Essentials of STEM CELL BIOLOGY

2nd Edition

Edited by **ROBERT LANZA**

John Gearhart • Brigid Hogan • Douglas Melton • Roger Pedersen
E. Donnall Thomas • James Thomson • Sir Ian Wilmut

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Preface to the Second Edition

The second edition of *Essentials of Stem Cell Biology* incorporates the latest advances in the field of stem cells, with new chapters on clinical translation, cancer stem cells, and direct reprogramming—including chapters by the scientists whose groundbreaking research ushered in the era of induced pluripotent stem (iPS) cells. While the second edition offers a comprehensive—and much needed—update of the rapid progress that has been achieved in the field in the last half decade, we have retained those facts and subject matter which, while not new, is pertinent to the understanding of this exciting area of biology.

Like the original volume, the second edition of *Essentials of Stem Cell Biology* is presented in an accessible format, suitable for students and general readers interested in following the latest advances in stem cells. The organization of the book remains largely unchanged, combining the prerequisites for a general understanding of embryonic, fetal, and adult stem cells; the tools, methods, and experimental protocols needed to study and characterize stem cells and progenitor populations; as well as a presentation by the world's leading scientists of what is currently known about each specific organ system.

No topic in the field of stem cells is left uncovered, including basic biology/mechanisms, early development, ectoderm, mesoderm, endoderm, methods (such as detailed descriptions of how to generate both iPS and embryonic stem cells), application of stem cells to specific human diseases, regulation and ethics, and a patient perspective by Mary Tyler Moore. The second edition also includes a Foreword by 2007 Nobel laureate Sir Martin Evans (who is credited with discovering embryonic stem cells). The result is a comprehensive reference that we believe will be useful to students and experts alike, and that represents the combined effort of eight editors and more than 200 scholars and scientists whose pioneering work has defined our understanding of stem cells.

Robert Lanza, M.D.
Boston, Massachusetts

Foreword

It is with great pleasure that I pen this foreword to the second edition of the *Essentials of Stem Cell Biology*. The field of stem cell biology is moving extremely rapidly as the concept and potential practical applications have entered the mainstream. Despite this worldwide intensity and diversity of endeavor, there remain a smaller number of definable leaders in the field, and this volume brings most of them together.

Although the concept of stem and progenitor cells has been known for a long time, it was the progress towards embryonic stem cells which lit the field. Mouse embryonic stem (ES) cells originally came from work aimed at understanding the control and progress of embryonic differentiation, but their *in vitro* differentiation, despite being magnificent, was overshadowed experimentally by their use as a vector to the germline, and hence as a vehicle for experimental mammalian genetics. This now has led to studies of targeted mutation in up to one third of gene loci, and an ongoing international program to provide mutation in every locus of the mouse. These studies greatly illuminate our understanding of human genetics.

Jamie Thomson, reporting the advent of the equivalent human embryonic stem cells, very clearly signaled that their utility would be neither in genetic studies (impractical and unethical in man), nor in fundamental studies of embryonic development (already catered for by mouse ES cells), but, by providing a universal source of a diversity of tissue-specific precursors, as a resource for tissue repair and regenerative medicine.

Progress towards the understanding of pluripotentiality and the control of cellular differentiation, that is basic fundamental developmental biology at the cell and molecular level, now stands as a gateway to major future clinical applications. This volume provides a timely, up-to-date state-of-the-art reference.

The ideas behind regenerative medicine, powered by the products of embryonic stem cells, reinvigorated study of committed stem and precursor cells within the adult body. The use of such stem cells in regenerative medicine already has a long history, for example in bone marrow transplantation and skin grafting. In both of these examples not only gross tissue transplantation, but also purified or cultured stem cells may be used. They have been extensively applied in clinical treatment, and have most clearly demonstrated the problems which arise with histoincompatibility. Ideally, in most cases, a patient is better treated with his own—autologous—cells than with partially matching allogeneic cells. An ideal future would be isolation, manipulation, or generation of suitable committed stem or precursor cell populations from the patient for the patient. The amazing advances of induced pluripotential stem cells point to the possibilities of patient-specific *ad hominem* treatment. This personalized medicine would be an ideal scenario, but as yet the costs of the technologies may not allow it to be a commercial way forward. The timelines are, however, likely to be long before the full promise of these technologies is realized, and there is every possibility that such hurdles will be circumvented. Quite properly, much of this book concentrates on the fundamental developmental and cell biology from which the solid applications will arise.

This is a knowledge-based field in which we have come a long way, but are still relatively ignorant. We know many of the major principles of cell differentiation, but as yet need to understand more in detail, more about developmental niches, more about the details of cell–cell and cell growth-factor interaction, and more about the epigenetic programming which maintains the stability of the differentiated state.

Professor Sir Martin Evans

Sir Martin Evans, PhD, FRS
Nobel Prize for Medicine 2007
Sir Martin is credited with discovering embryonic stem cells, and is considered one of the chief architects of the field of stem cell research. His ground-breaking discoveries have enabled gene targeting in mice, a technology that has revolutionized genetics and developmental biology, and have been applied in virtually all areas of biomedicine—from basic research to the development of new medical therapies. Among other things, his research inspired the effort of Ian Wilmut and his team to create Dolly the cloned sheep, and Jamie Thomson's efforts to isolate embryonic stem cells from human embryos, another of the great medical milestones in the field of stem cell research. Professor Evans was knighted in 2004 by Queen Elizabeth for his services to medical science. He studied at Cambridge University and University College London before leaving to become director of bioscience at Cardiff University.

Why Stem Cell Research

Medical research is endlessly exciting, by its very nature continuously uncovering new facts and principles that build upon existing knowledge to modify the way we think about biological processes. In the history of science, certain discoveries have indeed transformed our thinking and created opportunities for major advancement, and so it is with the discovery and isolation of pluripotential stem cells. Although appearing only briefly in mammalian development, they are a source of an organism's complete array of cell types at every stage of development, from embryogenesis through senescence, in health and in disease.

Scientists recognizing the remarkable opportunities pluripotential stem cells provide have, in a less than a decade, progressed from being able to isolate pluripotential stem cells from early embryos and grow the cells in the laboratory (Thomson *et al.*, 1998; Reubinoff *et al.*, 2000), to being able to generate them by reprogramming somatic cells using viral insertion of key transcription factors (Okita *et al.*, 2007; Takahashi *et al.*, 2007). These advances now make it possible, in principle, to use stem cells for cell therapy—to identify new molecular targets for disease treatment, to contain oncogenesis, to reconstruct or replace diseased tissues—and for gene therapy. New opportunities for expanding effective hematopoietic and other adult stem cell therapies appear in the literature almost daily, and increasing numbers of scientists, clinicians, and patient advocates are becoming excited about an impending revolution in non-hematopoietic cell-based medicine.

Embryonic stem (ES) cells will remain the gold standard for pluripotentiality research, but induced pluripotential stem (iPS) cells hold the promise of making personalized medicine a reality. By using them we can analyze the heterogeneity of complex human diseases, including the diverse causes of cell degeneration and cell death—information certain to help us develop new drugs. IPs cells will also help us understand adverse responses to new drugs by those small cohorts within larger patient populations who can stall or collapse otherwise successful clinical trials. Central to these studies will be the need to precisely manipulate cell fate and commitment decisions to create the tissues that are needed, but doing so will require much more information about the cocktails of transcription factors necessary to regulate cell differentiation (Zhou *et al.*, 2008).

Stem cell technology will also become invaluable in animal science, and perhaps even animal conservation (Trounson, 2008). One exciting new direction currently underway is to generate iPS cells in endangered species, and to re-establish these populations through chimerism in closely-related species.

The stem cell revolution was initially delayed by funding restrictions, arising from those with ethical concerns about using human embryos for research. The tide is turning, however, not only because of wider acceptance of the technology and appreciation for its potential importance, but also because of iPS cell technology, which obviates the use of human embryos. As a result, many agencies around the world are now funding stem cell research, and growing numbers of scientists and their students are entering the field. The result should be a global collaboration focused on delivering clinical outcomes of immense benefit to the world's population. We are just at the beginning of a very long road of work and discovery, but one thing is certain: stem cell research is vital and must go forward.

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FURTHER READING

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A New Path: Induced Pluripotent Stem Cells

Embryonic stem (ES) cells proliferate rapidly while maintaining pluripotency, namely, the ability to differentiate into all cell types. Human ES cells have been considered promising sources in cell transplantation therapies for various diseases and injuries, such as spinal cord injury, myocardial infarction, type I diabetes, and muscular dystrophy. The clinical application of human ES cells, however, faces difficulties regarding the use of human embryos, as well as tissue rejection following implantation. One way to circumvent these obstacles is to generate pluripotent stem cells directly from somatic cells. To this end, it is necessary to identify the factors that induce pluripotency in somatic cells. In 1960, Gurdon and his colleagues generated tadpoles by transferring the nuclei of intestinal cells from an adult frog into oocytes (Gurdon and Byrne, 2003). His successful cloning showed that pluripotency-inducing factors do indeed exist.

In 2006, induced pluripotent stem (iPS) cells were generated from mouse embryonic or adult fibroblasts by the retrovirus-mediated introduction of four transcription factors, Oct3/4, Sox2, c-Myc, and Klf4 (Takahashi and Yamanaka, 2006). These iPS cells are similar to ES cells in morphology, proliferation, and teratoma formation. Furthermore, mouse iPS cells were proved to be competent for adult chimeric mice and germline transmission. More recently iPS cells have been generated without Myc retroviruses. In addition to fibroblasts, iPS cells have been generated from mouse hepatocytes, epithelial cells, pancreatic cells, neural stem cells, and B-lymphocytes. These data demonstrated that pluripotency can be induced in various somatic cells, using only a few defined factors.

It is still not known how iPS cells can be generated from somatic cells by just a few transcription factors (Yamanaka, 2007; Jaenisch and Young, 2008). Oct3/4, Sox2, and Klf4 synergistically regulate the expression of pluripotency-associated genes, while suppressing lineage-specific genes in ES cells. They cannot access the target genes in somatic cells, because of the epigenetic modification of chromatin, and the presence of transcriptional suppressors. It is likely that these blockers cause the efficiency of iPS cell generation to be extremely low. The mystery which remains to be solved is precisely how these three transcription factors override various obstacles and reach the target genes in a small portion of transduced cells. Stochastic events may play a role in this phenomenon. Alternatively, a more sophisticated scenario may be involved in such transcription factor-mediated direct reprogramming.

In 2007, iPS cells were established from human fibroblasts by the introduction of the same four factors (Takahashi et al., 2007), or a slightly different combination (Yu et al., 2007). Because of the substantial differences between mouse and human ES cells, it was not known whether the same factors which we had identified in mice could also generate human iPS cells. However, it proved to be surprisingly easy to generate human iPS cells with the same four factors. Many other laboratories have now also generated human iPS cells. This rapid progress is attributable to the numerous recent findings obtained from human ES cell research. The culture requirements of human iPS cells and their appearance have been well characterized. Without this knowledge, human iPS cells could not have been generated so rapidly.

Human iPS cells are similar to human ES cells in many aspects. They form tightly-packed and flat colonies. Each cell exhibited morphology similar to that of human ES cells, characterized by large nuclei and scant cytoplasm. They expressed hES cell-specific surface antigens, including SSEAs, TRAs, and NANOG protein. Human iPS cells also had growth potential, gene expression patterns, telomerase activity, and an epigenetic status similar to those observed in human ES cells. Human iPS cells could differentiate into three germ layers through embryoid bodies and in teratomas. In addition, they have also been differentiated directly into neurons and beating cardiomyocytes *in vitro*. These data demonstrated that iPS cells can be generated not only from mouse, but also from human fibroblast cultures with the same defined factors.

In 2008, two groups succeeded in generating iPS cells from various patients, including those suffering from amyotrophic lateral sclerosis (ALS), Parkinson's disease, muscular dystrophy, and type I diabetes (Dimos et al., 2008; Park et al., 2008). These cells could provide unprecedented opportunities to understand how these diseases develop, to screen effective drugs, and to predict both side-effects and toxicity. One of the challenges in these kinds of *in vitro* applications is the establishment of methods to recapitulate the pathogenesis in somatic cells derived from the patients' own iPS cells. This might be more difficult than anticipated in diseases like ALS, in which it takes 10 or more years before patients develop symptoms.

Another future application of the iPS cells technology is in the field of regenerative medicine. Patient-specific iPS cells may make it possible to perform cell transplant therapy free from immune rejection. The banking of iPS

cells and differentiated cell progenies of various HLA types might be an alternative way to carry out regenerative medicine successfully (Nakatsuji et al., 2008). However, iPS cell technology still faces various safety issues that must be overcome prior to clinical application. The generation of iPS cells requires the dedifferentiation and reactivation of cell cycles. The same two phenomena take place during tumorigenesis. Although the recent demonstration of iPS cell generation by integration-free methods is an important step toward the clinical application, extensive basic research is still required to assure the safety of such iPS cells.

iPS cell technology is still in its infant stage (Nishikawa et al., 2008). There are many obstacles, as well as expectations. The molecular mechanisms should be thoroughly elucidated. The safety of using this technology should be extensively characterized. Effective protocols for directed differentiation into various lineages should be established. It is very difficult to predict where the technology will be five years from now. More and more talented scientists are getting involved in iPS cell research. I believe fair, but intense, competition among multiple laboratories will speed up the progress of the field. Breakthroughs will occur, which will boost the advancement of this field. In humans, it takes 20 years for infants become adults. iPS cells technology, although in its infant stage, may take a shorter time to mature and be ready for various clinical applications.

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"Stemness": Definitions, Criteria, and Standards

INTRODUCTION

Stem cells have recently generated more public and professional interest than almost any other topic in biology. One reason stem cells capture the imagination of so many is the promise that understanding their unique properties may provide deep insights into the biology of cells as well as a path toward treatments for a variety of degenerative illnesses. And although the field of stem cell biology has grown rapidly, there exists considerable confusion and disagreement as to the nature of stem cells. This confusion can be partly attributed to the sometimes idiosyncratic terms and definitions used to describe stem cells. Although definitions can be restrictive, they are useful when they provide a basis for mutual understanding and experimental standardization. With this intention, I present explanations of definitions, criteria, and standards for stem cells. Moreover, I highlight a central question in stem cell biology, namely the origin of these cells. I also suggest criteria or standards for identifying, isolating, and characterizing stem cells. Finally, I summarize the notion of "stemness" and describe its possible application in understanding stem cells and their biology.

WHAT IS A STEM CELL?

Stem cells are defined functionally as cells that have the capacity to self-renew as well as the ability to generate differentiated cells (Weissman *et al.*, 2001; Smith, 2001). 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). This simple and broad definition may be satisfactory for embryonic or fetal stem cells that do not perdure for the lifetime of an organism. But this definition breaks down in trying to discriminate between transient adult progenitor cells that have a reduced capacity for self-renewal and adult stem cells. It is therefore important when describing adult stem cells to further restrict this definition to cells that self-renew throughout the life span of the animal (van der Kooy and Weiss, 2000). Another parameter that should be considered is potency: Does the stem cell generate to multiple differentiated cell types (multipotent), or is it only capable of producing one type of differentiated cell (unipotent)? Thus, a more complete description of a stem cell includes a consideration of replication capacity, clonality, and potency. Some theoretical as well as practical considerations surrounding these concepts are considered in this chapter.

Self-renewal

Stem cell literature is replete with terms such as "immortal," "unlimited," "continuous," and "capable of extensive proliferation," all used to describe the cell's replicative 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. Most somatic cells cultured *in vitro* display a finite number of (less than 80) population doublings prior to replicative arrest or senescence, and this can be contrasted with the seemingly unlimited proliferative capacity of stem cells in culture (Houck *et al.*, 1971; Hayflick, 1973; Hayflick, 1974; Sherr and DePinho, 2000; Shay and Wright, 2000). Therefore, it is reasonable to say that a cell that can undergo more than twice this number of population doublings (160) without oncogenic transformation can be termed "capable of extensive proliferation." In a few cases, this criteria has been met, most notably with embryonic stem (ES) cells derived from either humans or mice as well as with adult neural stem cells (NSCs) (Smith, 2001; Morrison *et al.*, 1997). An incomplete understanding of the factors required for self-renewal *ex vivo* for many adult stem cells precludes establishing similar proliferative limits *in vitro*. In some cases, a rigorous assessment of the capacity for self-renewal of certain adult stem cells can be obtained by single-cell or serial transfer into acceptable hosts, an excellent example of which is adult hematopoietic stem cells (HSCs) (Allsopp and Weissman, 2002; Iscove and Nawa, 1997). Adult stem cells are probably still best defined *in vivo*, where they must display sufficient proliferative capacity to last the lifetime of the animal. Terms such as "immortal" and "unlimited" are probably best used sparingly if at all.

Clonality

A second parameter, perhaps the most important, is the idea that stem cells are clonogenic entities: single cells with the capacity to create more stem cells. This issue has been exhaustively dealt with elsewhere and is essential for any definitive characterization of self-renewal, potential, and lineage.[1] Methods for tracing the lineage of stem cells are described in subsequent chapters. Although the clonal "gold standard" is well understood, there remain several confusing practical issues. For instance, what constitutes a cell line? The lowest standard would include any population of

cells that can be grown in culture, frozen, thawed, and subsequently repassaged *in vitro*. A higher standard would be a clonal or apparently homogenous population of cells with these characteristics, but it must be recognized that cellular preparations that do not derive from a single cell may be a mixed population containing stem cells and a separate population of "supportive" cells required for the propagation of the purported stem cells. Hence, any reference to a stem cell line should be made with an explanation of their derivation. For example, it can be misleading to report on stem cells or "stem cell lines" from a tissue if they are cellular preparations containing of a mixed population, possibly contaminated by stem cells from another tissue.

Potency

The issue of potency maybe the most contentious part of a widely accepted definition for 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 (Slack, 2000). A case can be made, for clarity, that a unipotent cell is probably best described as a progenitor. Progenitors are typically the descendants of stem cells, only they more constrained in their differentiation potential or capacity for self-renewal and are often more limited in both senses.

Definition

In conclusion, a working definition of a stem cell is a clonal, self-renewing entity 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.

WHERE DO STEM CELLS COME FROM?

The origin or lineage of stem cells is well understood for ES cells; their origin in adults is less clear and in some cases controversial. It may be significant that ES cells originate before germ layer commitment, raising the intriguing possibility that this may be a mechanism for the development of multipotent stem cells, including some adult stem cells. The paucity 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 more widely believed, though still unsubstantiated, model for the origin of adult stem cells assumes that they are derived after somatic lineage specification, whereupon multipotent stem cells–progenitors arise and 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.

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 cystic blastocyst (Papaioannou, 2001). 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 (Tanaka *et al.*, 1998). 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 (embryonic day (E) 6.5 in mice), a population of cells near the epiblast can be identified as primordial germ cells (PGCs), which are subsequently excluded from somatic specification or restriction (Saitou *et al.*, 2002). 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 (Matsui *et al.*, 1992; Resnick *et al.*, 1992). 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 (Labosky *et al.*, 1994; Stewart *et al.*, 1994). 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 (Surani, 1998; Sorani, 2001; Howell *et al.*, 2001). 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.

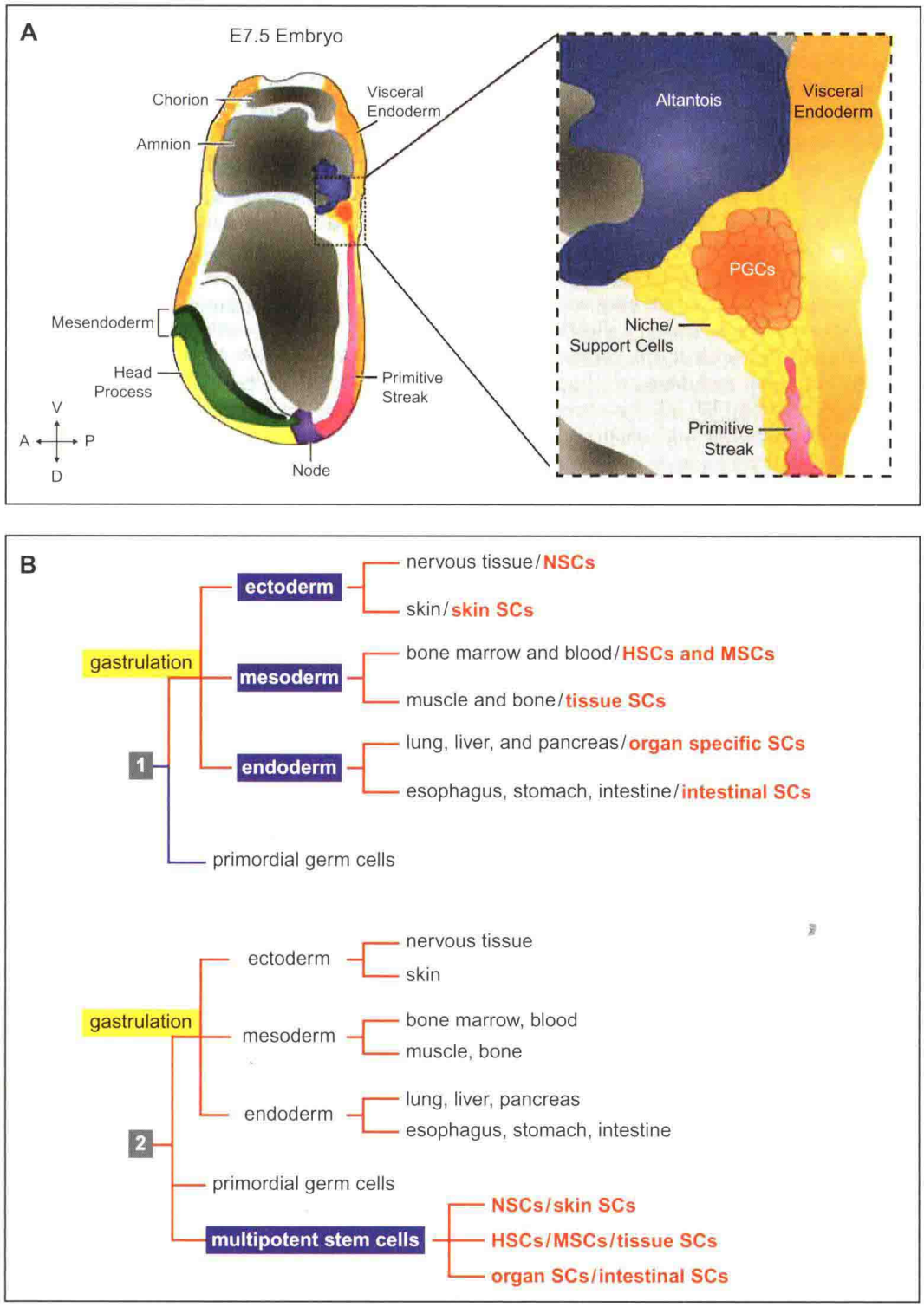

FIGURE 1 (A) Development of primordial germ cells. A schematic of an embryonic day 7.5 mouse embryo highlights the position of the developing primordial germ cells (PGCs) proximal to the epiblast. The expanded view on the right serves to illustrate the point that PGCs escape lineage commitment/restriction by avoiding the morphogenetic 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.

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 (Jiang *et al.*, 2002) 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