), it may be that the SP phenotype can be used to define stem cell-like entities in numerous tissue systems. If so, then the stage is set for accurate functional and molecular comparisons. In fact, the latter may ultimately be the best way to determine what it means to be a stem cell in general and in particular what differences, if any, there are among stem cells from different somatic tissues. An interesting step in this direction may be found in studies that suggest that a hematopoietic stem cell is already molecularly "primed" to amplify gene-expression programs that will be necessary for the function of mature blood cells (31, 32). One could imagine that such a tissue-specific primed state is not irreversible and could be modulated into other primed states; possibly in response to local environmental cues. Perhaps such reprogramming is occurring during the culture period used by Jackson *et al.* (19). If so, then this culture-induced reprogramming could explain the inefficiency of freshly isolated muscle SP cells when in competition with the bone marrow SP compartment observed by Gussoni *et al.* (20). If the above notions are correct, then the hypotheses of tissue-specificity or nonspecificity of somatic stem cells would both be correct and not mutually exclusive. In one very practical sense, it may therefore be prudent to refocus efforts aimed at *ex vivo* expansion of hematopoietic stem cells into efforts to reprogram easily obtainable somatic stem cell sources to a hematopoietically primed state.

Taken together and viewed from the hematopoietic perspective, both of these studies provide compelling evidence that an appropriate hematopoietic stem cell source can be found in at least one tissue not previously considered as hematopoietic. An additional and more extreme example may be found in a recent report that establishes hematopoietic activity in neural stem cell-derived neurospheres (33). An interesting possibility is that at least some of these muscle-derived or other cell populations may in fact have some hematopoietic activity in a normal unperturbed individual. Clearly, as discussed above, much remains to be done. These are exciting times, and the aforementioned studies provide food for thought. The table has been set, and dinner is almost ready.

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