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Chick Stem Cells

C.D. Stern

1 Introduction

A stem cell is defined as a proliferating cell with the ability to renew itself. In the adult organism, such cells are required to maintain a continuous supply of cells to compensate for cell loss throughout the lifetime of the individual and therefore include the basal proliferating cells that renew the epidermis, specialized cells that maintain the inner lining of the digestive system, progenitors of the spermatozoa in the male, hematopoietic progenitors responsible for ensuring a supply of blood cells, and probably the olfactory epithelium, which produces sensory neurons throughout life. The fertilized egg itself could be considered as a stem cell, since subsequent divisions generate all the somatic cells of the organism as well as germ cells.

In the embryo, there are surprisingly few cases in which the existence of stem cells has been unambiguously demonstrated, other than the progenitors of cells known to have this property in the adult. And yet, embryonic development can in some ways be likened to the maintenance of the hematopoietic (including the immune) system: both must generate many cells and ensure a correct balance of cell diversity to fulfill appropriate functions. Stem cells are therefore

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centrally implied by the title of this volume, which aims to stress the similarities between immunology and embryology as well as to underline the value of the avian embryo in both disciplines.

2 Cell Fate and Asymmetry of Cell Division

A common misconception is that stem cells must be multipotent; that is, they must give rise to different cell types and therefore they cannot be committed to any particular fate. However, this is not necessarily the case. In the adult, some of the best examples of stem cells, such as the basal cells of the skin, appear to be committed to a single fate: that of generating epidermal cells. Their division generates some progeny that continue to divide and other progeny that begin to differentiate and lose the potential to proliferate. Other examples, notably in the hematopoietic system, do include cells that are multipotent, and the initiation of the commitment of their progeny to differentiate is accompanied, at some stage, by selection from a specific subset of fates.

Is the decision between proliferation, differentiation and self-renewal made at the level of single cells or at the level of cell populations? These two modes imply very different mechanisms to ensure a continued supply of stem cells. In the former, each cell division is asymmetric: one daughter must retain the stem cell property while the other daughter enters into a pathway of differentiation. This mode is probably best exemplified by the basal layer of the epidermis (Potten and Morris 1988; Parkinson 1992), where at each cell division one daughter remains in the basal layer and continues to proliferate, retaining its stem cell properties and representing the founder cell of a single epidermal proliferative unit (EPU; see Potten and Morris 1988). In the case in which the decisions are made at the level of cell populations, cell divisions are not necessarily asymmetric; when a stem cell divides, it is conceivable that both daughters retain the stem cell character. This mode is advantageous when a decision from among several possible fates must be made and is probably the mechanism by which the hematopoietic system maintains itself (Sigal et al. 1992; Zipori 1992; Janssen 1993). However, there is little or no direct evidence for this.

The second mechanism requires some signal, external to the cell itself, ensuring that at least one cell retains its multipotency and stem cell character. One possible mechanism, favored by many workers on the bone marrow, is that there are specialized physical niches within the marrow which maintain cells in this special state (Parakh and Kannan 1993; Tamayo et al. 1993; Rosendaal et al. 1994). Cells divide and retain their multipotency and ability to self-renew as long as they remain in this niche, but when progeny leave it, they may lose one or both of these characteristics. In a sense, therefore, both modes of stem cell self-renewal are compatible with the existence of special spatial niches that maintain this characteristic, and the basal layer of the skin is probably one such niche; however, the second mechanism is absolutely dependent on external stimuli.

Stern, C.D. (1996) Chick stem cells. *Curr. Top. Microbiol. Immunol.* **212**, 193-204.

Perhaps the clearest, and therefore the most extreme, example of stem cells operating at the level of a cell population is that of the so-called embryonic stem cells (ES cells). When an early mammalian embryo (at a stage before the appearance of the blastocoele) is dissociated into single cells and these are placed in culture on a confluent monolayer of feeder cells, they continue to divide without differentiating for a very long time, greatly expanding the cell population. Treatment of such cultures with specific factors, such as retinoic acid, can induce their differentiation into many different recognizable cell types. Under some conditions, cells begin to aggregate into nodules, known as embryoid bodies. If cells from one such body, or indeed subconfluent ES cells, are injected into a young blastocyst, the cultured cells can contribute to any cell lineage in the embryo, including the germ cells. Indeed, it is this property that is exploited in the production of transgenic mice (see Joyner 1991; Robertson et al. 1992). The case of ES cells represents a clear demonstration that stimuli external to the cells can direct their continued self-renewal or the initiation of cell differentiation and the loss of the stem cell character.

Formally, therefore, a cell can only be defined as a stem cell if it always produces more stem cells. According to this strict definition, asymmetry of cell division is required; ES cells are therefore not "true" stem cells because their divisions are not necessarily asymmetric and because the control of their self-renewal is external to the cell being considered.

In the remainder of this review, I will concentrate first on asymmetric cell divisions and then briefly discuss several candidate cell populations that may have stem cell properties in the avian embryo, concentrating on a specific population of putative stem cells in the "organizer" region of the gastrulating chick embryo (Hensen's node) as a possible example of these. Finally, I will consider whether it will be possible to generate the avian equivalent of ES cells and the future prospects for constructing transgenic chicks.

3 Asymmetric Cell Divisions in Early Development

The "standard" view of cell division is of a cell that divides to generate two identical daughters. However, the nematode *Caenorhabditis elegans*, an organism in which the complete cell lineage during development is known, illustrates the fallacy of this view: out of the 949 somatic cell divisions that produce this animal, no fewer than 807 (85%) are asymmetric, giving rise to daughters that differ from one another in their fate. In this organism at least, asymmetry of cell division is the rule rather than the exception. One example of this is in the progeny of the N neuroblast. This cell divides to give rise to a daughter (A) that differentiates into a neuron and to another neuroblast which, at the next cell division, generates another neuron (B) and whose other daughter undergoes programmed cell death (Fig. 1).

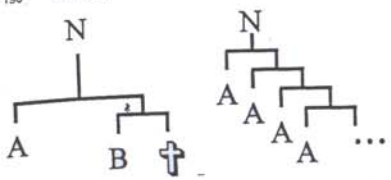


Fig. 1. Progeny of the N neuroblast

Fig. 2. Division of the N neuroblast in *unc-86* mutants

The asymmetry of division of the N neuroblast requires the expression of a POU domain containing transcription factor, *unc-86* (Finney and Ruvkun 1990). In *unc-86* mutants, rather than generating the above scheme, the N neuroblast acquires stem cell-like characteristics, so that at each of many subsequent divisions it gives rise to a neuron and another neuroblast (Fig. 2).

These results suggest that the *unc-86* gene product is required to prevent the self-renewal of the N progenitor; that is, it suppresses the stem cell-like character of the cell division. The *unc-86* gene is expressed in 57/302 neurons in different lineages, but always in just one of the two daughters of a cell division and not in the other daughter or the mother cell. However, it is not expressed in the remaining 750 asymmetric cell divisions in the animal, suggesting that other genes are also required to define the extent of this asymmetry.

Genes homologous to *unc-86* exist in vertebrates. One example is *brn-3*, with 85% homology within the POU domain. It is expressed in various neural organs in the chick, mouse, rabbit, monkey and human (Xiang et al. 1993; Gerrero et al. 1993). Another vertebrate POU domain gene with homology to *unc-86* is *Oct-2*, which, in addition to the nervous system, is expressed in B cells and has been implicated in the regulation of immunoglobulin gene transcription and B cell development (Feldhaus et al. 1994). However, it is not known whether the cells expressing either gene, or their progenitors, divide asymmetrically.

4 Stem Cells of the Vertebrate Nervous System

In the avian embryo, as in mammalian systems, there are cells that have the ability to self-renew in the skin and hematopoietic system as well as during bone development (osteocytes). There is also some evidence that skeletal muscle cell development includes a population wih the capacity to self-renew (Quinn et al. 1985). However, work from several groups indicates that, during early development of the avian and mammalian nervous systems, some of the cells there may

Stern, C.D. (1996) Chick stem cells. *Curr. Top. Microbiol. Immunol.* **212**, 193-204.

also possess stem cell properties. In the optic tectum of the chick, for example, cell lineage analysis reveals that one of the last divisions of a multipotent stem cell-like precursor gives rise to radial glia and that these cells may themselves have stem cell-like properties (Gray and Sanes 1992). Similarly, in the neural tube, some cells are believed to be equivalent to the invertebrate neuroblasts and also be capable of self-renewal (Senda et al. 1992), although direct evidence for this self-renewal is still lacking. The only exception is perhaps a recent study (Davis and Temple 1994) revealing the existence of such a self-renewing population of neuroblasts in the rat forebrain.

Perhaps the best evidence for stem cells in the developing nervous system comes from studies on the neural crest. Baroffio et al. (1991) and Dupin et al. (1993) first suggested, based on clonal analysis of avian neural crest cells, that these cells are multipotent and able to renew themselves, giving rise to multipotent descendants. Similarly in the mammalian neural crest, Stemple and Anderson (1992) have succeeded in isolating a stem cell-like cell population which is multipotent and whose descendants in vitro are also multipotent, because they give rise to both neurons and glia. Moreover, neural crest derivatives such as sensory neurons in the dorsal root ganglia can respond to stem cell factor, which induces their growth (Carnahan et al. 1994).

In all these studies, however, cells have been followed for only a few divisions, and it is therefore unknown whether the progenitors continue to have stem cell properties for a long time. In this sense, neural crest cells appear to be more akin to the ES cells of the mouse, where the stem cell property is only retained in vitro for either a few cell divisions or for as long as the culture conditions remain permissive. In one in vivo study, the multipotentiality of the avian neural crest has been demonstrated by short-term single cell lineage analysis (Bronner-Fraser and Fraser 1988), but there has been as yet no direct demonstration of self-renewal in vivo for neural crest cells.

5 Putative Stem Cell Progenitors of the Somites and Notochord of the Avian Embryo

Cells destined to form the somites and notochord of the embryo come from a small region in the anterior part of the primitive streak and Hensen's node (Selleck and Stern 1991; Schoenwolf 1992). How do they generate the large number of cells required to make all the somites and the whole length of the notochord? At the time of its formation, a somite comprises about 2000 cells (Menkes and Sandor 1969), and the cells divide about every 10h (Keynes and Stern 1988; Primmett 1988; Primmett et al. 1989). Since about 50 somites form from each side of the embryo, formation of the somitic mesoderm involves some 80 million cells. How is this vast number of cells generated over a few days from such a small region of the early gastrula stage embryo, which also gives rise to other structures at the same

time? And what mechanisms ensure a continuous supply of cells, such that segmentation can proceed smoothly, with new somites forming every 100 min? One possibility is that the presumptive somite cells of Hensen's node have stem cell properties; that is, they renew themselves at the same time that they give rise to committed progeny. And, given the large number of progeny that they must generate, if they are indeed stem cells, they must retain this property for a considerable time.

Since cells destined to form somites and notochord leave Hensen's node in a continuous fashion, some mechanism must punctuate the stream of cells so that groups of prospective somitic cells adhere together to form a somite. It is thought that the cell division cycle is involved in this punctuation on the basis of heat shock studies, measurements of cell cycle length, pharmacological experiments and analysis of expression of heat shock proteins (Primmett et al. 1988, 1989; Stern et al. 1988). The cell cycle was also suggested to be involved in some way in the elongation of the notochord (Selleck and Stern 1992b), but there is no direct evidence for this.

Selleck and Stern (1991) mapped the descendants of cells in Hensen's node (the anterior tip of the primitive streak) of the gastrulating embryo, using carbocyanine dyes (DiI, DiO) to mark small groups of cells and lysinated rhodamine-dextran to follow the progeny of single cells. One unexpected finding was that the node contains cells that contribute only to the medial halves of the somites. Their lateral halves were found to come from progenitors located about 100 µm further posteriorly in the primitive streak (Fig. 3). At the time, there was no obvious reason to expect this on functional or embryological grounds. But at about the same time an independent study by Ordahl and Le Douarin (1992) reported that the medial halves of somites normally contribute only to the axial musculature, while their lateral halves contribute to muscles of the body wall and limbs.

In addition to progenitor cells for the medial halves of the somites, Hensen's node also contains prospective notochord cells, situated in the anterior, median quadrant. Progenitors of the gut endoderm and of the floor plate of the neural tube are also present in the node, but more widely distributed. The intermediate region of the node situated between the medial somite precursors and the prospective notochord area contains cells that contribute to both notochord and somites, as

Fig. 3. A summary fate map of Hensen's node of the chick embryo at stage 4. Adapted from the results of Selleck and Stern (1991)

Stern, C.D. (1996) Chick stem cells. *Curr. Top. Microbiol. Immunol.* **212**, 193-204.

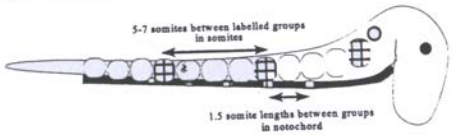
revealed by analysis of descendants of single marked cells (Selleck and Stern 1991). These are therefore pluripotent precursor cells. It was suggested (Selleck and Stern 1992a,b; Stern et al. 1992) that these pluripotent precursors give rise to committed progenitors of medial half somite and notochord, situated in the adjacent regions. These findings beg the question: do the pluripotent precursor cells in the intermediate region of the node have stem cell properties; that is, are they able to renew themselves? Such a mechanism would ensure the maintenance of a progenitor population of constant size which would be responsible for generating the large number of cells required to make notochord and somites spanning the entire length of the embryo.

5.1 Evidence for Stem Cells from Heat Shock Experiments and Cell Lineage Analysis

When chick embryos are given a single, short heat shock, an unexpected result is obtained: discrete anomalies of somite development are seen at regular intervals along the axis of the embryo, appearing every seven or so segments (Primmett et al. 1988). The anomalies consist of an abnormal (either large or small) number of cells being allocated to the defective somites. Because somite formation occurs sequentially, this suggests that heat shock affects some repeated cyclic process. A pair of somites forms every 100 min or so (Menkes and Sandor 1969), therefore the time interval between these anomalies corresponds to groups of cells that are about 10h apart. An obvious candidate for the repeated process is the cell division cycle. This was measured by [^{3}H] thymidine pulse and chase, which confirmed that presumptive somite cells divide every 10h (Primmett et al. 1989).

These findings suggest that cells that segment at the same time as each other divide relatively synchronously. This is consistent with measurements of the mitotic index of presumptive somite cells in the segmental plate (Stern and Bellairs 1984): a large peak of cells in the M-phase of the cycle is seen just before segmentation, at the anterior tip of the segmental plate, another in the middle and a third one at the posterior end of the plates. Since the segmental plate contains 13 presumptive somites (Jacobson and Meier 1986), these peaks of mitosis are separated by about seven somites.

Thus, the cell cycle is probably involved somehow in punctuating the continuous stream of cells into a discrete pattern of somites (Stern et al. 1988; Primmett et al. 1989). Further evidence for this comes from single cell lineage analysis of presumptive somite cells at earlier stages of development in Hensen's node (Selleck and Stern 1991, 1992b). When a single cell in the presumptive medial half somite region of the node is marked by intracellular injection of lysinated rhodamine-dextran (LRD), its progeny appears clustered in small groups, separated by about five to seven somites (Fig. 4). Interestingly, when the injected cell is in a region that contributes to the notochord, repeated clusters of labeled cells are also seen, but these are much closer together: 1.5–2 somite-lengths apart. This



DISTRIBUTION OF DESCENDANTS OF A SINGLE MARKED HENSEN'S NODE PROGENITOR CELL

Fig. 4. After injecting the lineage tracer LRD into a single cell in the intermediate region of Hensen's node, its precursors are sometimes found in regularly spaced clusters in the somites and/or notochord. The spacing is 5–7 somite lengths in the somitic mesoderm and 1.5 somite equivalents in the notochord. Based on the results of Selleck and Stern (1992b)

suggests that the notochord precursors divide about 2.5 times faster than the somite progenitors and predicts a doubling time of about 3.5–4h.

Since, as discussed above, there are cells in the intermediate region of the node that contribute to both notochord and somite, then it is expected that they will divide also at the faster rate of 3.5–4h and that the descendants slow down to a cycle time of about 10h if they become committed to a somite fate.

5.2 Evidence for Asymmetric Cell Division

Are the divisions of the putative stem cells in Hensen's node asymmetric? Three modes of cell division can be envisaged (in each of the three schemes shown in Fig. 5; the interrupted line shows the limits of the node where the dividing progenitor cells are located. The "progenitor" is considered at the last cell division before its daughters begin to leave the node).

In the first mode (Fig. 5A), the progenitor cell in Hensen's node behaves as a stem cell because it renews itself, but when it stops doing so it gives rise to two differentiating daughters which leave the node region to start the segmentation process. This scheme would not lead to periodic arrangements of the daughters but to one or at most two clusters of labeled cells at the site at which the daughters emerge. It is important to realize that in the period between the division shown and the next one of the progenitor cell, other progenitors (not labeled) also divide. This accounts for the continuous supply and the movement of cells from the node to more anterior regions of the embryo as segmentation proceeds.

In the second mode (Fig. 5B), the progenitor cell does not behave as a true stem cell. Even if both daughters do continue to divide, as in the first scheme, this mechanism will produce, at most, two clusters of labeled cells. In order to account for periodic labeled clusters in this and the previous scheme, unlabeled cells must become interspersed with the daughters of those emerging in a very precise way.

The third mode (Fig. 5C) is consistent with the results of the experiments described above, and also uniquely and simply accounts for the existence of the

Stern, C.D. (1996) Chick stem cells. *Curr. Top. Microbiol. Immunol.* **212**, 193-204.

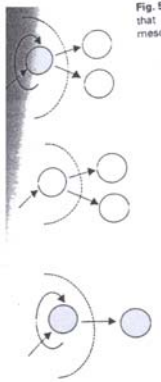


Fig. 5a–c. Three modes of cell division for progenitors in Hensen's node that can generate periodically spaced clusters of cells destined for mesodermal tissues of the embryo

stem cells as postulated. At each cell division of the progenitor cell, only one daughter (which itself continues to divide) leaves the node region and becomes a founder cell for just one cluster of labeled cells. The next division of the progenitor, still situated in the node as it regresses, will yield a similar cell, which will generate the next cluster. This scheme is the only one of the three presented which is, in a sense, inexhaustible, since it can continue to produce clusters of labeled cells as long as the progenitor continues to divide. The hypothesis proposes that the spacing between consecutive clusters is related directly to the cell division cycle of the progenitor cell.

Only two of the above schemes (the first and third) are compatible with a stem cell character of the progenitor cell. All three could, in principle, generate multiple periodic clusters of labeled cells from a single precursor as seen (Selleck and Stern 1991), but the third scheme seems the simplest because it does not require other neighboring cells to intersperse with dividing cells outside the node in a very precise and predictable way.

5.3 Hensen's Node Stem Cells and Mesoderm Induction

The discovery of pluripotent precursor cells in Hensen's node indicates that individual cells at the end of the primitive streak stage still contribute progeny to both ectodermal (floor plate, neural tube) and mesodermal (somites, notochord)

derivatives (Selleck and Stern 1991). How do cells decide between these fates? From studies in amphibians, it is generally believed that responsiveness to mesodermal induction is lost at the beginning of gastrulation (see Green and Smith 1991 for review). The situation in Hensen's node of the amniote embryo is therefore either different from *Xenopus*, or the appropriate region of the frog (dorsal lip of the blastopore) has not been adequately explored.

In amphibians, there is good evidence for the involvement of the peptide growth factor activin in mesodermal induction. Some of our recent results (Stern, Yu, Kakizuka, Kintner, Mathews, Vale, Evans and Umesono, unpublished data) suggest that activin could play a role in allocating cells to mesodermal fates during the later phases of gastrulation in the amniote embryo. First, we have cloned two activin receptors homologous to the amphibian ActRIIA and IIB. Transcripts of both genes are first expressed when the primitive streak appears; after this, cActRIIA is concentrated in Hensen's node of the full-length streak. Moreover, when different tissues are treated with activin and allowed to differentiate in culture, the primitive streak is found to be the most responsive tissue, giving rise to all mesodermal derivatives, including the most axial/dorsal types, in a concentration-dependent manner, as has been found in the frog blastula. These results argue that the role of activin-related signaling pathways in normal development may be confined to rather late stages of mesoderm formation. A study of the behavior of single cells in the node and their progeny in an amniote embryo will provide valuable information complementing present knowledge based on amphibian mesoderm induction and will be essential to provide direction for research on the molecular bases of mesoderm induction in amniotes.

6 Chick Embryonic Stem Cells? Towards the Production of Transgenic Birds

Despite the obvious desirability of an avian equivalent of the mouse ES cell system for the production of targeted mutations (see Shuman 1991), and many attempts to generate them, it has so far proved impossible to produce stable cell lines that remain multipotent and which, upon further passages in culture, retain the ability to populate an entire host embryo including the germ line. The reasons for this remain unclear. However, recent progress has been made in culturing cells on feeder layers which, when supplied with various cytokine supplements to the medium, continue to divide and resemble mammalian ES cells both morphologically and in terms of the expression of many molecular markers. When treated with substances like retinoic acid, they can be induced to differentiate and then appear to give rise to most if not all possible recognizable cell types (Pain et al. 1995; see also chapter by Samarut, this volume).

Although previous studies have found relatively limited colonization of early embryos by implanted cells (Watanabe et al. 1992), spectacular advances have been made recently, dramatically improving the efficiency of production of

Stern, C.D. (1996) Chick stem cells. *Curr. Top. Microbiol. Immunol.* **212**, 193-204.

embryos made with donor cells and a host preprimitive streak stage blastoderm. The method consists of treating the host embryo with X-rays to compromise the early blastodermal cells (Etches et al. 1993; Fraser et al. 1993). These two developments promise, in the not too distant future, the possibility of producing transgenic birds in almost routine manner. One of the great attractions of the avian system is that it allows sophisticated lineage analysis and manipulation of early and later embryonic cells. It is to be hoped that future studies will benefit from a combination of targeted mutations produced by the new technology and well-thought-out embryonic manipulations. With this combination, the avian embryo will no doubt become an even more powerful system to study early developmental mechanisms than many others in current use.

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