Stewart Sell *Editor*

Stem Cells Handbook

Second Edition

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Preface

Our understanding of embryonic cells has increased exponentially over the last 3 decades. It was only 30 years ago when embryonic stem cells were first cultured from mouse embryos. Fifteen years later, human embryonic stem cells were derived from human embryos that were donated from early blastocysts not needed for in vitro fertilization. In the 9 years since the publication of the first edition of *Stem Cells Handbook*, much has changed, yet much remains the same. Obviously, this second edition of *Stem Cells Handbook* concentrates on what has changed and provides a source for experts' critical reviews of their results in various aspects of stem cell research during the last 10 years. The chapters cover what stem cells are, how they contribute to diseases, such as cancer, how bad stem cells can be converted to good stem cells, and how good stem cells can be manipulated and used for therapy. What has not changed is the limited ability to use embryonic cells to treat disease. We hope that this book will help in reaching the goal of many FDA-approved uses of stem cells, both embryonic and adult.

This edition starts with an overview of stem cells in general and ethical problems that need to be addressed in any clinical use. Part I covers the properties of embryonic and fetal stem totipotential cells and how they may be manipulated. This includes how to get them, what signals maintain them as stem cells, how to differentiate them to selected tissue stem cells, and what immunological questions need to be answered if they are to be used for transplantation.

The area of greatest advance since the first edition is the development of methods to produce and apply iPSCs to generate cells that could be used to replace essentially any lost or diseased tissue in the body. The contribution of pluripotent stem cells in adult tissues to repair injury and replace amputated limbs in an experimental model opens Part II. Then we move on to a thorough look of the four critical steps in the use of iPSCs: obtaining them, expanding them, getting them to differentiate into functional tissue stem cells, and then successfully transplanting them. Finally, the vast commercial opportunities of iPSCs are presented.

Part III covers tissue-specific stem cells which are the cells in adult organs responsible for maintaining normal tissue renewal. Understanding how to manipulate normal tissue stem cells could lead to many approaches to preventing or curing various human diseases. The properties and characteristics of tissue stem cells is presented for individual organs or types of tissue and includes a discussion of the role of stem cells in aging.

Part IV deals with transplantation and translating therapeutic approaches, a critical stage of application of stem cell therapy. This includes transplantation of mesenchymal stem cells, use of stem cells in treatment of burns and wounds, as well as treatment of diseases of the eye and diabetes.

Part V examines the stem cell origin of cancer and cancer stem cells. The role of tissue stem cells as the cells of origin of cancer and how to target the signals that maintain cancer stem cells are discussed in general. Then approaches for targeting the stem cells of leukemia, liver and breast cancer, as well as a particular type of kidney cancer, nephroblastoma, for which cancer stem cells are readily identified, are adumbrated.

In closing, we have put together representative, timely, and substantive chapters covering critical aspects of current stem cell research, both basic and clinical. This is done with the full understanding that, given the rate of data accumulation, it is impossible to be all inclusive. Thus, there are many exciting and important aspects of stem cell research that are not covered in this book. What is in this book is a sampling of some of the most critical ongoing studies in stem cell research.

I would like to thank the numerous authors of the chapters in the book for their critical contributions. I owe a particular thanks to my coworkers in the laboratory: Zoran Ilic and Ian Guest, who keep things going productively. Then there are my mentors, who are too numerous to mention, but include Frank J. Dixon, William Weigle, Richard Farr, and Hank Fennel from the University of Pittsburgh; Benjamin Castleman, Robert Scully, and Byron Waksman (Massachusetts General Hospital), John Fahey (NIH), and Phillip Gell (U. Birmingham, England); as well as my long-time collaborators: Hyam Leffert (UCSD), Fred Becker (M.D. Anderson), Ed Smuckler (UCSF), and Gennadi Glinsky (Sanford-Burnham Inst.). Finally, I owe a special thanks to Barry Pierce, who taught me what stem cells are.

Albany, NY Stewart Sell, M.D.

Contents

Introduction to Stem Cells 1
Rob Burgess

Contemporary Ethical Issues in Stem Cell Research 29
Valerie Gutmann Koch, Beth E. Roxland, Barbara Pohl, and Sarah K. Keech

Part I Embryonic/Fetal Stem Cells

Stem Cells from Early Mammalian Embryos 41
Anna-Katerina Hadjantonakis, Svetlana Gavrilov, Panagiotis Xenopoulos, and Virginia E. Papaioannou

Quantitative Approaches to Model Pluripotency and Differentiation in Stem Cells 59
Dmitri Papatsenko, Huilei Xu, Avi Ma'ayan, and Ihor Lemischka

Growth Dynamics of Fetal Human Neural Stem Cells 75
Walter D. Niles, Dustin R. Wakeman, and Evan Y. Snyder

Immunobiology of Embryonic and Induced Pluripotent Stem Cell Transplantation 91
Eleanor M. Bolton, J. Andrew Bradley, and Craig J. Taylor

Part II Pluripotent Stem Cells

Urodele Limb Regeneration: Mechanisms of Blastema Formation and Growth 107
David L. Stocum

Mechanism of miR-302-Mediated iPS Cell Generation 119
Shi-Lung Lin

Bioreactor Expansion of Pluripotent Stem Cells 129
Jaymi T. Taiani, Mehdi Shafa, and Derrick E. Rancourt

Generation of Hepatocyte-Like Cells from Human Pluripotent Stem Cells 139
Fallon K. Noto and Stephen A. Duncan

Neural Differentiation from Pluripotent Stem Cells 149
Mahendra Rao and Nasir Malik

Generation of Anterior Foregut Derivatives from Pluripotent Stem Cells 161
Hans-Willem Snoeck

Commercial Opportunities for Induced Pluripotent Stem Cells 177
Amy Lynnette Van Deusen and Rania Nasis

Part III Tissue Stem Cells

Spermatogonial Stem Cells in Adult Mice and Men 203
Courtney Sachs and Marco Seandel

Characterization of the Hematopoietic Stem Cell Niche: Cellular and Molecular Analysis 211
Charles Durand, Thomas Häupl, and Pierre Charbord

Glandular Stem Cells (GSCs): Stem Cells in Glandular Organs 223
Ming Jiang, Karin Williams, and Simon W. Hayward

Human Mammary Epithelial Stem/Progenitor Cells 235
Patricia J. Keller, Lisa M. Arendt, and Charlotte Kuperwasser

Mammary Epithelial Stem Cells 245
Brian W. Booth, Daniel Medina, and Gilbert H. Smith

Gastric Stem Cell Biology: Proliferation Kinetics, Differentiation Hierarchies, and Role in Carcinogenesis 255
Sherif M. Karam

Progenitors of Islet Cells 271
Isabelle Houbracken, Josue Kunjom Mfopou, and Luc Bouwens

Stem Cell Populations Giving Rise to Liver, Biliary Tree, and Pancreas 283
Mark E. Furth, Yunfang Wang, Vincenzo Cardinale, Guido Carpino, Giacomo Lanzoni, Cai-Bin Cui, Eliane Wauthier, David Gerber, Tsunekazu Oikawa, Juan Dominguez-Bendala, Luca Inverardi, Domenico Alvaro, Eugenio Gaudio, and Lola M. Reid

Liver Regeneration in Health and Disease 311
Malcolm R. Alison and Shahriar Islam

Human-Induced Pluripotent Stem Cells, Embryonic Stem Cells, and Their Cardiomyocyte Derivatives: An Overview 321
Shiva Prasad Potta, Tomo Šarić, Michael Heke, and Jürgen Hescheler

Potential of Glial Cells 347
Magdalena Götz and Swetlana Sirko

Stem Cells and Aging 363
Heidi Scrable and Susan Ashrafzadeh-Kian

Part IV Stem Cell Transplantation

Translating Stem Cell Discoveries 377
Alan Trounson

Mesenchymal Stromal/Stem Cell Transplantation: From Tissue Regeneration to Immune Modulation 391
Peiman Hematti

Stem Cells and Tissue Engineering in Burns and Wounds 399
Patrick H. Blit, Anna I. Arno, and Marc G. Jeschke

Derivation of Retinal Pigmented Epithelial Cells for the Treatment of Ocular Disease ... 411
Dennis O. Clegg, Sherry T. Hikita, Qirui Hu, Dave Buchholz, Teisha J. Rowland, Britney Pennington, Roxanne Croze, Lyndsay Leach, Marlene Tsie, Lisa Conti, and Lincoln V. Johnson

Stem Cells and Diabetes ... 419
Der-I Kao and Shuibing Chen

Part V Stem Cells and Cancer

Cancer Is a Problem of Developmental Biology: Maturation Arrest and the Hierarchical Model of the Origin of Cancer from Stem Cells ... 429
Stewart Sell and Pier Mario Biava

Targeting the Stem Cell Plasticity of Tumor Cells ... 441
Luigi Strizzi, Katharine M. Hardy, Thomas M. Bodenstine, and Mary J.C. Hendrix

Molecular Evolution of Leukemia Stem Cells ... 449
Leslie A. Crews and Catriona H.M. Jamieson

Rodent Models for Assessing the Role of Stem Cells in Liver Development, Regeneration, and Carcinogenesis ... 459
Jennifer A. Sanders and Douglas C. Hixson

Breast Cancer Stem Cells: From Theory to Therapy ... 477
Sarah J. Conley and Max S. Wicha

Nephroblastoma/Wilms Tumor: A Corrupted Cap Stem Cell Caricature ... 491
Michael D. Hall and Alan O. Perantoni

About the Editor ... 511

Index ... 513

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Introduction to Stem Cells

Rob Burgess

Omnis cellula e cellula. (All cells come from cells.)

Rudolph Virchow (1858)

Stem cells are present within most if not all multicellular organisms and are the ultimate drivers of growth and regeneration. They and are defined as biological cells capable of self-renewal and the capacity to differentiate into a variety of cell types. They are considered to be the most critical biological components necessary for proper growth and development during embryogenesis. Yet they have also been demonstrated to play indispensable roles in adult species, providing a much needed source of cellular replenishment for virtually every mature, differentiated cell type. All stem cells originate from what one might consider the ultimate stem cell, the fertilized egg. As a *totipotent* entity, the fertilized egg has the capacity to drive the formation of all intra- and extraembryonic tissues during growth and development. It is during the process of embryonic maturation that *determination* occurs wherein a variety of more specialized stem cell types are generated with differing properties that allow for the development of specific tissues and organs. For example, embryonic stem cells have the *pluripotent* capacity to drive the formation of all tissues of the embryo proper, but not extraembryonic tissues such as the placenta or amniotic membrane. As the embryo matures, determined *multipotent* stem cells are produced which provide a limited, albeit extremely powerful ability to produce more differentiated cell types. An example of multipotency is the stem cell population of the hematopoietic system, which drives blood formation from a common precursor stem cell both during embryonic development and for a lifetime after birth. While hematopoietic stem cells (HSCs) are capable of differentiation into a variety of blood cell types, they do not contribute to other organ systems; this restricted differentiation capacity defines them as adult in origin. Eventually in the mature adult, *unipotent* stem cells reside in a few select systems such as the heart and central nervous system (CNS) which have the capacity to differentiate into only one mature lineage (Fig. 1).

Gleaning a firm understanding of the genetic and biochemical hierarchies involved in embryonic and adult stem cell differentiation will no doubt lead to new cutting-edge cell-based and non-cell-based therapeutic strategies. In addition, whereas stem cells play crucial roles in embryonic development and adult tissue maintenance, their powerful mitotic properties may potentially mediate cancer development. The ability of stem cells to rapidly propagate can be deregulated and derailed resulting in oncogenic and ultimately tumorigenic properties. The theory of the existence of cancer stem cells is rapidly emerging and may open the door to new avenues for cancer treatment. Gaining a firm understanding of how cancer stem cells contribute to tumorigenic and metastatic phenotypes is key to developing new technologies and methods for cancer diagnosis and treatment. For example, targeting therapeutic entities to cancer stem cells present within a large population of tumor cells may be a powerful technique to rid the body of certain forms of cancer. Part V of this book takes an intimate look at this controversial field, outlining data gleaned on the existence and properties of cancer stem cells present in breast cancer, melanoma, and Wilms' tumor.

A firm understanding of stem cell origins and biology is critical to the development of new modes of therapy. This chapter introduces the origins and basic concepts of stem cells, from embryonic to adult to cancer, and emphasizes key areas of stem cell research and focus that are described and highlighted by leaders in the field in following chapters. Particular attention is paid to the signaling cascades and genetic regulatory mechanisms underlying embryonic and adult stem cell development as well as the differentiation of stem cells into mature, cell type-specific lineages. Unless otherwise noted, the focus of this chapter and the majority of the book sections is on mammalian stem cells.

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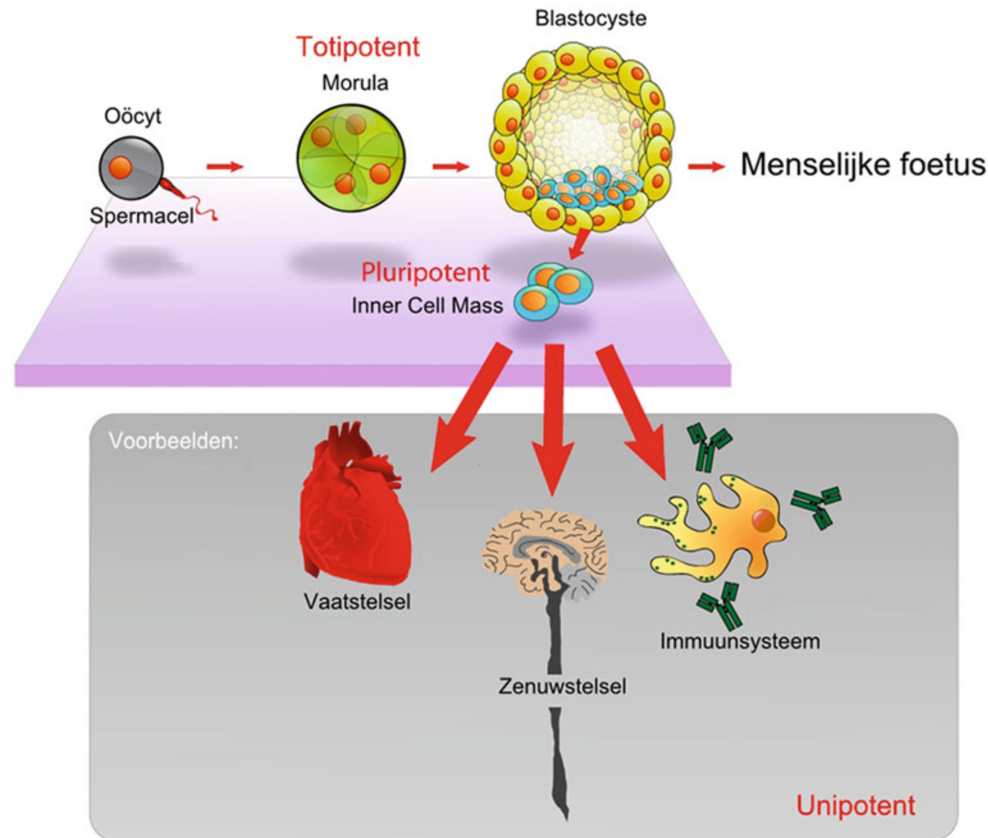

Fig. 1 The origin and specialization of stem cells. See text for details (Courtesy Wikimedia Commons; reprinted with permission)

A Brief History of Stem Cell Research

The first records of man contemplating the origin of life and human development can be traced back to when the ancient Greeks believed that living entities could arise spontaneously (*Generatio spontanea*). Aristotle (384–322 bc) did not agree with the theory of spontaneous generation, but he did believe that order could arise from disorder. This led him to hypothesize that the embryo originates from the mother's menstrual blood. Aristotle's hypotheses are well documented in Leslie Brainerd Arey's comprehensive textbook *Developmental Anatomy: A Textbook and Laboratory Manual of Embryology* [1]. The concept of spontaneous generation was widely accepted and prevailed for the better part of 2,000 years until the mid-1600s when the Italian physician Francesco Redi demonstrated that not all forms of life arise spontaneously in his infamous "six jar experiment" [2] (Fig. 2).

It was in the mid-1800s when Franz Leydig proposed that spontaneous generation in fact did not occur in any context and that all life comes from preexisting life (*omne vivum ex vivo*). Leydig was a German zoologist and comparative anatomist who specialized in the study of neural tissue at the University of Tubingen in Germany. In his seminal publication *Lehrbuch der Histologie des Menschen und der Tiere* he not only outlined crucial developments in the study of histology (including the groundbreaking research of Jan Evangelista Purkyne in 1837), he also emphasized his theory on the origin of life [3]. Purkyne was a Czech anatomist and physiologist who specialized in the study of the brain. His analysis of the histological properties of the cerebellum (he was the first in the world to use a microtome to study tissue slices) resulted in the discovery of Purkinje cells, large neurons possessing a high degree of branched dendrites. Although Robert Hooke is widely credited with the discovery of the cell, this observation is generally accepted as the first definitive documentation of cells. Leydig's theory that all life comes from preexisting life was expanded upon by Rudolph Virchow, a leading Prussian scientist who vehemently disagreed with the theory of spontaneous generation. Virchow carried out a number of experiments in nematodes to demonstrate the prerequisite that all cells come from preexisting cells (*Omnis cellula e cellula*) in 1858 and was a major advocate of this "cell theory." The research and theories of both Leydig and Virchow have withstood the test of time and laid the groundwork for the considerable advancement in cell biology research and stem cell research in

Fig. 2 Portrait of Francesco Redi, the Italian physician first to disprove the theory of spontaneous generation

particular. It was through studies on the role of microorganisms in fermentation in 1864 that Louis Pasteur finally and formally disproved the theory of spontaneous generation [4]. Almost 100 years later, in 1961, researchers James Till, a biophysicist, and Ernest McCulloch, a hematologist, inadvertently discovered the existence of adult stem cells in a suspension of murine bone marrow cells capable of indefinite proliferation. These cells were found to be transplantable and the first colony counting methodology to characterize stem cell numbers was established [5]. These early findings by Till and McCulloch have had perhaps the most significant impact on stem cell research and therapeutic advancements to date. Other key findings are temporally outlined in specific sections below and in Fig. 3. The discovery and characterization of particular types of embryonic and adult stem cells and their potential uses in regenerative medicine are described therein.

Embryonic Development and the Origin of Stem Cells

Over the last 100 years, a massive effort by developmental biologists has been directed at understanding the biochemical, molecular, and morphological mechanisms behind embryonic development, from fertilization through birth. It is only in the past 30 years, however, that significant advancements in understanding cellular potential and lineage commitment as a function of internal cues, environmental influences, and time have revealed unique mechanisms underlying embryogenesis. Research by countless developmental biologists has resulted in the amassing of a wealth of data and information delineating the unique morphological, signaling, and molecular events that drive embryogenesis in a variety of species. In order to understand the capacity of stem cells, it is necessary to review their origins from the perspective of early embryonic development.

Initial Events in Embryogenesis

During the process of embryonic development, the pivotal early event following fertilization is *cleavage*, a stage at which the single cell fertilized egg divides, setting the stage for multiple *symmetric* cell divisions primarily directed at increasing the size of the embryo by amassing large populations of undifferentiated cells in preparation for later cell type specialization. Cells resulting from early cleavage-stage symmetric divisions are known as *blastomeres*, and retain the genetic potential to divide and produce daughter cells that will eventually become specialized. The embryo proper becomes known as the blastocyst and consists of three unique groups of cells: the primitive ectoderm, epiblasts, and the trophectoderm. It is only the epiblast lineage which gives rise to the embryo proper and is a component of the *inner cell mass* (ICM), from which the embryo proper is formed. Transcriptional regulation of the development and anatomical organization of these three lineages is critical to setting up the morphological domains that will later give rise to endoderm, mesoderm, and ectoderm of the embryo itself. In fact, it has been well established that the identity of at least one of these three groups of cells is regulated by the homeodomain transcription factor Cdx2. This notion is illustrated by the fact that overexpression of Cdx2 in murine embryonic stem cells drives them to differentiate into trophoblasts and to exhibit characteristics related to trophoblast stem cells [6]. Interestingly, Cdx2 is expressed in an asymmetric manner at the morula stage on outside cells, thus setting the stage for trophectoderm formation even at this early time point in embryonic development [7, 8]. *Eomes*, a T-box transcription factor, has been placed downstream of Cdx2, yet gene targeting experiments in mice have revealed that neither gene is required for the formation of trophectoderm, which suggests that other factors are involved [8, 9]. Indeed, TEAD4 of the TEA domain transcription enhancer factor family has been placed upstream of Cdx2 with mutants exhibiting a more severe

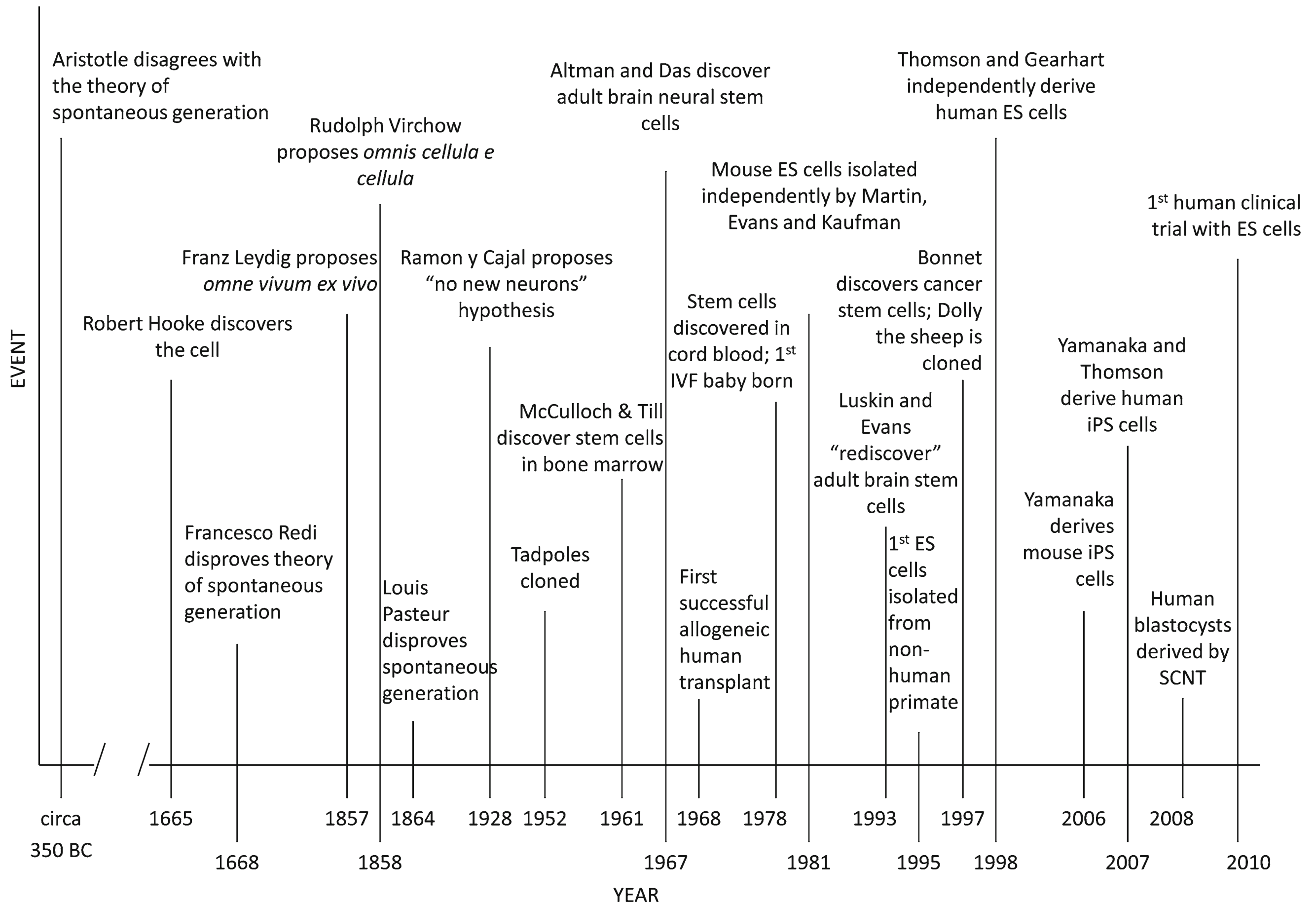

Fig. 3 Timeline of historical events related to stem cell research

phenotype that than of the latter transcription factor. Finally, Cdx2 has been shown to be a potent negative regulator of the pluripotent transcription factors Sox2, Nanog, and Oct4 in trophectoderm cells after blastocyst formation [8]. This is a classical example of transcriptional repression driving extraembryonic lineage commitment. Thus an important transcriptional cascade has been outlined involving multiple positive and negative factors which sets the stage for trophectoderm development. More descriptions of the powerful pluripotent transcription factors Sox2, Nanog, and Oct4 are outlined immediately below and throughout this book.

Inner Cell Mass Regulatory Identity

The ICM of mammalian blastocysts consists of pluripotent stem cells and gives rise to all cells of the embryo proper. The regulatory mechanisms underlying ICM formation have thus been well studied. As mentioned above, it is actually the epiblast subcompartment of the ICM from which the embryo is derived. Interestingly, perhaps the most high profile transcription factors that denote the pluripotency of ICM cells, specifically Nanog and Oct4, are expressed even at the earliest stages of cleavage in all cells (stochastically expressed), yet the expression of these factors becomes restricted to the ICM post-blastocyst formation. As mentioned above, this restricted expression pattern is dependent upon the activity of Cdx2. Thus it has been postulated that early lineage specification throughout the blastocyst begins with the upregulation of key trophectoderm targets and the repression of ICM-specific loci in outside cells. Later during embryonic maturation, the factors Oct4, Sox2, and Nanog actively repress trophectoderm specification and promote pluripotency in the ICM. Positive autoregulatory feedback of these factors subsequently allows for the maintenance of ICM lineage specification [10]. Finally, the growth factor receptor bound protein, Grb2, acts to simultaneously inhibit Cdx2 expression and activate Gata6 expression in a population of ICM cells that will later give rise to the primitive endoderm [11]. These are the first and perhaps most crucial positive and negative transcriptional events that set up both extra- and

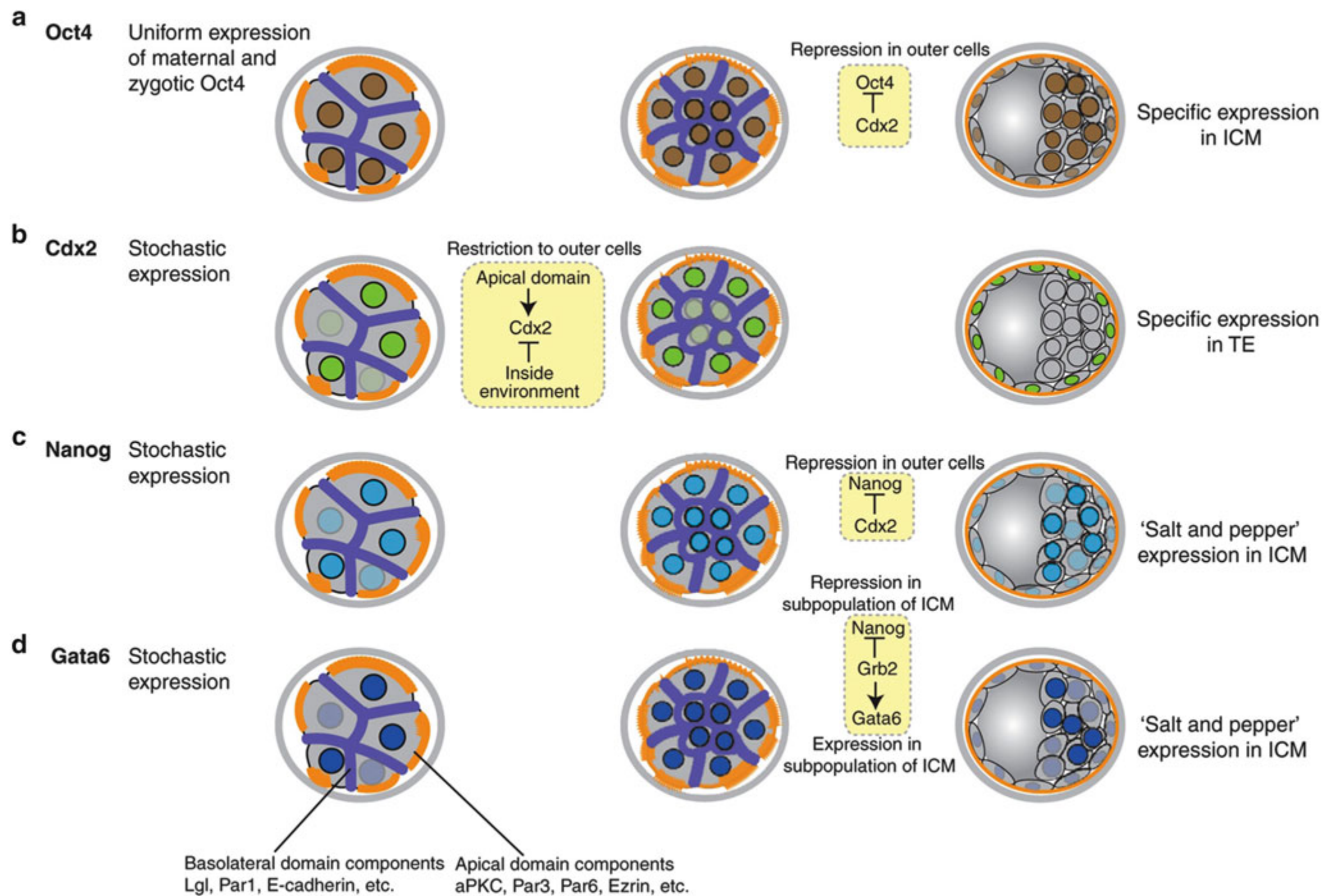

Fig. 4 Molecular players in the formation of early embryonic lineages. Four lineage-specific transcription factors, Oct4, Cdx2, Nanog, and Gata6, are important for the generation of the first three lineages in the blastocyst. The initial expression of these transcription factors is not restricted to specific cell populations. Lineage-specific expression is gradually established in association with the maturation of cellular structures (such as apical-basolateral cell membrane domains, intercellular junctions) and of positive and negative interactions among the transcription factors themselves. (**a**) Oct4: Oct4 protein is observed in all blastomeres throughout early cleavage stages. At the eight-cell stage, all blastomeres contain Oct4. At the blastocyst stage, Oct4 is gradually downregulated in the outer trophectoderm (TE) cells by Cdx2 through direct physical interaction and transcriptional regulation. (**b**) Cdx2: Cdx2 protein is detected beginning at the 8- to 16-cell stage, its initial expression appears to be stochastic. By the early morula to early blastocyst stages, Cdx2 expression is ubiquitous but higher in outer, apically polarized cells. Restricted expression in outer TE cells is established by the blastocyst stage. (**c**) Nanog and (**d**) Gata6: Nanog and Gata6 are detected from the eight-cell stage. Both proteins are expressed uniformly in all cells until the early blastocyst stage. Nanog expression is downregulated in outer cells by Cdx2 and in a subpopulation of the ICM by Grb2-dependent signaling. By contrast, Gata6 expression is maintained by Grb2-dependent signaling. By the late blastocyst stage, ICM cells express either Nanog or Gata6 exclusively (Courtesy Janet Rossant, Patrick P.L. Tam and *Development* (Rossant and Tam [12]); reproduced with permission)

intra-embryonic lineage specification, moving from a totipotent to pluripotent phenotype (Fig. 4 and for a comprehensive review see [12]).

Upon blastocyst formation, ICM cells have already lost totipotency as all cells within the ICM are demarcated as either epiblast or primitive endoderm in phenotype. This has been well documented by comparing Nanog (epiblast) to Gata4 and Gata6 (primitive endoderm) expression and is now known as the "salt and pepper" mosaic pattern in the ICM [11, 165]. The molecular and morphological transition from fertilization through *gastrulation*, defined as the process by which three primary germlayers are acquired, is perhaps the most crucial early-stage developmental process, defining the future of every cell type derived from the one-cell stage fertilized embryo. Gastrulation is the specific stage at which a morphological transition occurs whereby invagination of specific cells of the ICM sets up the formation of the three primary germ layers: endoderm, ectoderm, and mesoderm. The *endoderm* will give rise to the organs, the *ectoderm* to brain and other neural tissue and the *mesoderm* to muscle, bone, and vasculature. It is thus during and after gastrulation that stem cells begin to lose some of their capacity to differentiate into all embryonic and extraembryonic cell types whereby the majority of cells have transitioned from a

totipotent to pluripotent or multipotent capacity for differentiation. As mentioned above, this loss of potency and initiation of specialization is known as *determination*.

What transcriptional mechanisms are crucial to determination of the three primary germ layers? As outlined above, a percentage of ICM cells express the homeodomain transcription factors Gata4 and Gata6 as well as Lrp2. These factors drive the expression of genes crucial for and specific to the endodermal phenotype. The mesodermal and ectodermal layers also express unique transcription factors that drive the eventual maturation of cell types specific to these layers. For example, the basic helix-loop-helix (bHLH) transcription factor twist is expressed at the earliest stages of mesoderm formation [13]. At later stages, the bHLH protein paraxis is expressed in the paraxial mesoderm and is thought to be either a direct or indirect target of twist. Mouse mutants of paraxis have severe defects in somitogenesis and musculoskeletal patterning, most likely the result of aberrant transcriptional signaling in the mesodermal compartment which also affects patterning of neighboring ectoderm along the entire embryonic axis [14, 15]. The ectodermal layer expresses transcription factors in a more restricted fashion. For example, Pax3 is active in a narrow band of ectoderm contiguous with future neural folds yet is not present throughout the entire ectodermal layer at early stages [16]. Ultimately, it is the transcriptional regulatory mechanisms underlying determination events which drive gastrulation, ICM compartmentalization, and stem cell development, with stem cells becoming restricted and specialized to differentiate into mature lineages during and even post-embryonic development throughout adulthood.

The Establishment of Germ Cell Identity

No discussion of stem cell potentiality would be complete without a mention of germinal (germ) cell specification and maturation. *Germinal cells* exist in both the developing embryo and the adult and are totipotent in nature. During embryogenesis, cells committed to the germinal lineage populate the *genital ridge*, a component of the embryo that will develop into the future gonads. It is here that these cells will commit to a germinal lineage of either male or female germ cells; this commitment is defined by an XX or XY genomic makeup. Thus they give rise to sperm as well as egg cells through a process known as *meiosis* in which each daughter cell derived from a common progenitor contains only half of the requisite chromosomal complement needed for a viable developing embryo. The full complement is therefore provided upon fertilization with the transfer of either an X or Y chromosome driving sex determination. As early as 1970, researchers realized the capacity of these cells to differentiate into all cell types and were thus classified as totipotent. The tumorigenic potential of germ cells was demonstrated in these early studies through murine transplant experiments which produced *teratocarcinomas*, malignant teratomas containing a wide range of cell types representing both embryonic and extraembryonic tissues [17]. Recent studies in murine models have suggested three unique molecular events which are crucial to establishing germ cell specification: a full repression of the somatic cell phenotype; pluripotency reacquisition; and epigenetic reprogramming. What transcriptional mechanisms drive germ cell development? Not surprisingly it is some of the same factors that drive ES cell development and pluripotentiality. For example, after gastrulation, Oct4 expression becomes restricted to the primordial germ cells (PGCs) [18, 19]. Interestingly, it is much later that PGC specification is thought to occur, suggesting the existence of other factors upstream of Oct4 which drive PGC commitment. Yet Oct4 has been shown to be indispensable for PGC survival in mouse conditional knockout experiments [20]. Oct4 also specifically marks cells with pluripotential properties in human germ cell tumors [21]. Upon closer look, BLIMP1/PRDM1, a PR (RIZ) domain-containing transcription factor, has been shown to be expressed very early, specifically in epiblast cells that later commit to the PGC lineage [22]. These same epiblast cells express a multitude of homeobox-containing (Hox) genes known to specify cell type and even axial structure in other systems. There is a transient repression of the expression of pluripotency transcription factors Sox2, Zic3, and Nanog, yet these are upregulated later in development [23, 24]. This suggests that PGCs transiently take on a mesodermal fate, and pluripotentiality reemerges as development proceeds. A second PR domain-containing protein, PRDM14 is exclusively expressed in PGC precursors and mature PGCs. Mouse knockouts of either PR domain-containing transcription factor have severe developmental defects in germ cell development [24, 25]. PRDM1 has since been demonstrated to function upstream of PRDM14 and is indirectly negatively regulated by the RNA binding protein Lin28 [26]. These factors working in concert via their activation by bone morphogenetic proteins such as BMP4 act to drive the expression of a variety of transcription factors to drive and secure germ cell identity (Fig. 5).

For a thorough and excellent review of the transcriptional regulatory mechanisms underlying mammalian germ cell specification see Saitou and Yamaji [135]. As they are beyond the scope of this text oogenesis and spermatogenesis will not be covered here but in a related context, Marco Seandel, Assistant Professor of Cell and Development Biology in the Department of Surgery at Weill Cornell Medical College in New York, will discuss the development and function of adult spermatogonial stem cells in Part III, Chap. 14 of this book.

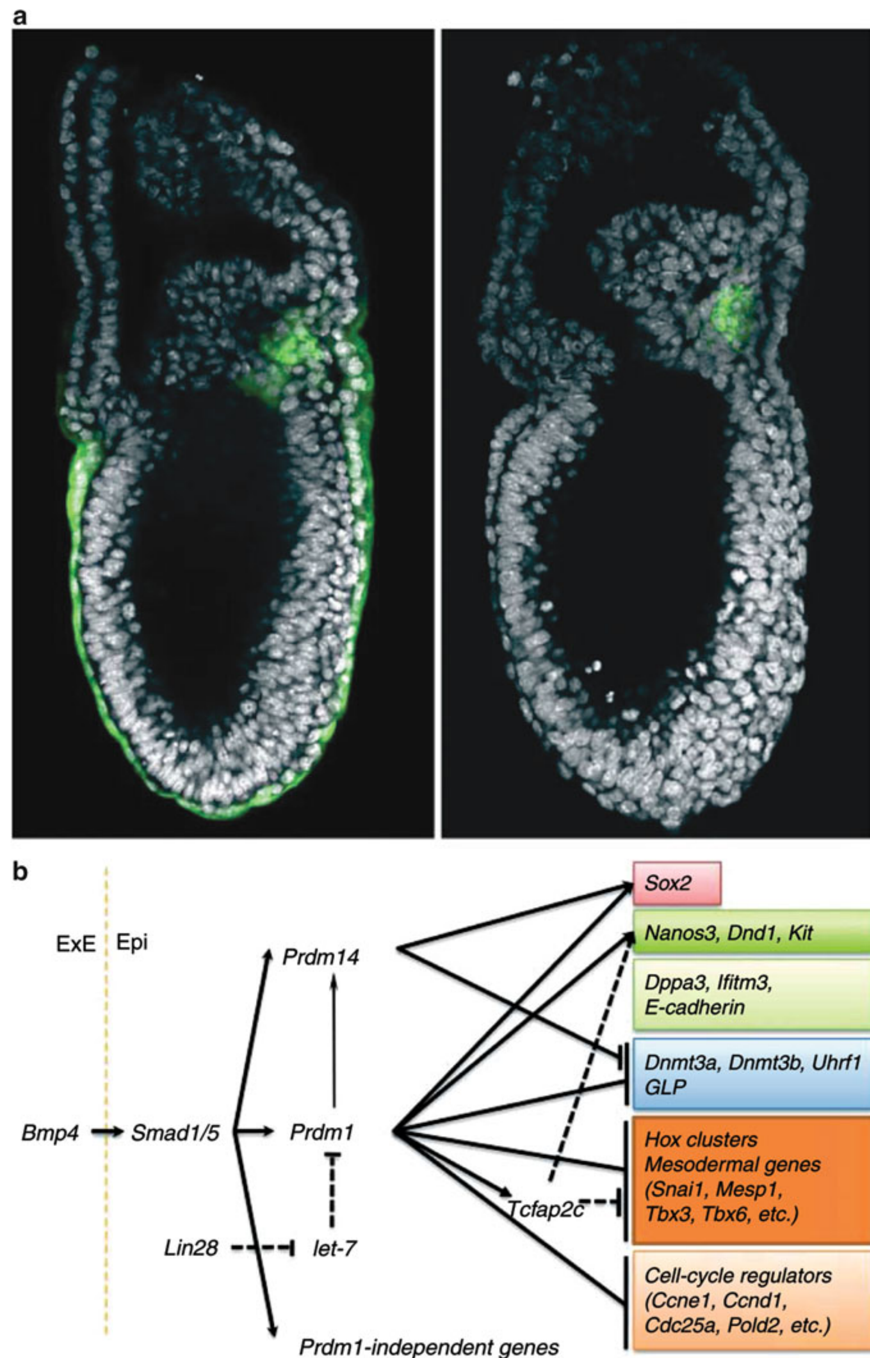

Fig. 5 (**a**) Expression of Prdm1 (left) and Prdm14 (right) in the LS stage embryo visualized by the Prdm1-mVenus and Prdm14-mVenus reporters respectively. Prdm1 is expressed in the nascent PGC precursors emerging from the most proximal part of the posterior epiblast as well as in the visceral endoderm. Prdm14 is exclusively expressed in the germ cell lineage and pluripotent cell lines. (**b**) A summary of genetic pathways for PGC specification

Embryonic Stem Cells

Perhaps the stem cell type that has received the most attention during the past 20 years is that of the *embryonic stem* (ES) *cell*. Embryonic stem cells are pluripotent in nature and derived from the ICM of blastocyst stage embryos. Mammalian ES cells were first isolated in 1967 in a seminal study by Robert Edwards and colleagues, who cultured rabbit blastocysts on feeder layers and demonstrated their capacity to differentiate into a variety of adult cell types representing hematopoietic, neural muscular, and connective tissue [27]. In 1981, murine embryonic stem cells were successfully isolated and cultured by two independent research teams, that of Gail Martin in the Department of Anatomy at the University of California—San Francisco and a team lead by Martin Evans and Andrew Kaufman in the Department of Genetics at the University of Cambridge [28, 29]. Martin's research showed that embryos could be successfully cultured

in vitro and embryonic stem cells derived there from which could be directed to differentiate into a variety of terminal, mature adult cell types. Her team accomplished this via whole embryo culture in serum followed by microdissection of the ICM and further expansion of isolated ICM cells in the presence of a fibroblast feeder layer. Individual colonies resulting from ICM cell plating and propagation were analyzed for pluripotential properties by assessing their ability to differentiate *in vitro* and to form embryoid bodies and teratomas in nude mice. Evans and Kaufman instead focused on the relatively low number of ICM cells present in a mammalian blastocyst and in particular how to increase this number to improve the chances of ES cell isolation and culture. Their group outlined a unique intra-uterine culture technique that allowed for increased ICM cell number, thus enabling successful ES cell isolation. Their technique was devised to delay embryonic implantation and involved hormonal manipulation of the pregnant mother through progesterone administration and ovary removal. This technique allowed for an increase in ICM cell number in utero. Embryos were subsequently isolated and cultured on arrested feeder cells in a manner similar to that employed by the Martin group and pluripotentiality confirmed. In 1987 and 1989, researchers Mario Capecchi, Martin Evans, and Oliver Smithies independently utilized mouse embryonic stem cells and genetic manipulation technologies to disrupt and thereby inactivate the hypoxanthine phosphoribosyl transferase (HPRT) locus [30, 31]. These techniques were later refined and the use of murine embryonic stem cells to inactivate specific loci through gene targeting experiments in mice has become one of the most widely used *in vivo* techniques for defining gene function in mammals. Capecchi, Evans, and Smithies went on to win the 2007 Nobel Prize in Medicine for this work.

What marks a truly pluripotential embryonic stem cell? During the 1990s a defined set of molecular and biochemical markers was established and accepted as minimum criteria for pluripotentiality. These include the stage-specific embryonic antigen cell surface markers SSEA-3, SSEA-4, TRA-1-60, and TRA-1-81. Of course, the presence of the key transcription factors Oct4 and Nanog also have been accepted as classical markers as well as drivers of pluripotentiality. In fact, these transcription factors not only define but are required for ES cell pluripotentiality. Oct4 knockout embryos fail to develop pluripotent stem cells, with the ICM instead skewing towards the extraembryonic trophoblast lineage [32]. Similarly, Nanog deficient ICMs failed to form epiblast cells, instead producing parietal endoderm-like cells [33]. These markers have enabled researchers to define minimum requirements for specific cell types to be considered -pluripotent, however, the ultimate proof is confirming the ability of a cell to contribute to lineages representing the three primary germ layers. In 1995, a research team led by James A. Thomson of the University of Wisconsin—Madison modified the classical embryonic and cell culture conditions employed for murine ES cell research over the last 17 years to successfully isolate primate embryonic stem cells. These cells were demonstrated to maintain a normal XY karyotype after 1 year of propagation and to have the capacity to differentiate into trophoblasts as well as derivatives of embryonic endoderm, mesoderm, and ectoderm. Two key differences were noted between the derivation of primate and murine ES cells. First, the cells were demonstrated to contribute to derivatives of both the ICM and extraembryonic trophectoderm. This suggests the possibility that the primate lines were isolated from an earlier developmental time point. Second, in the absence of feeders, primate ES cells were shown to undergo significant differentiation, even in the presence of leukemia inhibitory factor (LIF), a key supplement in the inhibition of murine ES cell differentiation in tissue culture. These findings implicated additional or other as yet unknown factors secreted by feeder cells required for maintaining a pluripotent phenotype [34]. In a seminal study 3 years later, Thomson's group employed similar methodologies to isolate human embryonic stem cells from embryos produced by *in vitro* fertilization methods and donated after informed consent. These cells exhibited morphological and marker presence features similar to ES cells derived from other species and were confirmed for pluripotential capacity (teratoma formation) and self-renewal. They also demonstrated high levels of telomerase activity indicating a replicative lifespan that will exceed that of somatic cells ([35] and for an example see Fig. 6).

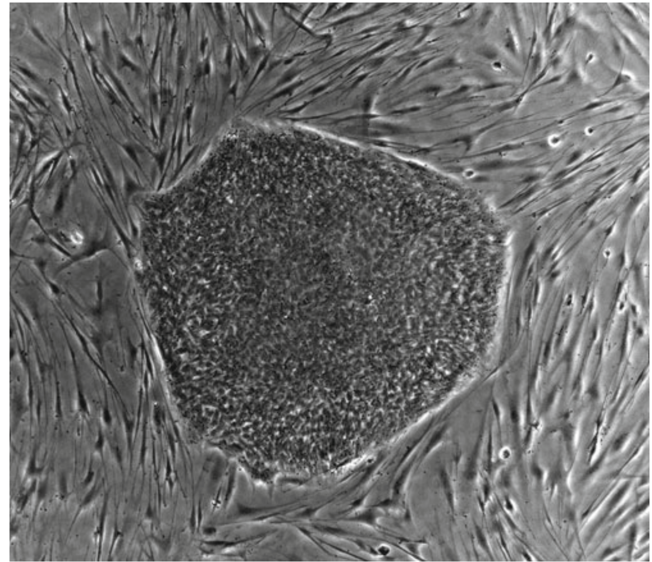

Fig. 6 Brightfield microscopy of a human embryonic stem (hES) cell colony grown from cell line SA02 on a mouse embryonic fibroblast (MEF) feeder layer (Courtesy Wikimedia Commons)

Thus, in a developing human embryo, ES cells can be successfully isolated from the ICM at the blastocyst stage in a similar manner to that of other species. The two classical hallmarks of ES cells are pluripotentiality and indefinite replication capacity. Their pluripotential nature means that these cells can give rise to differentiated derivatives of the three primary germ layers endoderm, mesoderm, and ectoderm including over 220 adult cell types. In Part I of this book, researchers Virginia Papaioannou, Professor in the Department of Genetics & Development at Columbia University Medical Center in New York, Ihor Lemischka, Director of the Black Family Stem Cell Institute and Mount Sinai Hospital in New York and Evan Snyder, Associate Physician of Pediatrics at the University of California—San Diego and Professor at Sanford Burnham Medical Research Institute outline three critical areas of research pertaining to embryonic stem cell origin and identity respectively: (1) The existence and derivation of embryonic stem cells from early mammalian embryos (Chap. 3); (2) A discussion of the key signals driving "stemness" (Chap. 4); and (3) The growth and differentiation dynamics of human fetal neural stem cells (Chap. 5).

It is thus the indefinite replication capacity of ES cells, if cultured properly such as in the presence of feeder cells and/or various growth factors, that allows for a powerful research and potential cell transplant therapeutic reagent source. Albeit controversial due to the use of human embryos, the use of ES cells in cell replacement therapies has been a main goal of ES cell research over the last 20 years. Some anomalies which could be addressed include immune system and hematopoietic diseases, neurological disorders such as Parkinson's Disease, spinal cord injuries, and juvenile diabetes. And existing human ES cell lines may provide a valuable unlimited resource for the development and implementation of cell-based drug screening platforms.

Adult Stem Cells

Adult stem cells can be defined as undifferentiated cells, often found among mature organs or tissues, which undergo self-renewal and have the capacity to differentiate into some or all of the specialized cell types of that organ or tissue system. Adult stem cells are considerably restricted in differentiation capacity, having already become both determined and committed to ultimately become or drive the production of specified mature lineages. They are hence defined as either multipotent or unipotent. Adult stem cells are sometimes referred to as *somatic stem cells* ("soma" means body) thus distinguishing them from stem cells of embryonic or germinal origin. Beginning with the early studies in bone marrow by McCulloch and Till, the past 50 years have seen the discovery of a multitude of somatic stem cells and the characterization of their potential to populate tissues and organs with much needed specialized cells. Adult stem cells have now been discovered in a variety of tissues and organ systems including brain, bone marrow, the vasculature and peripheral blood, skeletal muscle, cardiac tissue, hepatic tissue, ovarian epithelium, gut teeth, and testis. These cells may provide a valuable resource for the treatment of numerous medical disorders. Table 1 lists some of the more high profile adult stem cells discovered and characterized to date.

In each of the tissues mentioned above there exists a *stem cell niche*, an ideal microenvironment, within which adult stem cells reside. This niche is conducive to both stem cell propagation, and in some instances, differentiation. It should also be noted that adult stem cells have a finite capacity to undergo cellular division, and differentiation capacity is considerably limited to a few or one lineage(s). These two properties are the most notable differences between adult and embryonic stem cells. The following sections provide brief introductory synopses for some of the more high profile adult stem cell types studied. Greater detail on these and other categories of adult stem cells is found in later chapters of this book.

Hematopoietic Stem Cells

HSCs can be defined as a heterogeneous population of multipotent stem cells that can differentiate into the myeloid or lymphoid cell types of the adult blood system. During early vertebrate embryonic development HSCs exist as extraembryonic hemangioblasts which differentiate into both endothelial cells and erythrocytes to drive the development of the yolk sac vasculature. What are referred to as "adult" HSCs arise later in development and are unrelated to hemangioblasts, yet similar signaling and transcriptional control mechanisms that drive early HSC formation during embryogenesis are thought to play a role later in fetal development and even in the adult. In fact, as early as 1978 Schofield contemplated the existence of a HSC residing within a specific "niche" of the bone marrow postnatally where complex signaling crosstalk provides the cues needed for both stemness and proper differentiation [36]. The crucial "stemness" nature of the hematopoietic system is now known to be required throughout life for the constant generation of the different blood cell types. Over the last 40 years this niche concept has been expanded upon, with a delineation of concise crosstalk between bone marrow endosteal and vascular niches driving the development of HSCs and their function in the developing and mature blood system. The *endosteum*, the region interfacing bone marrow with bone, is infiltrated with *osteoblastic* cells that secrete numerous cytokines thought to drive the development, maintenance, and behavior of HSCs through the "endosteal niche." For example, thrombopoietin and angiopoietin are thought to enhance

Table 1 Sources of adult stem cells and their differentiation capacity (Adapted from the National Institutes of Health resource for stem cell research)

Tissue of origin	Adult stem cell type	Mature lineage produced	Reference
Blood	Circulatory Skeletal	Adipocyte Osteocyte	Kuznetsov et al. [174]
Bone marrow	Angioblast (endothelial precursor)	Mature endothelia and newly formed blood vessel	Kocher et al. [173]
	Hematopoietic stem cell (HSC)	Hepatocyte Cholangiocyte	Alison et al. [155] Theise et al. [140]
	Human marrow stromal	Stromal-derived cell engrafted in rat brain	Azizi et al. [156]
	Mesenchymal stem cell (MSC)	Adipocyte Chondrocyte Osteocyte	Pittenger et al. [136]
	MSC	Neuron	Woodbury et al. [149]
	MSC	Neuron	Sanchez-Ramos et al. [138]
	MSC	Adipocyte Bone marrow stromal cell Cardiomyocyte Chondrocyte Myocyte Thymic stromal cell	Liechty et al. [177]
Bone marrow (fetal)	HSC	HSC Red blood cell lineages White blood cell lineages	Baum et al. [157]
Brain	Neural stem cell (NSC)	Muscle cell	Galli et al. [163]
Brain (adult and neonatal)	Neural progenitor cell (NPC)	Astrocyte Neuron Oligodendrocyte	Palmer et al. [135]
Brain (fetal)	Human central nervous system stem cell (hCNS-SC)	Astrocyte Neuron Oligodendrocyte	Uchida et al. [144]
Fat	Stromal vascular cell fraction of processed lipoaspirate	Adipocyte precursor Osteocyte precursor Chondrocyte precursor Myocyte precursor	Zuk et al. [152]
Liver (fetal)	HSC	Hematopoietic progenitor cell (HPC) Red blood cell lineages White blood cell lineages	McCune et al. [154] Namikawa et al. [134]
Pancreas	Nestin-positive islet-derived progenitor cell (NIP)	Pancreatic Hepatic	Zulewski et al. [153]
Umbilical cord	HPC	Most red and white blood cell lineages	Broxmeyer et al. [158]
	HSC Mesenchymal progenitor cell (MPC)	Most red and white blood cell lineages Osteoblasts Adipocytes	Erices et al. [162]

HSC quiescence [37, 38]. These osteoblasts also express membrane-bound ligands such as Jagged and N-cadherin, and chemokines including stromal derived factor, SDF-1. These signaling molecules have been demonstrated to drive stem cell self-renewal and myelopoiesis [39–41]. The homing and migration properties of HSCs within the bone marrow can be largely attributed to regulation by the chemokine stromal derived factor (SDF-1), although this factor is not restricted to the endosteal niche but rather is secreted by a variety of cell types, including endothelial cells of the vascular niche, stromal cells, and osteoblasts. The TGFB superfamily of bone morphogenetic proteins TGFB1, BMP2, and BMP7A are released as a result of osteoclast bone breakdown and have been suggested in *in vitro* studies to cause HSCs to quiesce [42, 43]. In addition, even high endosteal ionic concentrations can regulate HSC behavior, with elevated levels of endosteal calcium promoting surface migration [44]. A component of the endosteal niche, the sympathetic

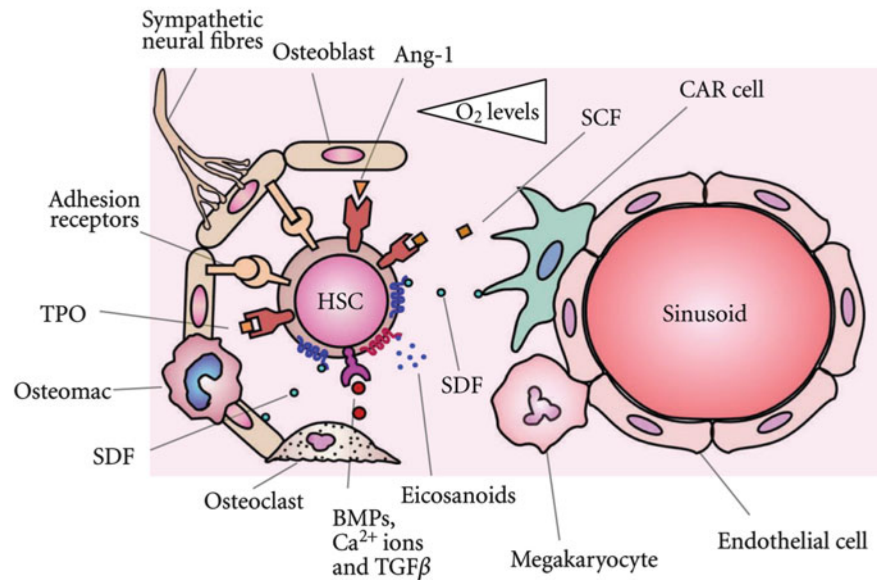

Fig. 7 The interplay between endosteal and vascular niches in the control of hematopoietic stem cells. Cells of both the endosteal and vascular niches communicate and the balance of signaling molecules between the two niches along with signaling from oxygen levels and small molecules regulates HSC behavior (Courtesy Andrew J. Lilly and *Stem Cells International* (Lilly et al. [178]); reprinted with permission)

nervous system (SNS) has also been demonstrated to provide signaling cues for HSC mobilization from the bone marrow [45]. A second key component of the regulatory environment is known as the "vascular niche," as it has been well documented that the bone marrow vasculature also plays a role in HSC maintenance. As mentioned above, extraembryonic vasculature is formed from hemangioblasts, yet the endothelial components of this vasculature have been shown to give rise to HSCs demonstrating close developmental regulation. In adults it has been shown that the vasculature of the liver and spleen can drive hematopoiesis in these organs [46, 47]. The signaling components of the vascular niche that regulate HSC proliferation, maintenance, and differentiation capacity are less well defined than for the endosteal niche, but it is clear that endothelial cells are crucial components of the vasculature that drive hematopoiesis *in vivo*. The cytokine receptor gp130, aka IL6-ST, ILbeta, and CD130, has been shown to be a crucial factor expressed by endothelial cells in the vascular niche to promote hematopoiesis. This has been definitively confirmed in conditional mouse knockouts deleting gp130 from both HSCs and endothelial cells [48]. These mice exhibited hypocellular bone marrow and died within a year after birth. In addition, bone marrow transplants from gp130 deficient mice to normal irradiated mice restored normal hematopoiesis, but the converse transplant failed to yield the same result [49]. Thus the glycoprotein gp130 acts as a key regulator of hematopoiesis, exerting its effect from the vascular niche. The localization of HSCs within the bone marrow has also been shown to be driven by the vascular niche. CAR reticular cells proximal to the sinusoidal endothelium have been demonstrated to play a role in the migration and localization of HSCs, specifically the attraction of HSCs via the secretion of SDF-1 [50]. HSC proliferation is also directly influenced by factors secreted from the CAR cell lineage residing in the sinusoidal endothelium, aptly named for CXCL12 abundant reticular cells. Strikingly, it has been demonstrated that the vast majority of HSCs, upwards of 97%, are proximal to CAR cells within both the bone marrow and endosteum, suggesting an intimate relationship between the two cells types from a signaling and perhaps migratory perspective. Stem cell factor, SCF, a cytokine aptly named for its pro-proliferative effects, has been shown to be secreted by CAR cells within this region [51]. Finally, irrespective of the endosteal or vascular niches, it should be noted that both small bioactive signaling molecules such as Eicosanoids and a hypoxic environment directly affect HSC behavior. Prostaglandins, for example, the most widely studied subgroup of the Eicosanoids, have been shown to drive increased expression of CXCR4 on the surface of HSCs, thereby enhancing migratory capacities [52]. Under hypoxic conditions, when oxygen levels drop below a certain threshold in the bone marrow, hematopoiesis has been shown to increase [53, 54]. Figure 7 illustrates the crosstalk between endosteal and vascular niches as well as other factors to drive HSC behavior.

Many other signaling factors emanating from both the endosteal and vascular niches have been shown to affect hematopoiesis and are beyond the scope of this introductory chapter. For a comprehensive review of the HSC niche and the interplay between the HSC, endosteal, and vascular niches, see Lilly et al. 178]. Part III of this book focuses exclusively on tissue stem cells. Specifically, Pierre Charbord of the Institut National de la Recherche et Santé Médicale (INSERM) in Tours, France will discuss the HSC niches further and expand on this brief introduction through a detailed analysis of the molecular and developmental pathways that drive hematopoiesis.

Liver Stem Cells

The hepatic system has one of the most unique and widely studied adult stem cell niches, and actually consists of numerous niches, both extra- and intrahepatic, which depend upon a considerable amount of proximal and distal signaling to drive the different stages of stem cell maturation (Fig. 8). Much of the paracrine signaling that occurs mimics the defined molecular crosstalk that drives the classical epithelial-to-mesenchymal transition during early embryonic development. The reciprocal signaling that occurs between parenchymal and mesenchymal cells in the developing and adult liver is based upon gradients of paracrine signals that regulate cellular identity. Feedback loop signaling also plays a role in later lineage specification, with both positive and negative signaling cues emanating from dying cells and actively proliferating hepatoblasts to promote not only proliferation, but also to set up terminal lineage commitment [55]. Both paracrine and feedback loop signaling ultimately allow for the generation of hepatic stem cells, hepatoblasts, angioblasts, and committed progenitors, each of which are precursors for the various cell types needed for proper liver function. Below is a brief summary and description of the cell types arising chronologically during the differentiation cascade (Fig. 8).

1. *Hepatic stem cells*: Hepatic stem cells are multipotent in nature and have the capacity to give rise to hepatoblasts, committed progenitors and ultimately mature adult cells. The location of hepatic stem cells varies depending upon age and can be found in the ductal plates of fetal and neonatal livers. Later in life, these cells restrict to the canals of Hering and remain there throughout adulthood [56–62]. Morphologically, hepatic stem cells have a high nuclear-to-cytoplasmic ratio and are about 8 μm in diameter. They represent up to 2% of the parenchymal (non-connective tissue) cell population of postnatal human livers. Some classic markers for hepatic stem cells include EpCAM, NCAM, sonic, and Indian hedgehog and the transcription factors Sox9, Sox17, and FoxA2. The Wnt-beta catenin signaling pathway has been shown to be crucial for activation of EpCAM and driving of the hepatic stem cell phenotype and Wnt1 specifically has been demonstrated

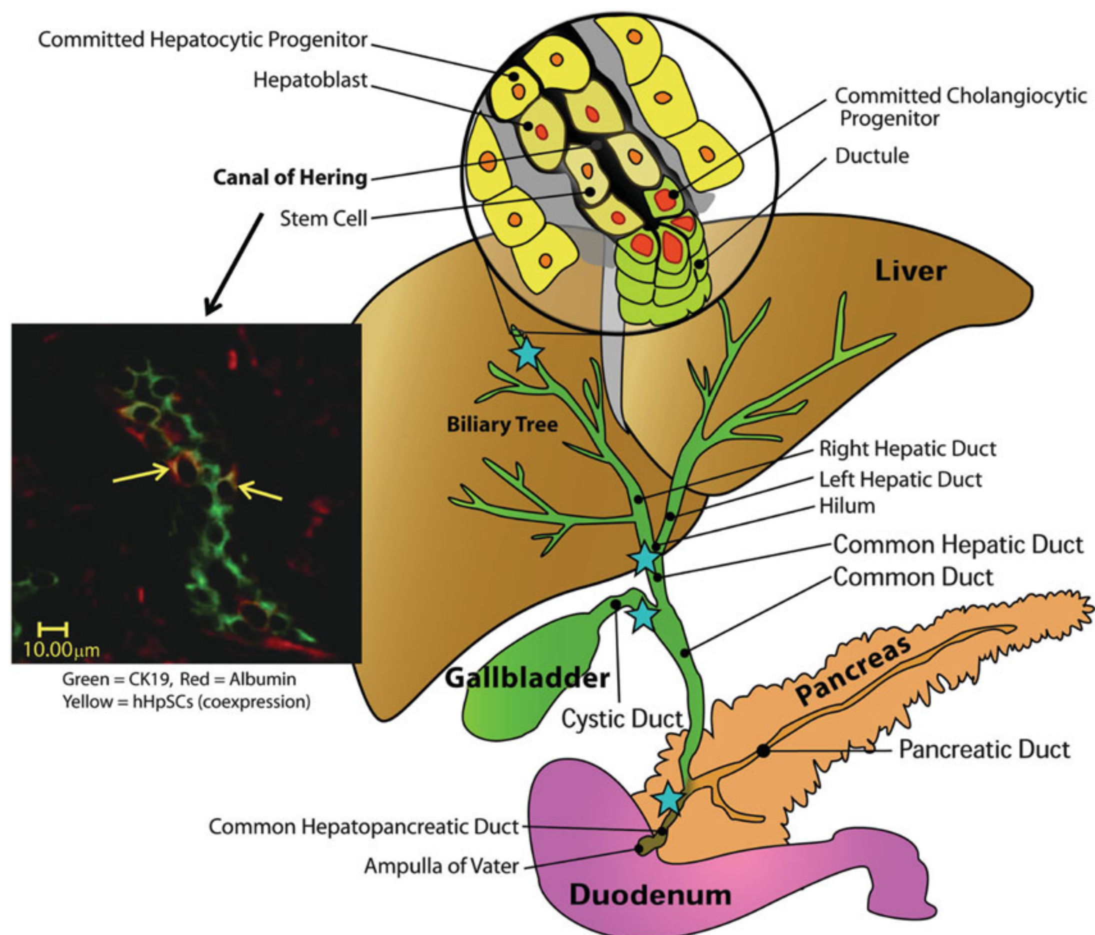

Fig. 8 Schematic image of liver, the biliary tree, and pancreas and their connections with the duodenum. The blue stars indicate sites at which there are high numbers of peribiliary glands, the stem cell niches of the biliary tree (Courtesy Rachael Turner and *Hepatology* (Turner et al. 2011) [73]; reprinted with permission)

to be required for the efficient regeneration of liver by oval cells after hepatic injury [63, 64].

2. *Hepatoblasts*: Hepatoblasts are undifferentiated bipotent stem cells which arise from the foregut endoderm during embryonic development, specifically form hepatic stem cells, and can give rise to mature hepatocytes and biliary epithelial cells [65]. In a manner similar to that for hepatic stem cells, their localized presence in the liver is dependent upon age, with widespread presence in the parenchymal region of fetal and neonatal livers and later clumped in the canals of Hering in adults [58]. Terminal differentiation of hepatoblasts requires a gradient of activin A and TGFb [66, 67]. They are identified morphologically as oval cells and express the markers OV-6, albumin, and cytokeratins CK-19 and CK-7 [68]. In contrast to hepatic stem cells, hepatoblasts do not express markers for mesenchymal cells or hematopoietic endothelial cells and exhibit up to fivefold more telomerase activity than hepatic stem cells. These are two distinguishing factors for the highly related cell types.
3. *Progenitor cells*: Two types of committed progenitor cells, *intermediate hepatocytes* and *small cholangiocytes,* arise from hepatic stem cells throughout the liver and within the bile ducts, lose stem cell marker expression such as NCAM and begin to express either biliary or hepatocytic terminal markers. Both cell types are typically unipotent, giving rise to either mature hepatocytes or cholangiocytes, respectively. Intermediate hepatocytes range in size from 12 to 15 μm in diameter, are polygonal in shape and tend to be present throughout fetal and neonatal liver tissue, expressing albumin and other terminal markers. Small cholangiocytes are smaller at 6–8 μm in diameter, cuboidal in shape, and tend to co-localize with hepatic stem cells in the ductal regions and canals of Hering. Marker expression is widespread for these cells and includes cystic fibrosis transmembrane conductance regulator (CFTR, humans only), anti-apoptotic proteins annexin V and bcl2 as well as endothelin receptors A and B [69]. Interestingly, small cholangiocytes have been shown to proliferate in response to H1 histamine receptor stimulation by activation of the IP3/CaMK I/CREB pathway [70].
4. *Proliferative adult cells*: Following commitment to a specific terminal lineage, adult hepatic cells, known as *small hepatocytes*, express terminal differentiation markers yet retain their ability to divide for on average 6–7 generations as confirmed by *in vitro* studies [71]. In fact, when cocultured with differentiated hepatocytes, these cells have been demonstrated to express terminal markers such as α-fetoprotein, albumin, and Mrp1 and form organoids exhibiting a fully differentiated transporter expression phenotype [72]. *Large cholangiocytes* also retain some proliferative capacity, and are present primarily in the ductal regions. They express the terminal markers CFTR, aquaporin 4, aquaporin 8, and others and play a primary role in regulating ductal bile secretion and absorption. Thus while small cholangiocytes tend to play a role in generating sufficient cell numbers via potent proliferative capacity large cholangiocytes are more focused on secretory and absorption functions. For a more thorough comprehensive review of the various stages involved in the development and differentiation of hepatic terminal lineages, please see the excellent review by Rachel Turner and colleagues at the University of North Carolina—Chapel Hill [73].

What are the clinical implications of adult liver stem cells? Cell and tissue replacement therapies could become routine and a reality for a variety of liver degeneration disorders utilizing the various stages and types of liver-specific stem cells as cell replacement candidates. It is widely known that the liver by itself is indeed capable of undergoing varying degrees of regeneration after partial hepatectomy or due to toxic injury-driven loss of pericentral cells. A more thorough analysis of the liver stem cell phenotype, pathways involved in its generation and the use of liver stem cells in clinical scenarios will be covered in detail in Chap. 22 by Malcolm Alison, Professor of Stem Cell Biology and Lead, Centre for Diabetes at the Blizard Institute of Cell and Molecular Science.

Neural Stem Cells

One of the primary characteristics of the CNS is that, unlike the liver its tissues do not regenerate (discussed above). Damage occurring as a result of disease or injury is usually permanent and its deleterious effects chronic. The foundation of this hypothesis was Santiago Ramon y Cajal's concept of "no new neurons" in the adult [74]. Conceptualized over 80 years ago, this idea was widely accepted until 1967 (initial findings occurred in 1961) when Joseph Altman and Gopal Das of Massachusetts Institute of Technology in Boston observed mitotic activity in the brains of adult guinea pigs. Altman further observed, using tritium radiolabeled thymidine, that these mitotic neural cells differentiated into a mature neuronal phenotype they termed "microneurons" [75]. As fate would have it the Altman/Das findings went largely ignored until 30 years later when adult neurogenesis was "rediscovered." In the 1990s, neurogenesis in the adult human brain was again confirmed [76–78]. These findings have led to a rebirth in the study of neurogenesis utilizing neural stem cells as key tools for deciphering the biochemical and molecular signaling events that drive neural lineage determination and commitment postnatally. They have also driven an intense effort at applying neural stem cell plasticity for therapeutic gain, i.e., CNS repair.

The beginning of CNS maturation during embryonic development, termed *neural induction,* is a temporal point at which *neural stem cells* (NSCs) or NSC-like precursor cells