

Regulatory Mechanisms in Stem Cell Biology

Review

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Introduction

Stem cells are a subject of intense and increasing interest because of their biological properties and potential medical importance. Unfortunately, the field has been difficult for the nonspecialist to penetrate, in part because of ambiguity about what exactly constitutes a stem cell. A working definition is useful in order to pose the important questions in stem cell biology. However, since different people define stem cells in different ways (for examples, see Hall and Watt, 1989; Potten and Loeffler, 1990), formulating a generally acceptable definition can lead to a conclusion similar to that of U. S. Supreme Court Justice Byron White's in regard to pornography: "It's hard to define, but I know it when I see it." A minimalist definition is that stem cells have the capacity both to self-renew and to generate differentiated progeny. Although this is in many respects inadequate, it immediately highlights some important problems: How at each cell division is a stem cell able to pass on its "stem" properties to at least one of its two daughters? And what determines whether stem cell divisions will be self-renewing, or differentiating?

In considering these and related questions, we will draw primarily on examples provided by stem cells in the mammalian hematopoietic and nervous systems, as well as by *C. elegans*. The focus on hematopoiesis and neurogenesis reflects the fact that these systems are the ones in which stem cells have been most rigorously and directly identified. Hematopoietic stem cells (HSCs) have been isolated using antibodies to cell surface antigens (Spangrude et al., 1988), and their functional properties have been established by transplantation into lethally irradiated host animals under conditions where the progeny of a single stem cell can be identified ("clonogenic" assays; for review, see Morrison et al., 1994). The self-renewal properties of these cells have been demonstrated by serial transfer into secondary recipients.

The brain has not traditionally been considered a stem cell system because of the dogma that this tissue is incapable of regeneration. Recently, however, there has been a rediscovery of Altman's original observations (Altman, 1969) that some regions of the adult brain exhibit ongoing neurogenesis, and this has been accompanied by a surge of activity in identifying the progenitor cells responsible for both embryonic and postnatal neural development (for reviews, see Alvarez-Buylla and Lois, 1995; Gage et al., 1995; Weiss et al., 1996). Stem cells in the neural crest (Stemple and Anderson, 1992) and embryonic central nervous system (CNS) (Davis and Temple, 1994; Johe et al., 1996; Reynolds and Weiss, 1996) have been identified using in vitro assays in which

the differentiation and self-renewal capacity of single progenitor cells have been demonstrated by subcloning experiments. It is not yet clear, however, whether any of these neural stem cells can generate all the different classes of neurons found in the adult CNS or PNS, nor is it clear whether the stem cells isolated from adult brain tissue manifest their multilineage differentiation capacity under physiological conditions in vivo.

The existence of stem cells in the gut (Potten and Loeffler, 1990), gonads (Dym, 1994), skin (Lavker et al., 1993), and olfactory epithelium (Monti Graziadei and Graziadei, 1979) has been demonstrated indirectly by mosaic in vivo lineage-marking experiments, anatomical studies, or in vitro experiments. Although the standard of proof defined for HSCs or neural stem cells has not yet been achieved, one can proceed on the assumption that stem cells exist in these tissues. It has also been proposed that stem cells exist in the liver (Sigal et al., 1992), a tissue which can regenerate in response to injury, although this is controversial (Wilson, 1996) because under most conditions differentiated cell types reenter the cell cycle and contribute the preponderance of regeneration.

Properties of Stem Cells

A number of properties besides self-renewal and differentiation potential are frequently ascribed to stem cells, including the ability to undergo asymmetric cell divisions, exhibit extensive self-renewal capacity, exist in a mitotically quiescent form, and clonally regenerate all of the different cell types that constitute the tissue in which they exist (Hall and Watt, 1989; Potten and Loeffler, 1990). Below, we illustrate how many of these properties are exhibited by stem cells in some tissues or organisms, but not in others. This helps to distinguish the most fundamental questions in stem cell biology from questions that are highly relevant but specific to certain systems. It also illustrates the difficulty in arriving at a universally applicable definition of a stem cell. While some readers will undoubtedly take issue with this point of view, a certain tolerance of ambiguity in the definition of stem cells is necessary in order to remain focused on the mechanistic questions and avoid semantic arguments.

Symmetric Versus Asymmetric Divisions

Stem cells are often thought to undergo repeated, intrinsically determined asymmetric cell divisions that produce one differentiated (progenitor) daughter and another daughter that is still a stem cell (Figure 1A). While there are clear examples of such lineages in *Hirudo medicinalis*, *Drosophila melanogaster*, and *Caenorhabditis elegans*, in mammalian systems there is stronger evidence that stem cells divide symmetrically (Figures 1B and 1C). Symmetric divisions allow the size of the stem cell pool to be regulated by factors that control the probability of self-renewing versus differentiative divisions (for more detailed discussion, see Potten and Loeffler, 1990).

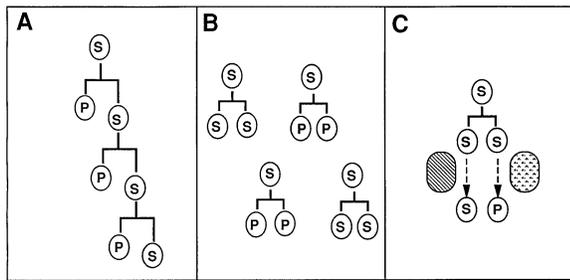


Figure 1. Possible Patterns of Cell Division in Stem Cell Lineages “S” indicates stem cell; “P” indicates a committed or restricted progenitor cell.

(A) All divisions are obligatorily asymmetric and controlled by a cell-intrinsic mechanism. Note that no amplification of the size of the stem cell population is possible in this type of lineage.

(B) A population of four stem cells is shown in which all divisions are symmetric, but half the time are self-renewing. The steady-state behavior of this population is indistinguishable from that of a population of stem cells like that shown in (A). However, the probabilities of self-renewing versus differentiative divisions could in principle be different than 0.5 (see Potten and Loeffler, 1990, for further discussion).

(C) A lineage in which individual stem cell divisions are asymmetric with respect to daughter cell fate, but not intrinsically so, as in (A). The daughters behave differently owing to different local environments (shaded ovals). Examples of all of the patterns in (A)–(C) are found in nature, including combinations of (B) and (C).

Self-Renewal Capacity

Murine HSCs do not have unlimited self-renewal potential, although a subset is able to self-renew for the lifetime of a mouse (for review, see Morrison et al., 1994). However, in larger, longer-lived animals, such as humans, it is not at all clear that HSCs self-renew for an entire lifespan; rather, successive subsets of stem cell clones may become activated with increasing age (Abkowitz et al., 1990). Even in small, shorter-lived organisms, there is clear evidence that stem cells have lifetimes less than that of the entire animal. For example, one of the two somatic stem cells in the *Drosophila* ovary dies or differentiates after about 26 days (Margolis and Spradling, 1995). Thus, not all stem cells have unlimited self-renewal potential.

In tissues where serial transplantation of isolated cells is not technically possible, it is often difficult to assess the self-renewal capacity of putative stem cells *in vivo*. The mere existence of progenitor cells in an adult tissue is not *de facto* evidence that these cells have undergone extensive self-renewal, as is sometimes assumed, because they may simply have persisted in quiescent form. There are, moreover, clear cases of stem cells that exist only transiently during development, such as fetal and embryonic HSCs. Oocyte production ceases by birth, while that of sperm continues into adulthood, yet both cells derive from primordial germ cells (PGCs) whose stem cell properties are indistinguishable in males and females early in gestation (Donovan, 1994). Thus, not all stem cells self-renew into adulthood, and not all adult stem cells reflect self-renewal of fetal cells. Finally, in some cases, adult stem cells may derive neither by self-renewal nor by persistence of fetal cells, but rather may represent a distinct stem cell class that develops from

a transient fetal stem cell population (Morrison et al., 1994). This makes the entire concept of self-renewal capacity “for the lifetime of the organism” precarious as a criterion for stem cells.

Mitotic Quiescence

Another property shared by some, but not all, stem cells is that they divide slowly or rarely. This is thought to be true for stem cells in the skin (Lavker et al., 1993) and bone marrow (Morrison and Weissman, 1994). Other kinds of stem cells, however, divide more rapidly. Somatic stem cells in the *Drosophila* ovary and mammalian intestinal crypt stem cells have been estimated to divide every 12 hr (Potten and Loeffler, 1990; Margolis and Spradling, 1995). It may be generally true that stem cells in adult tissues are more likely to cycle slowly, but this quiescence is not an obligatory property of stem cells.

“Mother of All Cells”

Another characteristic attributed to stem cells is the ability to regenerate clonally the entire adult tissue from which they derive, meaning all cell types that constitute that tissue (Potten and Loeffler, 1990). In practice, this is an extremely difficult criterion to satisfy. Even in the hematopoietic system, for example, certain classes of blood cells—such as some kinds of T cells—are only produced during fetal life and are maintained in the adult by proliferation of committed cells (Ikuta et al., 1990). Therefore, adult HSCs can replace most, but not all, blood cells found in the adult tissue (reviewed in Morrison et al., 1994). The mature olfactory epithelium consists of neurons and sustentacular (glial) cells, but retroviral lineage analysis has shown that only the neurons are regenerated from stem cells in the basal layer (Caggiano et al., 1994). These examples illustrate cases where stem cells regenerate only a subset of the differentiated cell types in a given tissue. We suggest that stem cells include all self-renewing progenitor cells that have the broadest developmental potential available within a particular tissue at a particular time.

Some authors do not consider all self-renewing pluripotent progenitors to be stem cells, reserving this category only for the subset with the “most primitive” characteristics. This results in a trend to restrict incrementally the stem cell definition to smaller and smaller subsets of cells. The concept of a most primitive progenitor is inherently ambiguous because it often is based on largely untested expectations about the properties that correlate with primitiveness. If we are to understand the biology of self-renewal and pluripotency, then all self-renewing pluripotent progenitors in a given tissue should be studied.

Regenerative Capacity

It has been argued that only regenerative tissues can have stem cells. The most significant problem with this definition is that certain tissues or at least certain cell types exhibit regenerative capacity only during limited windows of ontogeny (e.g., the spinal cord [Sechrist et al., 1995], or female germ line [Donovan, 1994]). It seems arbitrary to exclude certain classes of progenitor cells from consideration simply because they display their regenerative capacity at one stage of development but not at others. The failure of regeneration in the adult may be due not to the absence of pluripotent, self-renewing cells, but to the inability of the injured tissue

to accommodate or promote their differentiation, as may well be the case in most areas of the brain (Alvarez-Buylla and Lois, 1995; Gage et al., 1995; Weiss et al., 1996).

These considerations reinforce the idea that there are basic common properties of stem cells that extend across diverse species, tissues, and developmental stages: the capacity to self-renew and to generate progeny that are fated to differentiate into mature cells. This raises the question of whether there are common molecular mechanisms, shared by all stem cells, that underlie these properties. Other properties, such as the ability to divide asymmetrically, to undergo extensive self-renewing divisions, to exist in a quiescent rather than mitotically active state, and to generate a multiplicity of differentiated derivatives, are exhibited by some classes of stem cells, but not by others.

Control of Self-Renewal

Self-renewal potential is the most fundamental property of stem cells. However, to understand self-renewal it is not sufficient simply to understand how stem cell proliferation is controlled, because not all cell divisions involve self-renewal. Are there specific signals that couple mitogenesis to maintenance of the stem cell state? Or are proliferation and maintenance of the stem cell state regulated independently by distinct signals? These issues are important because although the size of the stem cell pool remains nearly constant in many tissues under steady-state conditions, it can expand rapidly in response to tissue damage (Harrison and Lerner, 1991; Paulus et al., 1992; Lavker et al., 1993; Grisham and Coleman, 1996).

Extrinsic Regulation of Self-Renewal

What limits the number of stem cells under steady-state conditions? One possibility is that stem cells can only exist in a restricted microenvironment in each tissue, which provides factors that maintain them and excludes factors that induce differentiation (Trentin, 1970). For example, intestinal epithelium stem cells appear to be localized to a narrow ring of tissue near the base of the crypts (Potten and Loeffler, 1990). If the amount of space in such microenvironments (or "niches") is limited, the number of stem cells would be limited by the number that can fit in that space. Stem cells generated in excess of the available space would differentiate (Williams et al., 1992). Evidence for such a mechanism is scant in mammals, but in *C. elegans* the self-renewal of germ line stem cells requires proximity to the distal tip cell (Kimble et al., 1992), which produces a ligand that promotes stem cell divisions (see below). Not all stem cell systems, however, utilize such local control mechanisms. For example, PGCs self-renew while migrating to the genital ridges (Tam and Snow, 1981).

The proliferation of stem cells also increases in response to tissue damage. For example, in the sensory epithelia of the nose (Monti Graziadei and Graziadei, 1979) and the inner ear (Forge et al., 1993), damage to the primary sensory neurons induces the proliferation of cells that regenerate the lost neurons. In principle, the induction of division in such systems could be promoted either by the release of mitogens from dying cells, or

by relief from inhibitors normally produced by healthy neurons (or both); no evidence yet exists to distinguish among these possibilities. It is also assumed that such feedback control of stem cell proliferation is local, either by direct signaling to the stem cells or by indirect signaling via intermediate progenitor compartments (discussed in more detail in Potten and Loeffler, 1990).

Identity of Factors That Control Stem Cell

Self-Renewal and Their Mechanisms of Action

In *C. elegans*, the germ line stem cells require activation of the Notch-related receptor GLP-1 to retain self-renewal potential. The ligand for GLP-1, LAG-2, is membrane bound and expressed only by the neighboring distal tip cell (Henderson et al., 1994). In *glp-1* mutants, germline stem cells not only cease self-renewing mitoses, but also undergo meiosis and differentiate into gametes (Crittenden et al., 1994). Thus, LAG-2 appears to be necessary both to maintain proliferation and prevent differentiation of stem cells. By contrast, genetic studies of *Notch* (a *glp-1*-related gene) in *Drosophila* have been interpreted to suggest that its primary role is to maintain cells in an undifferentiated state, whether or not those cells are actively dividing (Artavanis-Tsakonas et al., 1995). Consistent with this, activated forms of mNotch, a murine homolog of GLP-1, inhibit differentiation of myogenic and neurogenic cell lines without a detectable effect on cell proliferation (Kopan et al., 1994; Nye et al., 1994). However, lineage-specific expression of an activated form of human *Notch*, *tan-1*, is found in tumors of primitive lymphoid cells in humans (Ellisen et al., 1991). Taken together, these data suggest that Notch and its homologs can regulate proliferation or maintenance of the undifferentiated state, or both, depending on context.

Although a number of growth factors can drive quiescent HSCs into cycle, despite a vigorous search no factors have yet been identified that (singly or in combination) are capable of maintaining self-renewing divisions of these stem cells *in vitro*. In the nervous system, EGF promotes proliferation of stem cells from the adult CNS (Reynolds and Weiss, 1992), and basic fibroblast growth factor (bFGF) promotes the self-renewal of embryonic as well as adult CNS stem cells (Gritti et al., 1996; Johe et al., 1996). bFGF also promotes proliferation of primordial germ cells in culture (Resnick et al., 1992), although it also appears to broaden their developmental potential (Donovan, 1994). While these studies have been performed *in vitro*, they demonstrate that factors do exist that can cause stem cells to self-renew repeatedly when they would otherwise remain quiescent or differentiate.

Stem cell self-renewal can also be negatively regulated by locally acting or long-range factors. In tissues where stem cells have a restricted location, locally acting factors have been sought. For example, proliferation of primordial germ cells and intestinal crypt stem cells is thought to be inhibited by local sources of transforming growth factor β (TGF β) (Godin and Wylie, 1991; Podolsky, 1993). Both short- and long-range feedback mechanisms are hypothesized to regulate negatively HSC self-renewal (Zipori, 1992). Macrophage inhibitory protein 1 α , constitutively produced by macrophages, has been shown to inhibit the proliferation of multipotent progenitors (Graham et al., 1990); whether this inhibition occurs

locally or at long range is not yet clear. Since HSCs are segregated among different bones and organs throughout the body, at least some factors that regulate self-renewal must act at long range for the stem cell pool to be regulated in a coordinated fashion.

In summary, factors that regulate stem cell self-renewal can induce or inhibit proliferation, and can act locally or at long range. Few of the factors involved have been identified. In cases where factors have been identified, it is usually not known what cells produce them, or how their production is regulated. It will be interesting to determine whether there are systematic differences in stem cell regulation between tissues with relatively invariant architecture, like intestinal crypts, and those with more flexible architecture, like the hematopoietic system.

Do Stem Cells Have Intrinsic Limitations on Their Self-Renewal Capacity?

The self-renewal capacity of certain stem cells may exceed the extent of self-renewal that they actually undergo *in vivo*. Does that mean that self-renewal capacity is unlimited, or are there limitations on self-renewal capacity even when that capacity exceeds actual self-renewal fate? The hematopoietic system clearly exemplifies that not all pluripotent stem cells have equivalent self-renewal capacities. Individual HSCs can exhibit either transient (< 8 weeks) or long-term (> 16 weeks) self-renewal capacity (Harrison and Zhong, 1992). This difference was proposed to depend on the environment encountered by intrinsically similar cells (Uchida et al., 1993). However, fractionation of HSCs by surface marker expression has revealed distinct subpopulations that exhibit different self-renewal capacities even when the cells are exposed to equivalent environments *in vivo* (Morrison and Weissman, 1994), implying that these differences are cell intrinsic.

The molecular basis of self-renewal capacity remains to be elucidated. Even in cases where this has been shown to be an intrinsic property of stem cells, the molecules need not act in a purely cell-autonomous way. For example, differential expression of adhesion molecules could cause different HSC subpopulations to home to different bone marrow microenvironments that specify different self-renewal fates. Entirely cell-autonomous mechanisms may, however, be at work as well. Telomerase expression widely correlates with self-renewal potential in many cell types (Morrison et al., 1996a; Yasumoto et al., 1996). Recently, about 70% of fetal liver or bone marrow HSCs, but only rare non-self-renewing multipotent progenitors, were shown to exhibit telomerase activity (Morrison et al., 1996a). Unlike tumor cells, HSCs are not immortal (Ogden and Micklem, 1976), and human HSCs show decreasing telomere length with increasing age (Vaziri et al., 1994). Thus, telomerase may regulate self-renewal capacity by reducing the rate at which telomeres shorten. Stem cells with long telomeres could, nevertheless, be caused to differentiate and exit the stem cell pool by other factors.

Maintenance of the Uncommitted State by Intrinsic Factors

There is strong evidence for cell-intrinsic factors that can maintain the uncommitted nature of the stem cell

state without influencing proliferation. Germline progenitors in the *C. elegans* embryo undergo asymmetric divisions that maintain the germline lineage and produce a series of progenitor cells that become committed to various somatic fates (for review, see Guo and Kempfues, 1996). This asymmetric segregation of daughter cell fates appears to be determined by the nuclear protein PIE-1, which is maternally inherited and asymmetrically distributed to the germline daughter cells (Mello et al., 1996). PIE-1 represses the transcription of embryonic genes that cause commitment to particular somatic fates (Seydoux et al., 1996). Thus, one mechanism for maintaining the stem cell state is to actively repress genes required for commitment. Transmission of this state to daughter stem cells would require a mechanism for maintaining expression of such active repressors.

Evidence for Asymmetric Cell Divisions

As mentioned earlier, it is often assumed (incorrectly) that all stem cell lineages necessarily involve intrinsically asymmetric divisions (Figure 1A). There are several well-documented examples of such lineages in invertebrates, including *C. elegans* germline blastomeres (Mello et al., 1996; Seydoux et al., 1996) and *Drosophila* neural precursors (Rhyu et al., 1994; Spana et al., 1995). However, in mammals, there are very few examples of asymmetric stem cell divisions. In the ferret cerebral cortex, time-lapse films have revealed that some progenitor cells divide to generate one daughter that remains in the ventricular zone, and another that migrates away, presumably to differentiate to a neuron (Chenn and McConnell, 1995). Such asymmetric divisions are correlated with an orientation of the mitotic spindle perpendicular to the surface of the ventricle. The further observation that a mammalian homolog of Notch1 is asymmetrically distributed on some ventricular zone cells prior to cytokinesis (Chenn and McConnell, 1995) suggests that at least some molecules are unequally distributed to the daughter cells (although it does not mean that the orientation of this distribution is independent of environment). Asymmetric divisions of multipotent hematopoietic progenitors have also been observed in clone-splitting experiments (Mayani et al., 1993).

Molecular Determinants of Asymmetry. In *Drosophila* neuroblasts, asymmetric cell divisions are dependent upon correct mitotic spindle orientation, as well as on the asymmetric distribution of several proteins, such as *numb* and *prospero* (reviewed in Doe and Spana, 1995). The asymmetric distribution of *numb* and *prospero* is in turn controlled by additional regulators, such as *inscuteable* (for review, see Doe, 1996). Mammalian homologs of *numb* have been isolated (Verdi et al., 1996; Zhong et al., 1996), and one is asymmetrically distributed in some cortical progenitor cells (as well as in cells in other, non-neural tissues) (Zhong et al., 1996), suggesting that some asymmetric divisions in mammals may also be intrinsically determined. Distinct molecular determinants of asymmetric cleavages have also been identified in *C. elegans* and yeast (reviewed in Horvitz and Herskowitz, 1992; Guo and Kempfues, 1996), but whether these have been conserved in mammals as well is not yet known. Apparently asymmetric divisions can also reflect intrinsically symmetric divisions that place the daughter cells in different environments that confer different fates (Figure 1C). While such a mechanism has

been shown to control the fate of somatic blastomeres in *C. elegans* embryos at the four-cell stage (Priess and Thomson, 1987; Mickey et al., 1996), direct evidence for such a process in vertebrates is lacking.

Are Asymmetric Cell Divisions the Rule or the Exception? Despite the recent attention to asymmetric stem cell divisions, the available evidence favors a predominance of symmetric divisions in mammalian stem cell systems (Figure 1B). In strictly asymmetric stem cell lineages (Figure 1A), no regulation of stem cell number is possible. But there is ample evidence for such changes in the size of stem cell populations in mammals, implying that symmetric divisions must occur. The absolute number of fetal liver HSCs doubles daily during mid-gestation (Morrison et al., 1995), and during adult life in mice there is a more than five-fold increase in the absolute number of long-term self-renewing HSCs (Morrison et al., 1996b). Primordial germ cells undergo at least five rounds of symmetric self-renewing divisions while they migrate into the genital ridges during fetal development (Tam and Snow, 1981).

Some mammalian stem cell populations may undergo both symmetric and asymmetric divisions, depending on their circumstances. Indeed, neural progenitors in the ferret cortex undergo both symmetric and asymmetric divisions (Chenn and McConnell, 1995). The relative proportion of symmetric divisions appears to change over time, with symmetric divisions predominating at early time points when the stem cell pool would be expected to be expanding (Chenn and McConnell, 1995; Takahashi et al., 1996). Whether this indicates that a single cell can switch from a symmetric to an asymmetric mode of cell division is not yet clear.

Control of Stem Cell Survival

As mentioned earlier, the persistence of stem cell populations throughout adulthood likely depends on the survival of quiescent cells, as well as on the ability of cycling cells to self-renew. Evidence for quiescent stem cells has been presented in the liver (reviewed in Grisham and Coleman, 1996), the brain (Morshead et al., 1994), and in bone marrow (Morrison and Weissman, 1994). However, it is still not clear whether such apparently quiescent cells are really in G_0 or whether they are just moving very slowly through G_1 . Are there factors that promote stem cell survival, but not necessarily self-renewal? By itself, steel factor (also known as stem cell factor) promotes the survival, but not the proliferation, of HSCs (Keller et al., 1995) and primordial germ cells (Dolci et al., 1991; Godin et al., 1991); however, the regulation of these effects is likely to be complex, since steel factor is not required for the survival of HSCs and can synergize with other factors to promote stem cell proliferation (Ikuta et al., 1991; Resnick et al., 1992). Intestinal crypt (Leigh et al., 1995) and liver stem cells (Fujiio et al., 1994) are also regulated by steel factor. These data raise further questions about the regulation of steel factor expression and its combinatorial action with other factors. As more factors are identified, the control of stem cell survival is likely to become an increasing focus of investigation.

Control of Stem Cell Differentiation

This section will address the main outstanding questions concerning the differentiation of stem cells. What sets

the repertoire of potential fates available to a stem cell in a given tissue? How do stem cells choose to exit the stem cell state and begin to differentiate? In cases of multipotent stem cells, how is the choice of a particular differentiated fate made?

Determination of the Repertoire of Potential Stem Cell Fates

The overall developmental potential of a stem cell is defined by all the types of differentiated progeny it can ultimately give rise to. How is this property encoded in the stem cell in molecular terms? One possibility is that multipotent stem cells might express a set of transcription factors which individually specify different lineages or combinations of lineages. For example, mutations in the *ikaros* gene, which encodes a zinc finger protein present in HSCs, prevent the development of multiple lymphoid derivatives (Geogopoulos et al., 1994). However, it is not yet clear whether *ikaros* acts in HSCs themselves, or is independently required in multiple lymphoid sublineages at later stages of development. The entire developmental repertoire of a given multipotent stem cell could also, in theory, be specified by a single determining factor that sits at the top of a regulatory hierarchy. A targeted mutation in the bHLH transcription factor SCL prevents the development of all hematopoietic derivatives (Porcher et al., 1996), but it is not yet known whether SCL is expressed in HSCs, and, if so, required for their formation, self-renewal, or differentiation. From an evolutionary standpoint, mutations that increased the developmental repertoire of stem cells could lead to increased cellular diversity in a tissue by "duplication and modification" of cell types.

In tissues where different cell types are generated from a multipotent progenitor on a relatively precise schedule, such as the retina, multipotent cells may be competent to generate only one or two specific fates in a given period of development (for review, see Cepko et al., 1996). For example, all retinal cell types derive from multipotent progenitors (Turner and Cepko, 1987), but the competence of these progenitors to respond to environmental signals changes over time (Cepko et al., 1996). There are clear cases where competence is determined by the expression of receptors necessary to respond to fate-determining signals, but this need not always be so; in principle, competence may also be determined by expression of signal transduction molecules or transcription factors. However, there are few specific examples of this type.

How Do Stem Cells Initiate the Differentiation Process?

The differentiation of stem cells involves both exit from the uncommitted state and entry into a particular developmental pathway. Evidence from *C. elegans* indicates that these two aspects are independently controlled. Exit from the stem cell state requires loss of PIE-1, a zinc finger protein that represses the expression of genes involved in commitment to differentiation (Mello et al., 1996; Seydoux et al., 1996). This loss occurs by asymmetric distribution of PIE-1 to stem cell daughters at each blastomere division. However, the absence of PIE-1 in somatic blastomere daughters is insufficient to initiate a program of differentiation: positive-acting transcriptional regulators, such as SKN-1 (Bowerman

et al., 1993), are also required to promote entry into a particular somatic lineage.

It is not yet clear whether exit from the stem cell state and initiation of differentiation are also independently controlled in mammals. At one extreme, differentiation might be a “default” pathway executed by the stem cell when it is removed from a microenvironment that promotes maintenance of the uncommitted state. At the other extreme, specific signals might promote differentiation and consequently exit from the stem cell state. There is evidence that both mechanisms operate in the nervous system. In vitro, CNS stem cells undergo self-renewing divisions in bFGF, but upon withdrawal of this growth factor they rapidly differentiate to neurons (Johe et al., 1996). On the other hand, the differentiation of cultured neural crest stem cells to autonomic neurons is promoted by BMP2 (Shah et al., 1996; see below). These examples leave open the question of whether the effect of such environmental signals is to regulate transcription factors that maintain the stem cell state (analogous to PIE-1), or factors that promote entry into particular lineages, or both. In either case, such factors are likely to be subject to both negative and positive regulation by environmental signals, which may explain the different effects of such signals on cell fate decisions by CNS and PNS neural stem cells.

How Do Multipotent Stem Cells Select a Particular Differentiation Pathway?

The choice of fate by a multipotent stem cell could, in principle, be controlled from inside or outside the cell. There is ample evidence from invertebrate systems that such choices can be determined nonautonomously by local cell-cell interactions. For example, in *C. elegans*, an EGF-like signal produced by the gonadal anchor cell specifies the fate of vulval precursor cells (for review, see Kenyon, 1995). Similarly, in *Drosophila*, the choice between cone (glial) and photoreceptor cell fates is determined by a transmembrane ligand, BOSS, presented by the R8 photoreceptor (Zipursky and Rubin, 1994). While these examples concern cells that do not exhibit the self-renewal capability necessary to fit our definition of stem cells, they nevertheless provide important examples of how extrinsic signals can regulate fate determination in multipotent progenitors.

Selective Versus Instructive Actions of Growth Factors on Mammalian Stem Cells. In mammalian systems, there is considerable evidence that growth factors and cell-cell interactions can influence the outcome of fate decisions by multipotent progenitors at the population level. This raises a problem not encountered in invertebrate systems where the fates of individual cells are easily monitored. Specifically, growth factors could influence individual stem cells in a selective or instructive manner (Figure 2). In a selective mechanism, the stem cells commit to a particular lineage independently of the growth factors, and the factors act subsequently to control the survival or proliferation of such committed progenitors (Figures 2A and 2B). In an instructive mechanism, the growth factor causes the progenitor to choose one lineage at the expense of others (Figures 2C and 2D). In hematopoiesis, the relative contributions of these two mechanisms remain controversial (see Metcalf, 1991; Mayani et al., 1993). Forced expression of *bcl-2* in an

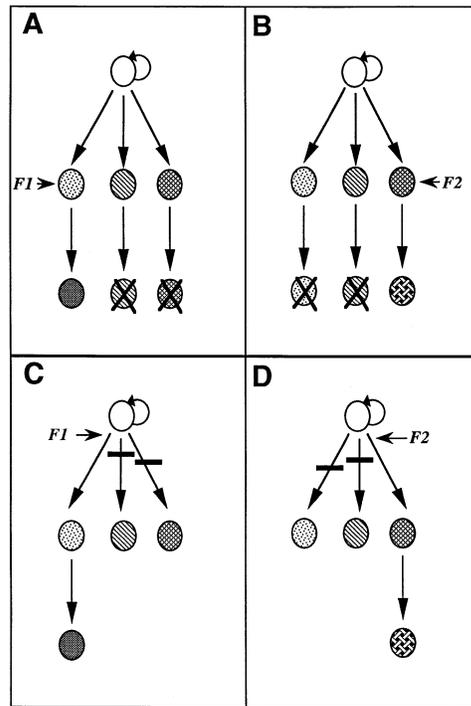


Figure 2. The Difference Between Selective and Instructive Mechanisms of Growth Factor Influences on Stem Cell Fate Decisions

(A and B) Selective mechanism in which two different factors (F1 and F2) allow the survival and maturation of lineage-committed progenitors generated by a cell-autonomous mechanism; “X” indicates death of the other progenitors. Erythropoietin appears to work in this manner (Wu et al., 1995).

(C and D) Instructive mechanism in which the factors cause the stem cell to adopt one fate at the expense of others. Glial growth factor and BMP2 appear to work in this manner on neural crest cells (Shah et al., 1994, 1996).

immortalized hematopoietic progenitor cell line yielded multilineage differentiation in the absence of cytokines, implying that these growth factors act selectively (Fairbairn et al., 1993). In the neural crest, by contrast, serial observation of individual clones in vitro has indicated that differentiation to each of three cell types—autonomic neurons, Schwann (glial) cells, and smooth muscle—can be instructively promoted by three signals: BMP2, GGF (a neuregulin), and TGFβ, respectively (Shah et al., 1994, 1996). Similarly, the differentiation of CNS stem cells to astrocytes is instructively promoted by CNTF (Johe et al., 1996). It remains to be determined whether growth factors influence stem cells in the nervous system and hematopoietic system in fundamentally different ways, or whether instructive differentiation signals for HSCs have simply not yet been identified owing to lack of appropriate assays.

Instructive Factors Can Influence Differentiation Choices Whose Outcomes Are Stochastic. Instructive environmental signals may increase or decrease the probabilities of choosing a particular fate, rather than promote or repress them in an all-or-none manner. In nematodes, the binary decision between ventral uterine (VU) and anchor cell (AC) fates by neighboring precursor cells is controlled by lateral signaling, mediated by the

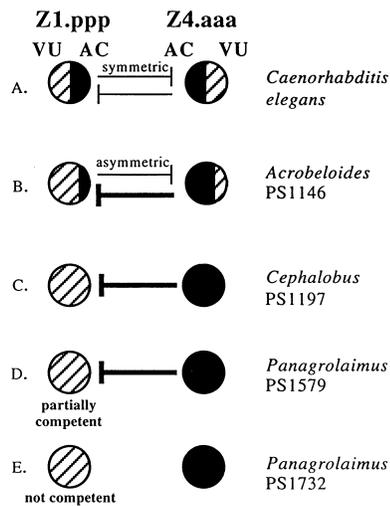


Figure 3. Phylogenetic Variation in the Control of a Binary Cell Fate Decision in Nematodes

In each case (A–E), a choice between ventral uterine (VU) and gonadal anchor cell (AC) fates is made by adjacent precursors (called “Z1.ppp” and “Z4.aaa”). In *C. elegans* (A), the decision is stochastic with a 50:50 probability and nonautonomously controlled by lateral signaling. In *Acrobeloides* (B), lateral signaling exerts a partial bias on a stochastic decision, so that the probability is about 80:20. In *Cephalobus* (C), the decision is deterministic yet nonautonomously controlled, while in *Panagrolaimus* PS1732 (E) it is both deterministic and autonomously controlled. (D) represents an intermediate case between (C) and (E) where the decision is deterministic, but displays autonomy only some of the time in laser-ablation experiments. Although the precursor cells involved do not meet our criteria for a stem cell, they illustrate how the same cell fate decision can be either stochastic or deterministic and controlled by autonomous or nonautonomous mechanisms. Reprinted with permission (from Felix and Sternberg, 1996).

NOTCH-like protein LIN-12 and its ligand LAG-2 (Figure 3). In some species, such as *Cephalobus*, this cell–cell interaction produces a deterministic (invariant) outcome (Figure 3C): the same precursor always adopts the VU fate in every animal of the species (Felix and Sternberg, 1996). In others (*Acrobeloides*), a similar cell–cell interaction produces a stochastic (probabilistic) outcome exhibiting bias (Figure 3B): one precursor becomes the anchor cell roughly 80% of the time (Felix and Sternberg, 1996). Finally, in *C. elegans*, the outcome is stochastic and unbiased: each precursor has a 50:50 probability of adopting either fate (Figure 3A). In all three cases, the cell–cell signaling is instructive, since in the absence of one precursor the other always adopts the AC fate (Felix and Sternberg, 1996). Thus, in different species, instructive signaling can exert a range of bias strengths on stochastic cell fate decisions. Similarly, it has been proposed that the engagement of MHC molecules with either the CD4 or CD8 coreceptors may exert a bias on a stochastic decision by T-cell progenitors between helper and killer cell fates (Davis and Littman, 1994).

It is sometimes assumed that if differentiation is stochastic and unbiased, a cell-autonomous mechanism must be at work. However, in *C. elegans*, the unpredictability of the outcome of the AC/VU decision derives from the equivalent strength of the reciprocal inhibi-

tory interactions between AC/VU precursors (Felix and Sternberg, 1996) (Figure 3A). Similarly, where cell-autonomous mechanisms have been inferred from the apparently stochastic behavior of hematopoietic progenitors in vitro (see Suda et al., 1983; Mayani et al., 1993), the cells are usually cultured in complex media containing serum and other sources of undefined factors, and the collective influence of such environmental factors could cause the cells to behave in an apparently unpredictable (stochastic) manner.

Autonomous Control of Cell Fate. A selective action of environmental factors implies that the initial choice of differentiated fate by a stem cell is controlled by a cell-autonomous mechanism. Such an intrinsic mechanism may yield a stochastic outcome, as has been suggested for HSCs, or a deterministic outcome. In yeast, the mating-type switch is a cell-autonomous fate decision that appears stochastic at the population level, but is deterministic for individual cells according to their previous history (Herskowitz, 1989). In early *C. elegans* embryos, the assignment of somatic blastomere fate is determined in an autonomous and deterministic manner by the asymmetric partitioning of transcription factors at successive cleavages (Bowerman et al., 1993; Hunter and Kenyon, 1996). Currently there are no clear examples of such cell-autonomous mechanisms operating in a mammalian stem cell.

There are, of course, many examples of transcription factors required for the development of particular mammalian lineages. Although once expressed these factors may impose a cell-heritable and autonomous state of determination on a progenitor cell, the initial decision to express such factors may be nonautonomously controlled. For example, the bHLH transcriptional regulator myoD is able to confer a cell-heritable state of myogenic determination, owing to its autoregulatory properties, when transfected into cultured fibroblasts (Weintraub et al., 1991). However, in vivo, the expression of this protein in somitic mesoderm is induced by a combination of signals from neighboring tissues, such as the notochord and neural plate (reviewed in Molkenin and Olson, 1996). Moreover, the execution of the muscle differentiation program in determined myoblasts is still regulated by growth factors (Molkenin and Olson, 1996). Thus, the involvement of lineage-specific transcription factors does not imply that either selection or execution of specific fates are autonomously controlled.

Order and Pattern in the Segregation of Different Lineages from Stem Cells

In principle, multipotent stem cells could generate different derivatives in a random manner (Figures 4A and 4C), or according to a predictable sequence or hierarchy (Figures 4B and 4D). There is evidence for both mechanisms in different systems. In grasshopper, the midline neuroblast sequentially produces neurons, glia, and neurons again (Condrón and Zinn, 1994). In the vertebrate retina, different cell types emerge on a predictable schedule (Cepko et al., 1996), although whether individual progenitors generate their differentiated progeny in a fixed order is not yet clear. In contrast, clone-splitting experiments in vitro have suggested that there is no perceptible order or pattern to the emergence of different lineages from multipotent hematopoietic progenitors (Suda et al., 1983), although since no lymphoid

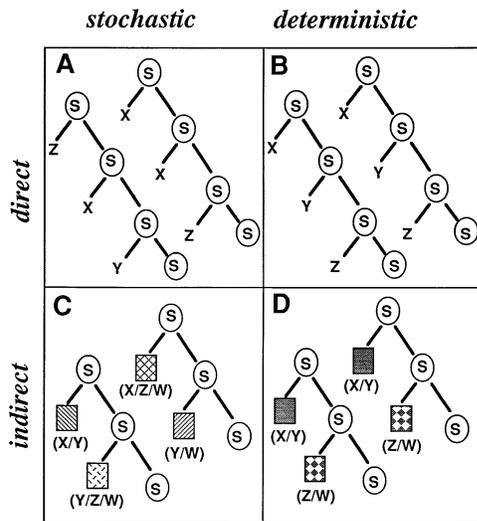


Figure 4. Alternative Modes of Differentiation by Multipotent Stem Cells

In each panel, two equivalent stem cells in a population are shown. In a “direct” mode (A and B), the immediate progeny of stem cell divisions are committed to a single fate. This mode frequently operates in invertebrates. In an “indirect” mode (C and D), stem cell progeny are partially restricted to a subset of potential fates. This mode operates in hematopoiesis. In either case, the segregation of different lineages can exhibit no perceptible order or pattern (“stochastic;” A and C), or can occur according to a defined sequence or hierarchy (“deterministic;” B and D). For convenience, all examples are shown with asymmetric stem cell divisions; however, symmetrically dividing stem cells could operate with each mode as well. Furthermore, hierarchical restrictions, as shown in (B) and (D), could occur by progressive loss of developmental potentials from partially restricted intermediates, rather than by sequential production from a self-renewing stem cell. Finally, all four modes could be controlled either cell-autonomously or nonautonomously. (For an example of a stochastic decision that is nonautonomously controlled, see Figures 3A and 3B.)

differentiation was detected it is not clear whether these conclusions apply to HSCs.

A related question is whether the immediate progeny of stem cells are committed to a single fate (“direct” differentiation; Figures 4A and 4B), or restricted to a subset of fates (“indirect” differentiation; Figures 4C and 4D). CNS stem cells generate some progeny fated to produce only neurons (Davis and Temple, 1994), but whether these unifant cells are truly committed was not determined. Committed neuronal progenitors have been identified in the PNS (Lo and Anderson, 1995), but whether these are directly generated from stem cells is not yet clear. In the hematopoietic system, progenitors committed to single lineages (e.g., B cell or T cell) have been shown to be derived from partially restricted lymphoid progenitors (Galy et al., 1995; Wu et al., 1996). Analogous partially restricted progenitors have been suggested to exist in the neural crest based on *in vitro* clonal analyses (Le Douarin et al., 1991), but whether these cells are truly committed to a subset of lineages has not been rigorously tested by exposure to appropriate instructive signals. The existence of partially restricted intermediates raises the additional question of whether their developmental potentials are assorted

randomly (Figure 4C), or in an ordered, hierarchical manner (Figure 4D). The hematopoietic system may employ both strategies, depending upon the stage of lineage diversification (Suda et al., 1983; Wu et al., 1996). An ordered or hierarchical segregation of lineages at the cellular level may reflect the action of transcription factors that coordinately specify multiple sublineages; for example, there are lymphoid progenitors restricted to B and T sublineages (Wu et al., 1996) and several transcription factors, such as *ikaros* and *E2A*, required for both sublineages (for review, see Kehrl, 1995).

Formation of Stem Cells

Stem cells in the hematopoietic system, nervous system, gonads, liver, and intestine form *de novo* during fetal life. The progenitors of stem cells are sometimes referred to as pre-stem cells. Pre-stem cells can be defined as cells whose progeny contribute to tissues other than that derived from the particular stem cell they generate, and that produce stem cells only during a defined interval of development. While the sites of stem cell formation during mammalian fetal development are generally known, the identities of the pre-stem cells are usually not known; furthermore, little is known about the events that regulate the acquisition of stem cell competence.

Are there any genes identified that are required for the formation of stem cells? In *Drosophila*, asymmetrically dividing CNS progenitors, which are in many ways like stem cells, delaminate from a group of neuroectodermal precursor cells. Within this group, the bHLH transcription factors *ACHAETE-SCUTE* confer competence to generate the progenitor (Campuzano and Modolell, 1992). A single progenitor is selected from the group of competent cells by lateral inhibition, mediated by Notch proteins and their ligands (Ghysen et al., 1993). Recent data indicate that a similar process underlies the selection of neuronal precursors during primary neurogenesis in *Xenopus* (Chitnis et al., 1995; Ma et al., 1996). Although such amphibian neuronal precursors have not been defined as stem cells, a similar mechanism may be employed in the mammalian CNS, where stem cells have been clearly identified. Genes encoding both transcription factors and extracellular signals that are involved in the formation of the hematopoietic system have been identified (Maeno et al., 1996; Porcher et al., 1996), but whether these act at the level of stem cell formation is not yet known. Genetic screens in zebrafish may identify more such molecules (for review, see Zon, 1995).

There is evidence that different classes of stem cells can exist simultaneously in the same tissue. Stem cells from different positions along the cephalocaudal axis of the gut exhibit position-specific differences in terms of the differentiated cells they give rise to. When explants from different portions of the intestine were transplanted subcutaneously, the regional differences appeared to persist, providing some evidence that the differences may be intrinsic to the stem cells (Rubin et al., 1992). There is also evidence for regional differences among central nervous system progenitor cells. Mouse basal ganglion progenitors, but not ventral mesencephalic progenitors, were able to differentiate into striatal

cells upon transplantation into rat striatum, suggesting that the progenitors differed in their ability to adopt the fates of their new tissues (Campbell et al., 1995). Such differences are correlated with the region-specific expression of transcriptional regulators in the neuroepithelium from the earliest stages of brain development (for review, see Puelles and Rubenstein, 1993), suggesting an intrinsic component to such progenitor cell diversity. On the other hand, there are several cases where neural precursors adopt a correct identity when transplanted from one region into another (reviewed in Temple and Qian, 1996), suggesting that intrinsic differences may not always irreversibly commit such cells to a given fate.

The developmental potential of stem cells for a given tissue can differ in time as well as in space. Fetal liver HSCs are thought to give rise to adult bone marrow stem cells (Fleischman et al., 1982). Yet fetal liver stem cells are able to give rise to several classes of blood cells that adult bone marrow stem cells do not themselves produce (Ikuta et al., 1990; reviewed in Morrison et al., 1994). These differences are intrinsic to the stem cells since they persist even when fetal liver stem cells are transplanted into adult bone marrow, or when both stem cell types are transplanted into culture. The mechanisms underlying such stage-specific differences in developmental potential are not known.

Perspective

In this review, we have tried to raise and address some of the key mechanistic questions in stem cell biology. A few salient points emerge. First, molecules that maintain the stem cell state are beginning to be identified: ligands of Notch family receptors do this from outside the cell, and factors like PIE-1 do it from within. At least some of these mechanisms appear conserved in mammals. Second, we are beginning to gain insight into the mechanisms that may regulate stem cell self-renewal capacity, such as expression of telomerase. Third, it is now clear that at least some stem cells can be instructed to choose one pathway of differentiation, at the expense of others, by growth factors. In other systems, however, stem cells may make this choice stochastically, and growth factors may act mainly as survival factors or mitogens for committed cells. Understanding the interplay between extracellular and intracellular regulatory factors in controlling lineage determination remains an important challenge for the future.

A great deal of effort in the near term is likely to be invested in identifying self-renewal and survival factors for stem cells in various tissues. This in turn will allow investigation of the way in which these factors interact with cell-intrinsic molecules to maintain the uncommitted state and transfer it to daughter cells at each stem cell division. Some of the most interesting future questions will involve understanding the control of stem cell behavior at the population level, e.g., in tissues undergoing regeneration in response to injury. What feedback mechanisms operate to maintain the steady state in such tissues, to initiate the regenerative response and to restore the system back to steady state once regeneration is achieved? Stem cells in the adult brain present

a particularly intriguing subject for study. What is the normal function of these cells? Can the system be manipulated to exploit the regenerative potential implied by the existence of these cells, as a recent study (Craig et al., 1996) suggests? The answers to such questions will advance our understanding of basic developmental mechanisms, and may open new avenues for therapeutic intervention in humans.

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