CHAPTER 2

'Stemness': Definitions, Criteria, and Standards

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2.1 WHAT IS A STEM CELL?

Stem cells are functionally defined as having the capacity to self-renew and the ability to generate differentiated cells. More explicitly, stem cells can generate daughter cells identical to their mother (self-renewal), as well as produce progeny with more restricted potential (differentiated cells). Such a simple and broad definition may be satisfactory for embryonic or fetal stem cells that do not persist for the lifetime of an organism but it breaks down when trying to describe other types of stem cells (e.g., adult stem cells). Another functional parameter that should be included in a definition of stem cells is potency, or its potential to produce differentiated progeny. Does the stem cell generate multiple differentiated cell types (multipotent or pluripotent) or is it only capable of producing one type of differentiated cell (unipotent)? Thus, a more complete functional definition of a stem cell includes a description of its replication capacity and potency.

2.2 SELF-RENEWAL

Stem cell literature is replete with terms such as 'immortal,' 'unlimited,' 'continuous,' to describe a cell's replication capacity. These rather extreme and vague terms are not very helpful, as it can be noted that experiments designed to test the 'immortality' of a stem cell would by necessity outlast authors and readers alike. Such terms are probably best avoided or used sparingly.

Most somatic cells cultured *in vitro* display a finite number (less than 80) of population doublings prior to replicative arrest or senescence, in contrast to the seemingly unlimited proliferative capacity of stem cells cultured *in vitro*. Therefore, it is reasonable to say that a cell that can undergo more than twice this number of population doublings (i.e. 160) without oncogenic transformation can be termed 'capable of extensive proliferation.' In a few cases, this

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criterion has been met, most notably in embryonic stem (ES) cells derived from either humans or mice, as well as in adult neural stem cells (NSCs).

For adult stem cells, an incomplete understanding of the factors required for self-renewal *ex vivo* exists, thus the ability to establish similar proliferative criteria based on *in vitro* culture is limited. Therefore, the proliferative capacity of adult stem cells is currently best defined *in vivo*, where they should display sufficient proliferative capacity to last throughout the lifetime of the animal. In some cases, a rigorous assessment of the capacity for self-renewal of certain adult stem cells has been obtained by single cell or serial transfer into acceptable hosts, an excellent example of which is adult hematopoietic stem cells (HSCs).

2.3 POTENCY

The issue of potency may be the most difficult parameter to incorporate into a widely accepted definition of stem cells. A multipotent stem cell sits atop a lineage hierarchy and can generate multiple types of differentiated cells, the latter being cells with distinct morphologies and gene expression patterns. At the same time, many would argue that a self-renewing cell that can only produce one type of differentiated descendant is nonetheless a stem cell. A case can be made that a unipotent cell is best described as a progenitor cell for clarity of terminology. Progenitors are typically the descendants of stem cells, only they are more constrained in their differentiation potential or capacity for self-renewal, and are often more limited in both senses.

2.4 CLONALITY

Replicative capacity and potency are functional parameters. Clonality is a characteristic that describes how a population of cells, usually in culture, was derived. A clonal population is generated from a single cell, such as a stem cell, with the capacity for self-renewal. Clonality becomes very important when one asks what constitutes a cell line. Although the clonal 'gold standard' is well understood, there remain several confounding issues when applied to stem cells.

The lowest standard of defining a cell line would be to include any population of cells that can be grown in culture, frozen, thawed, and subsequently repassaged *in vitro*. A higher standard would be to limit the definition to a clonal or apparently homogeneous population of cells, but it must be recognized that cellular preparations that are not derived from a single cell may be a mixed population. Such preparations may contain both stem cells and other cells, some of which may be required to support the propagation of the purported stem cells. Hence, any reference to a stem cell line should include an explanation of its derivation. For example, it can be misleading to report on stem cells or stem cell lines if they were prepared from a tissue containing

multiple cell types because the possibility that the culture is contaminated with stem cells from another tissue (e.g., blood vessels) exists.

2.5 DEFINITION

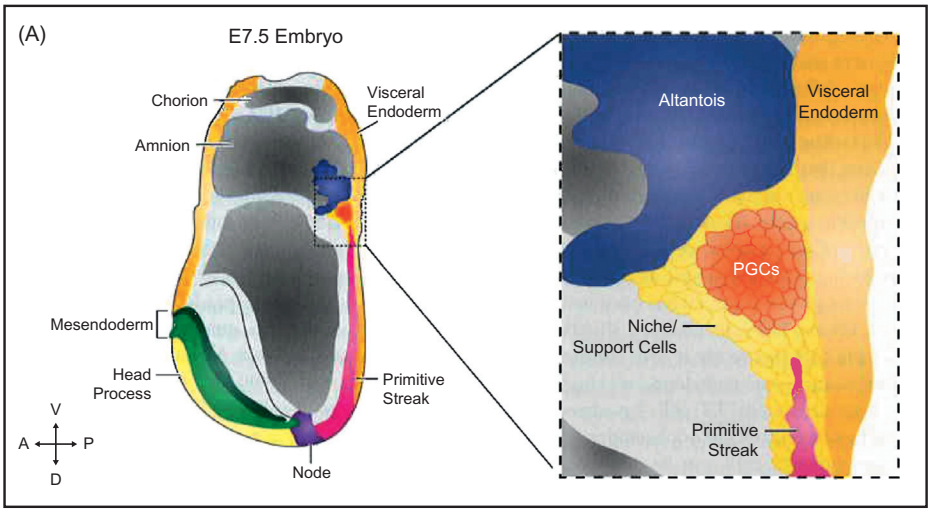
In conclusion, a working definition of a stem cell line is a clonal, self-renewing cell population that is multipotent and thus can generate several differentiated cell types. Admittedly, this definition is not applicable in all instances and is best used as a guide to help describe cellular attributes.

2.6 WHERE DO STEM CELLS COME FROM?

The origin or lineage of stem cells is well understood for ES cells, but the origin of adult stem cells is less clear and in some cases controversial. Of significance may be the observation that ES cells originate prior to germ layer commitment, raising the intriguing possibility that avoidance of commitment to a developmental pathway may be a mechanism by which multipotent stem cells arise. The lack of information on the developmental origins of adult stems cells leaves open the possibility that they too escape lineage restriction in the early embryo and subsequently colonize specialized niches, which function to both maintain their potency as well as restrict their lineage potential. Alternatively, the prevailing model for the origin of adult stem cells hypothesizes that they arise after somatic lineage specification and then colonize their respective cellular niches. In this section, I briefly summarize the origin of stem cells from the early embryo and explain what is known about the ontogeny of adult stem cells focusing attention on HSCs and NSCs.

2.7 STEM CELLS OF THE EARLY EMBRYO

Mouse and human ES cells are derived directly from the inner cell mass of preimplantation embryos after the formation of a blastocyst. This population of cells would normally produce the epiblast and eventually all adult tissues, which may help to explain the developmental plasticity exhibited by ES cells. In fact, ES cells appear to be the *in vitro* equivalent of the epiblast, as they have the capacity to contribute to all somatic lineages and in mice to produce germ-line chimeras. By the time the zygote has reached the blastocyst stage, the developmental potential of certain cells has been restricted. The outer cells of the embryo have begun to differentiate to form trophectoderm, from which a population of embryonic trophoblast stem cells has also been derived in mice. These specialized cells can generate all cell types of the trophectoderm lineage, including differentiated giant trophoblast cells. At the egg cylinder stage of embryonic development, commonly referred to as embryonic day 6.5 (E6.5) in mice, a population of cells near the epiblast (Figure 2.1) can be identified as primordial germ cells (PGCs), which



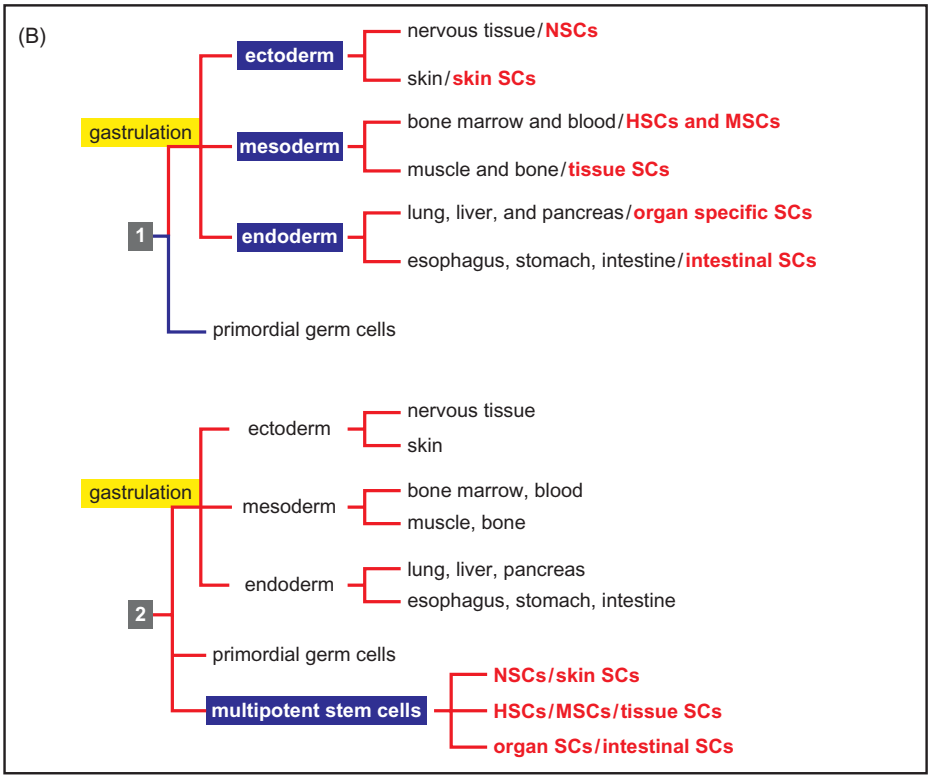


FIGURE 2.1

(A) Development of primordial germ cells. A schematic of an embryonic day 7.5 mouse embryo highlights the position of the developing primordial germ cells (PGCs) proximal to the epiblast. The expanded view on the right serves to illustrate the point that PGCs escape lineage commitment/restriction by avoiding the morphogenetic

are subsequently excluded from somatic specification or restriction. PGCs migrate to and colonize the genital ridges, where they produce mature germ cells and generate functional adult gametes. PGCs can be isolated either prior or subsequent to their arrival in the genital ridges and, when cultured with appropriate factors *in vitro*, can generate embryonic germ (EG) cells. EG cells have many of the characteristics of ES cells with respect to their differentiation potential and their contribution to the germ-line of chimeric mice. The most notable difference between ES and EG cells is that the latter may display (depending upon the developmental stage of their derivation) considerable imprinting of specific genes. Consequently, certain EG cell lines are incapable of producing normal chimeric mice.

Importantly, no totipotent stem cell has been isolated from the early embryo. ES and EG cells generate all somatic lineages as well as germ cells but rarely if ever contribute to the trophectoderm, extraembryonic endoderm, or extraembryonic mesoderm. Trophectoderm stem (TS) cells have been isolated, and these only generate cells of the trophectoderm lineage. It remains to be seen whether cells can be derived and maintained from totipotent embryonic stages. Although our understanding of cell fates in the early embryo is incomplete, it appears that the only pluripotent stem cells found after gastrulation are PGCs (with the possible exceptions of multipotential adult progenitor cells and teratocarcinomas). It may be that PGCs escape germ layer commitment during gastrulation by developing near the epiblast and subsequently migrate to positions inside the embryo proper. This developmental strategy may not be unique to PGCs, and it raises the interesting possibility that other stem cells might have similar developmental origins. Alternatively, it may be the case that adult stem cells are derived from PGCs. Although intriguing, it is important to stress that this idea lacks experimental evidence.

2.8 ONTOGENY OF ADULT STEM CELLS

The origin of most adult stem cells is poorly understood. With the issue of adult stem cell plasticity at the forefront, as described in this section, studies designed to elucidate the ontogeny of adult stem cells may help to reveal their specific lineage relationships and shed light on their plasticity and potential. Information on the origins of adult stem cells would also help to

◀ effects of migrating through the primitive streak during gastrulation. (B) Putative developmental ontogeny of stem cells. In lineage tree 1, the development of stem cells occurs after the formation of germ layers. These stem cells are thus restricted by germ layer commitment to their respective lineage (e.g., mesoderm is formed, giving rise to hematopoietic progenitors that become hematopoietic stem cells). Lineage tree 2 illustrates the idea that stem cells might develop similarly to PGCs, in that they avoid the lineage commitments during gastrulation and subsequently migrate to specific tissue and organ niches.

define the molecular programs involved in lineage determination, which may in turn provide insights into methods for manipulating their differentiation. To this end, I summarize what is known about the development of adult stem cells within the context of the hematopoietic and neural systems.

The development of hematopoietic cells in mice occurs soon after gastrulation (E7.5), although HSCs with the same activities as those in the adult have only been observed and isolated at midgestational stages (E10.5). These observations suggest that the embryo has a unique hematopoietic lineage hierarchy, which may not be founded by an adult type HSC. Thus, hematopoiesis appears to occur at multiple times or in successive waves within the embryo, and the emergence of an HSC may not precede or be concomitant with the appearance of differentiated hematopoietic cells.

The first site of hematopoiesis in the mouse is the extraembryonic yolk sac, soon followed by the intraembryonic aorta–gonad–mesonephros (AGM) region. Which of these sites leads to the generation of the adult hematopoietic system and, importantly, HSCs is still unclear. Results from non-mammalian embryo-grafting experiments, with various findings in the mouse, suggest that the mammalian embryo, specifically the AGM, generates the adult hematopoietic system and HSCs. Interestingly, the midgestational AGM is also the region that harbors migrating PGCs and is thought to produce populations of mesenchymal stem cells, vascular progenitors, and perhaps hemangioblasts.

In the absence of studies designed to clonally evaluate the lineage potential of cells from the AGM, and without similarly accurate fate mapping of this region, it remains possible that all of the adult stem cell types thought to emerge within the AGM arise from a common unrestricted precursor. This hypothetical precursor could help explain reports of nonfusion-based adult stem cell plasticity. The observed lineage specificity of most adult stem cells could likewise be attributed to the high-fidelity lineage restriction imposed on them by the specific niche they colonize or are derived from. Simple ideas such as these have not been ruled out by experimental evidence, underscoring both the opportunity and the necessity for further study of the developmental origins of adult stem cells.

A key lesson from studies of the developing hematopoietic system is that the appearance of differentiated cells does not tell us where or when the corresponding adult stem cells originate. Definitive lineage tracing, with assays of clonogenic potential, remains the method of choice for identifying the origin of stem cells. Another potential pitfall revealed by these studies is that the definition of the stem cell can influence whether and how it is identified.

The development of NSCs begins with the formation of nervous tissue from embryonic ectoderm following gastrulation. Induction of the neural plate is

thought to coincide with the appearance of NSCs as well as restricted progenitor types. The exact frequency and location of stem cells within the developing neuroepithelium remains unknown; specific markers must be discovered to fully unravel this question. An emerging view in the field is that embryonic neuroepithelia generate radial glia that subsequently develop into periventricular astrocytes, and that these cells are the embryonic and adult NSCs within the central nervous system. Developing and adult NSCs also appear to acquire positional and temporal information. For example, stem cells isolated from different neural regions generate region-appropriate progeny. In addition, several studies suggest that temporal information is encoded within NSCs, that earlier stem cells give rise more frequently to neurons, and that more mature stem cells preferentially differentiate into glia. Moreover, more mature NSCs appear incapable of making cells appropriate for younger stages when transplanted into the early cerebral cortex.

Taken together, observations to date suggest that the nervous system follows a classical lineage hierarchy, with a common progenitor cell generating most if not all differentiated cell types in a spatially- and temporally-specific manner. Rare stem cells may also exist in the nervous system, perhaps not of neural origin, which have greater plasticity in terms of producing diverse somatic cell types and lacking temporal and spatial constraints. Several caveats must be considered when describing the developmental origins of NSCs. First, disrupting the neuroepithelia to purify NSCs may have the undesirable effect of dysregulating the spatial patterning normally acquired by these cells. Second, growth of purified NSCs *in vitro* may reprogram the cells through exposure to non-physiological conditions during culture. Both of these problems can be addressed either by *in vivo* lineage tracing or by prospectively isolating NSCs and transplanting them into acceptable hosts without intervening culture. Such experiments, carefully done, might answer questions important for stem cell biology but also for neuroembryology and development. These questions include which features of the developmental program are intrinsic to individual cells, which differentiation or patterning signals act exclusively to instruct specific cell fates, and how developmental changes in cell-intrinsic programs restrict the responses of progenitors to cell-extrinsic signals.

2.9 HOW ARE STEM CELLS IDENTIFIED, ISOLATED, AND CHARACTERIZED?

The ways that stem cells are identified, isolated, and characterized are the key methodological questions in stem cell biology, so much so that subsequent chapters are devoted to addressing these problems in detail. Here, I briefly

outline standards and criteria that may be employed when approaching the challenge of identifying, isolating, and characterizing a stem cell.

2.10 EMBRYONIC STEM CELLS

The basic characteristics of an ES cell include self-renewal, multilineage differentiation *in vitro* and *in vivo*, clonogenicity, a normal karyotype, extensive proliferation *in vitro* under well-defined culture conditions, and the ability to be frozen and thawed. In animal species, *in vivo* differentiation can be assessed rigorously by observing whether transferred ES cells contribute to all somatic lineages and produce germ-line chimerism. However, experimentation ethics prohibit the same experiments being performed with human ES cells; consequently, human ES cells are assayed for their ability to generate embryoid bodies and teratomas containing differentiated cells of all three germ layers. Moreover, because a stringent *in vivo* assessment of pluripotency is impossible, human ES cells must exhibit expression of well-known molecular markers of pluripotent cells. Such markers are factors that are expressed consistently and are enriched in human ES cells.

Another experimental substitute for whole-animal chimerism is to evaluate the ability of human ES cells to contribute to the development of specific tissues when transplanted into discrete developmental regions of nonhuman adults or embryos. A complementary analysis might include transplanting human ES cells into nonhuman blastocysts and evaluating their contribution to various organs and tissues of the resulting embryo, although producing human/nonhuman chimeras at this earliest stage of development has raised ethical concerns.

Finally, a practical consideration for all ES cells is the number of times the cells have been passaged *in vitro*. Although it is important to establish the capacity of ES cells to proliferate extensively, it is equally important to preserve stocks of cells that have been passaged only a few times so that experimental findings observed on working stocks of ES cells can be verified with low-passage cells to screen for artifacts that can be introduced during *ex vivo* expansion.

2.11 ADULT STEM CELLS

The basic definition of an adult stem cell is that the culture is derived from a single cell (clonal) that self-renews and generates differentiated cells. The most rigorous assessment of these characteristics is to prospectively purify a population of cells (usually by cell surface markers), transplant a single cell

into an acceptable host without any intervening *in vitro* culture, and observe self-renewal and reconstitution of either a tissue, organ, or lineage as appropriate for the adult stem cell type. Admittedly, this type of *in vivo* reconstitution assay is not well defined for many types of adult stem cells. Thus, it is important to define a set of functional assays that accurately reflect the cells' developmental potential and can be performed on *in vitro* cultures. Above all, clonal assays should be the standard by which fetal and adult stem cells are evaluated because this assay removes any doubt that an observation is the result of contamination of a culture with other cell types.

Two concepts about the fate or potential of stem cells have moved to the forefront of adult stem cell research. The first is plasticity; the idea that restrictions in cell fates are not permanent but are flexible and reversible. The most obvious and extreme example of reversing a committed cell fate comes from experiments in which a terminally differentiated somatic cell generates an entire animal following nuclear transfer, or cloning. Nuclear transfer experiments have demonstrated that differentiated cells, under the appropriate conditions, can be returned to their most primal state. Thus, it may not be surprising if conditions are found for more committed or specified cells to dedifferentiate and gain a broader potential. A related concept is that of transdifferentiation. Transdifferentiation is the generation of functional cells of a tissue, organ, or lineage that are distinct from that of the founding stem cell. Important issues here are whether the cells proposed to transdifferentiate are clonal, and whether the mechanism by which they form the resulting cell type requires fusion. Experiments designed to carefully evaluate these possibilities will yield further insight into the basic nature of stem cells.

2.12 STEMNESS: PROGRESS TOWARD A MOLECULAR DEFINITION OF STEM CELLS

Stemness refers to common molecular processes underlying the core stem cell properties of self-renewal and the generation of differentiated progeny. Although stems cells in different cellular microenvironments or niches will by necessity have different physiological demands and therefore distinct molecular programs, there are likely to be certain genetic characteristics that are both specific to and shared by all stem cells. Through transcriptional profiling, many genes that are enriched in ES cell, TS cell, HSC, and NSC populations have been identified. By extending transcriptional profiling to other stem cells and more organisms, it may be possible to develop a molecular fingerprint for stem cells. Such a fingerprint could form the basis of a molecular definition of stem cells that, when combined with functional characteristics, would provide a more comprehensive set of criteria for understanding their

unique biology. Perhaps more importantly, transcriptional profiling could eventually become the primary tool by which new stem cells are identified and isolated.

The goal of having a comprehensive definition of stemness is far from being accomplished, but preliminary findings for specific stem cells have been described. Transcriptional profiling of stem cells has suggested that several distinct molecular characteristics are shared. Stem cells appear to have the capacity to sense a broad range of growth factors and signaling molecules and to express many of the downstream signaling components involved in the transduction of these signals. Signal transduction pathways present and perhaps active in stem cells include TGF, Notch, Wnt, and Jak/Stat family members. Stem cells also express many components involved in establishing their specialized cell cycles, either related to maintaining cell cycle arrest in G1 (for most quiescent adult stem cells) or connected to progression through cell cycle checkpoints promoting rapid cycling (as is the case for ES cells and mobilized adult stem cells).

Most stem cells also express molecules involved in telomere maintenance and display elevated levels of telomerase activity. Considerable evidence exists that stem cells have significantly remodeled chromatin due to the activity of DNA methylases, transcriptional repressors of histone deacetylase, and Groucho family members. Another common molecular feature is the expression of specialized posttranscriptional regulatory machinery that is influenced by RNA helicases of the Vasa type. Finally, a shared molecular and functional characteristic of stem cells appears to be their resistance to stress, mediated by multidrug resistance transporters, protein-folding machinery, ubiquitin, and detoxifier systems.

Although in its infancy, the search for a molecular signature to define stem cells continues. We have begun to understand in general terms what molecular components are most often associated with stem cells. In the future, it may be possible to precisely define stem cells as a whole and individually by their tell-tale molecular identities. Until that time, stemness remains a concept of limited utility with tremendous potential.

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FOR FURTHER STUDY

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