Mirjana Pavlović · Ksenija Radotić

Animal and Plant Stem Cells

Concepts, Propagation and Engineering

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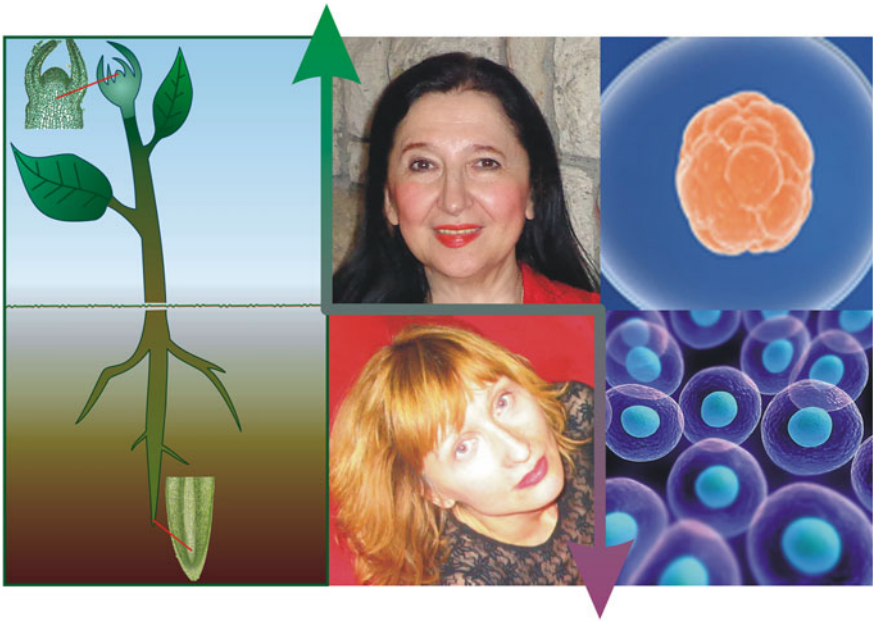

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Preface

This book provides a unique parallel view on animal and plant stem cells from various aspects. The book maximizes reader's insight into research and application in its endeavor to understand the nature of these cells; their sources and categories; engineering of these cells, reprogramming of their functions, and their role as novel cellular therapeutic approach. Written by the author who has already published books and articles in this field, this new one focuses on all aspects of stem cells that were omitted in previous (such as expansion, propagation in culture, metabolic aspects) and gives the specific, multifaceted insight into the world of stem cells this time enriched with contribution of the second author who is the expert in plant cell domain. For plants, it is characteristic indeterminate growth pattern, requiring specific features of their stem cells. In contrast, the animal body plan is mostly defined during embryonic development, and adults generally lack pluripotent stem cells. These features make certain crucial differences between plant and animal stem cells, although there are many similarities in their structure and mechanisms of functioning. This course enhances reader's understanding of plant and human ordinary stem cells, their similarities and differences. It introduces the concepts of emergence of cancer stem cells and different modalities in targeted cancer stem cell therapies. The book treats both theoretical and practical aspects of stem cell research and application and covers many different applications with their advantages and limitations. It is a valuable source of fresh information for academics and researchers, giving an intriguing insight into molecular mechanisms of animal and plant stem cell regulation and their usage for therapeutic applications. It will be a great source of information for students at different level of their education in the fields that require medical and bioengineering background, since it includes cases

that illustrate and explain mechanisms, interactions, targeted effects, and multimodal therapeutic approaches. This work explores the intersection between animals and plants and explains their co-operative role in life. Academics, researchers, and those who want to expand their knowledge in this field will find this to be an exceptional source of references.

Boca Raton, FL, USA — Mirjana Pavlović
Belgrade, Serbia — Ksenija Radotić

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Mirjana Pavlović
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Contents

Abstract

Stem cells are a noncoherent group of cells that have little in common. Despite the fact that these diverse cell types are regarded as belonging to the same category, they do not share molecular markers. The definition of stemness is therefore descriptive, relating to potentials of the cells rather than to the actual properties that they harbor. This situation is confusing and causes unnecessary debates in this field of research. It is therefore of paramount importance to find a new molecular definition of stemness that would consist of the cellular machineries, which constitute the stem cell state. Going through this broad area, the authors are showing interesting aspects of the life and behavior of two kingdoms: animal and plant and their stem cells.

Chapter 1
Introduction

Stem cell concept in animals and plants: what is stem cell, and what is stemness?

Stem cells are nowadays topical research issue due to their potential medical use and due to the ethical debate around human embryonic stem cells. However, from biological point of view, stem cells have even wider significance as central players in the development of complex animal and plant organisms. There are surprising similarities and striking differences between stem cells found in plants and in animals. How were they discovered, and what are their general implications for stem cell biology?

Stem cells function as the source of new cells to build tissues and organs giving the specific features to the development of diversity of complex biological organisms. They are building blocks for the other cells and tissues in animal and plant kingdoms. Through their three fundamental features (self-renewal, proliferation/differentiation, and plasticity) animal stem cells play a crucial role in regeneration, replacement and repair of damaged and lost tissues. The essential feature of stemness is the existence of unique phenotypic molecular markers and specific functionality that can be measured and detected after stem cell has reached the end in its differentiation: maturation into normal, fully functional cell. This function is also based upon developmental changes of stem cells through the process of trans-differentiation, as a part of plasticity feature, meaning the plasticity is a capacity for trans-differentiation [1–5].

It still remains unrevealed whether stem cells have specific stemness factors that make them pluripotent, or they are simply any kind of cell that divides and is blocked from the next step of differentiation.

Furthermore, as Dov Zipori mentioned, once, they are rather state than entity if we are looking for the strict definition, in time and space (Fig. 1.1) [1–3]. This would fit very well with their heterogeneity within the specific source [4–6]. And really, it appears to be a heterogeneous population of cells, residing in the niche, some of which are very primitive and some of which already express lineage commitment [1–5]. They are maintained in the niche of different animal organs and tissues, regulated by spectrum of molecules and signals from different mature

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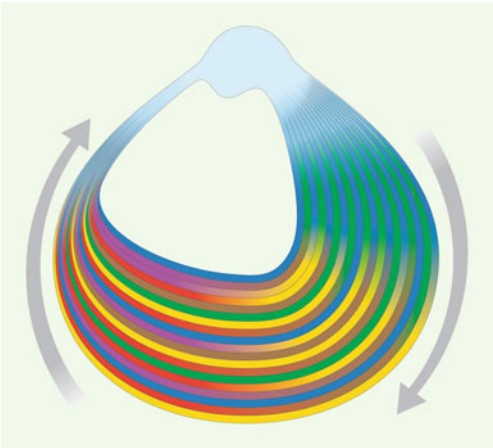

Fig. 1.1 The model of stem cell concept according to Dov Zipori

adjacent cells [4–6]. This cross talk between stem cells and their niches is essential for their self-renewal capacity [6, 7]. Transcriptional profiling experiments are important in understanding the molecular nature of both animal and plant stem cells in depth as they can reveal a reason for heterogeneity.

Relatively recently, the "evolutionary stem cell paradigm" based on the relationship between anaerobiosis and stemness in animal stem cell population was proposed by Ivanovic's group [6].

It is possible (by genetically marking stem cells), to show that nearly all cells of a mature plant, descend from small groups of stem cells located in their growing apices, in other words, plant stem cells [8]. Experiments with mutant plants and selective cell killing have shown that plant stem cells are maintained by signals from other, adjacent, cells. Thus, this feature is shared with animal stem cells and helps them to adjust stem cell proliferation to the needs of the plant organism. The functional similarities of stem cells in plants and animals probably have evolved independently as solutions to the problem of balancing the need to grow with the need to produce specialized cells, which often cannot divide any further [8]. Probably the most fundamental questions in comparing animal and plant stem cells is: Do stem cells work the same way in plants as they do in animals? As indicated, there are similarities and there are differences, as well.

Thus, the mechanisms that control whether a cell continues to function as a stem cell or starts to differentiate, for instance, show some similarities in plants and animals [9]. One gene that is conserved between plants and animals and has a central role in deciding whether a cell continues to divide or differentiate, encodes the Retinoblastoma (Rb) protein. When activated, Rb represses genes required to replicate DNA, in addition to other less well-characterized functions that lead to cell differentiation [10]. In Arabidopsis, if the gene encoding Rb is inactivated in the root meristem, the descendants of root stem cells cannot differentiate; conversely, if

Rb is artificially activated in the stem cells, they stop dividing and differentiate [11]. Similarly, Rb appears to promote exit from stem cell state in animals [9]. However, the regenerative mechanisms, are fundamentally different between two kingdoms [9, 12]. We are hopeful that this book will answer some aspects of those fundamental questions and probably induce further consideration in the light of experience and scientific vision for future studies.

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Part I
Bioengineering and Animal Stem Cells

Mirjana Pavlović

Chapter 2
Current Status and Perspectives in Stem Cell Research: The Concept of Normal Stem (NSC) and Cancer Stem Cell (CSC)

Science is organized knowledge. Wisdom is organized life.
Immanuel Kant

Abstract This chapter intends to give the audience a basic idea on normal and cancer stem cells, as two essential types of cells in stem cell phenomenology connected by the same feature—"stemness." This is an introductory conceptual consideration of what these cells are and where are their similarities and differences. The chapter discusses normal and abnormal mechanisms that are working in both type of these cells and make them different. General features of both cell types are given in condensed manner. The development of methodology for their isolation, purification, and segregation from other cell types in normal and cancerous tissues is presented. New methods such as magnetic beads separation, magnetic levitation, detection of microspheres in CSC, the confirmation of CSC entity through injection of the cells in NOD/SCID mice, are described. The impact of the concepts upon development of a new movement in cancer therapy, cancer stem cell targeted therapy is explained.

2.1 Introduction

Today, stem cell research is dedicated to better understanding of the fundamentals of normal stem cells (NSC) from different sources as well as cancer stem cell (CSC) concept. In order to understand CSC concept it is necessary to look into fundamental features of NSC [1–6].

Both types of cells are connected with the feature of "stemness," which in essence covers existence of one primitive cell with potential for renewal and trans-differentiation into advanced progenitors. And, while in a normal stem cell these progenitors mature into final, morphologically and functionally determined products, in CSC (due to activity of other mechanisms) we have resulting, immature cells of the tissue from which the cancer evolves, as a direct consequence of its tumorigenic functionality.

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2.2 Stemness, Migration, Circulation, and Seeding

In animals, the signaling molecules used within stem cell niches are often the same used to organize growth and tissue patterning during embryogenesis, such as homologues of the Notch, Wingless, and Hedgehog proteins from Drosophila [1, 2]. However, normal stem cell is seen by the eye of stem cell expert as the state of stemness, which assumes renewal, proliferation, and differentiation into final mature state [3]. The property of pluripotency is believed to depend at least in part on the way the chromatin is organized, that is, how the DNA is packed in the nucleus and how this affects the access of regulatory proteins to genes required for cell differentiation. As will be mentioned at the other place, polycomb proteins play an important role in regulating the chromatin to repress differentiation genes and therefore maintain the pluripotency of animal stem cells [2]. Cancer cell is also renewing, but does not differentiate completely, and therefore, never reaches mature state neither according to morphological nor functional criteria. Both types of cells give heterogeneous populations with different potential of stemness [4–7]. It seems that phenomenon of stemness is very well conserved through evolution within the range from *planaria* across *protozoa* and *ancestral prokaryotes* to *mammals* in animal world and in the plants between meristems (stem) cells of plants [8]. Thus, both sorts of humane stem cells: normal and cancerous, express phenomena of "resistency to drugs and radioactive irradiation" in the context of early evolutionary origin of stemness. Migration, circulation, and seeding into distant locations are also the features of both types of these cells.

2.3 Mobilization

Let us take, for example, normal, adult very small embryonic-like stem cell (VSEL), originated from hypoxic niches of many organs in mammals including bone marrow (BM). Morphologically, VSEL cell is smaller several times than hematopoietic stem cell (HSC), has high N/C ratio and embryonic body with the cells of all three embryonic leaflets within cytoplasm [9]. Specifically stained with fluorescent dyes, it shows all markers of stemness and phenotypic difference (with respect to markers) according to HSC, as well as according to other adult stem cells. These cells can be mobilized from their "niches" through binding with growth factor (G-CSF/Neupogen/Filgrastim) upon dosage-dependent manner, and thus dragged into peripheral blood that can be collected by apheresis. Once dragged into the blood, these cells can be selected and quantified, by Flow Cytometry (FC), Fluorescence Acquired—sorting, (FACS) which allows for segregation of "good mobilizers" and bad ones, thus serving as one of the criteria for stem cell therapy [9, 10]. That feature (mobility) have also CSCs which, even without growth factor added, migrate/metastasize into distant organs carried by blood as circulating tumor cells (CTC) [11]. The most probably among CTC, there are CSCs as a minor fraction within entire population, but with very high tumorigenicity.

2.4 Similarities and Differences Between Embryonic and Adult Stem Cells

Both types are non-specialized cells, therapeutically efficient, and they can cure some diseases if adequately applied. One of the essential features that distinct adult from embryonic stem cells is the lack of pluripotent stem cells with unlimited transformation into cells and organs in the repertoire of adult stem cells, which disables organism to fully regenerate the organs, but only participate in repair and repopulation with cellular elements in the case when regeneration would be desirable [11, 12]. The invention of induced pluripotent stem cell (iPSC) from somatic cells thus has replaced the need for embryonic stem cell in adult diseases scenarios [13]. The pluripotent stem cells are mostly characteristics of amphibia and plant meristem, where they can regenerate in first case limb and in the latter, leaf.

2.5 Similarities and Differences Between Normal and Cancer Stem Cell

Cancer stem cells (CSCs) are tumor cells that have the principal properties of self-renewal, clonal tumor initiation capacity, and clonal long-term repopulation potential [12–22]. CSCs reside in niches, which are anatomically distinct regions within the tumor microenvironment. These niches maintain the principle properties of CSCs, preserve their phenotypic plasticity, protect them from the immune system, and facilitate their metastatic potential [18, 21]. Since CSCs survive many commonly employed cancer therapies, we examine the prospects of targeting different components of CSC as preferable therapeutic targets. CSC has similarities with, and it is also fundamentally different than normal stem cell. Thus, Table 2.1, summarizes similarities and differences between normal and CSCs. It is clear that beside the similarities which have been described, there are also fundamental differences between them, especially with respect to:

- Homeostatic regulation of surrounding tissue regulation (lost in CSC)
- Growth regulation which is controlled by cellular and molecular components of the niche in normal stem cells and by internal mechanisms in CSC
- Signal responses upon growth factors that exist in normal and do not exist in CSC
- Apoptotic responses (existing in NSC and non-existing in CSC)
- Limited replication in NSCs and lack of limitations in CSC)
- Angiogenic supportive network—solid Tu (non-existing in NSCs and a present in CSC)
- Tissue invasion (noncharacteristic for NSCs and typical for CSCs)
- Differentiation of resulting daughter cells (present in NSC and decreased or nonexisting in CSCs)
- Aberrant methylation (lacking in NSCs and present in CSCs).

Table 2.1 Normal stem cells versus cancer stem cells

Endogenous & Exogenous Cues	Normal Stem Cells	Cancer Stem Cells
Homeostatic Regulation of Tissue Regeneration Signals	Maintained	Lost
Genetic Plasticity	High	High
Growth Regulation	Niche-driven	Self-sustained
Antigrowth Signal Response	Yes	No
Apoptosis Signal Response	Yes	No
Limitation to Replication	Yes	No
Angiogenic Sustainability	No	Yes
Tissue Invasion & Metastasis	No	Yes
Differentiation of Resultant Daughter Cells	Yes	Impaired or None
Aberrant DNA Methylation	No	Yes
Anaerobic Respiration	Yes	Yes
Heterogeneous population of cells	Yes	Yes
Different Sets of miRNA involved	Yes	Yes

Further comparison indicates:

- hypoxic nature (anaerobic respiration) of both types of stem cells,
- heterogenous populations, and
- different sets of miRNA with participation in epigenetic regulation.

2.6 Resume Based upon Similarities and Differences Between NSCs and CSCs

CSC of mammals including humans, shares a certain number of characteristics with the adult normal stem cells (NSC). Although CSCs are present with participation of only 0.1% of the whole tumor, they can regenerate original tumor and migrate through blood vessels spreading the cancer into secondary locations [20]. It is very difficult to localize this thin cell fraction within the tumor, since for a long time the scientists did not know about their existence, and therefore, there were no methods developed to the work with them. It was looking like a needle in a hay stuck.

However, with the application of magnetic beads today the isolation of purified CSCs samples especially after specific markers were determined and is a relatively easy procedure.

2.7 What and Which Molecular Markers of CSCs We Know Today?

Beside genetic, epigenetic, and biochemical markers, in the past decade almost all phenotypic markers of protein nature, are identified—mostly of the cluster of designation type (CD), which do not change during cancerogenesis and perpetuate through clonal expansion, building up characteristic phenotype and funding the platform for precise isolation, examination, and targeted treatment (Table 2.1).

2.8 Isolation of CSCs Using Magnetic Beads

Isolation is now possible by magnetic beads coated with antibodies raised against specific CSC markers and by using the magnet which drags the cell/bead complex bound to the magnetic beads through antibodies. The cells dragged to the wall of the tube by the magnet from outside, are washed, and separated from antibodies with appropriate buffer, being now purified in the solution, ready for further expansion if needed or are instantly used, if planned so (Quiagen).

2.9 Theories of Origin of CSCs

In the studies related to cancer there are two fundamentally different theoretical explanations for emergence of the CSCs, none of which can explain all the features of the cancer: (1) *Stochastic theory* or the *Theory of clonal evolution* and *hierarchic theory* or the *Theory of cancer stem cell* [22]. According to the first theory, clonally expanded cells originate from one clone, are all equally changed/mutated and of the same tumorigenicity, while according to the Hierarchic Theory, only one cell—CSC is on the top and it is orchestrating further development of tumorigenicity. Surrounding cells are of different degrees of differentiation, but they do not have that tumorigenic strength to renew the cancer. Both theories are equally inspiring for further understanding of the concept of targeted therapy. However, in order to develop more efficient treatment of the cancer, it is of critical importance to determine which of the theories is correct [22–25]. If most of the cells can proliferate and metastasize, then, virtually, all the cells must be eradicated in order for

the disease to be cured, while the specific elimination of CSC would be enough if the theory of clonal evolution is only myth.

2.10 Current Tests for Determination of the Presence of CSCs

Today we have two types of tests for determination of the presence of CSCs: in vitro and in vivo. In vivo is already described as the repopulation of breast and pancreas tumor tissue built up from only CSCs in the body of immunodeficient NOD/SCID mice, while in vitro tests detect occurrence of microspheres during the growth of CSCs in colonies [23–28].

2.11 Sorting and Isolation of CTC Using the Method of Magnetic Levitation

Before we continue with CSCs, let us see what are circulating tumor cells (CTC) and what their significance is. CTCs originate from a primordial tumor mass and they are entering the peripheral circulation. CTCs are crucial for understanding the biology of metastasis, and they are also playing a vital role in diagnosis, prognosis, follow-up of the disease and individual therapy [22]. However, they are also rare in blood and due to that, problematic for isolation. Besides, viability of CTC can be easily compromised under high stress while we are deliberating them from the surface. Their heterogeneity regarding expression of the biomarkers makes their isolation very challenging; efficacy of isolation and specificity of contemporary applications is in need for improvement. Nanostructured substrates appeared as promising biosensor platform since they produce better isolation sensitivity with regards to the price of isolation of CTCs. Method of magnetic levitation has, however, emerged as one of the newest approaches to isolation of these cells [22]. The immediate question: are among those cells also CSCs, is not yet answered. Magnetic levitation, or magnetic suspension is the method by which the object is in suspension without any other support except the support of the magnetic force [22]. Magnetic force is used to contradict the effect of gravitational acceleration and any kind of acceleration. Cells have components of micro- and nanoscale as well as material contributing to their fundamental features of density and magneticity. Both types of cells, eukaryotic and prokaryotic, can levitate and every cell will have its unique levitation profile [22]. That is how CTCs differ from other cells by density and magnetic features, which helps their segregation from overall mass. This is sorting of cancerous cells without labeling and eventual centrifugation since during centrifugation there is the release of ROS and fragmentation of DNA,

consecutively. The costly aspect of labeled antibodies today is not such a problem, since there are many methods for expansion of normal and cancer stem cells one of which is natural—hypoxia [1].

2.12 The Emergence/Origin and Development of CSC: Mechanisms

What mechanisms are leading to establishment of CSC?

a. ***Genetic***

Definitely, it is the first that we would think about, but here we shall not talk about genetic mechanisms since they are field per se and require another book. Retinoblastoma gene, genes of Lynch syndrome, the genes changed in the presence of specific viruses such as Ebstein-Barr and Varicella Virus, are all genetic mechanisms working on the design of the CSC [28–33].

b. ***Epigenetic***

Mammalian embryonic development is tightly regulated process that, from a single zygote, produces a large number of cell types with widely divergent functions. Distinct cellular differentiation programmes are facilitated by tight transcriptional and epigenetic regulation [28]. However, the contribution of epigenetic regulation to tissue homeostasis after the completion of development is less well understood. The research on the effects of epigenetic dysregulation on adult stem cell function is in progress. Current evidence indicates that depending on the tissue type and the epigenetic regulator affected, the alterations range from minor to stem cell malfunction and disruption of tissue homeostasis, which may predispose to diseases such as cancer. Therefore, maybe more intriguing then genetic are these epigenetic mechanisms in the range from aberrant methylation of DNA to the histone modifications [28–33]. Epigenetic phenomenology is a broad term and these mechanisms broadly contribute to emergence of CSC [33–49].

2.13 Aberrant Methylation

DNA methylation has an important role in epigenetic regulation of genes during development and during different illnesses. DNA methylation is the process by which methyl groups are binding to DNA. We know that aberrant methylation is most frequently described as hyper-or hypomethylation. Methylation is also possible in the normal non-methylated CpG islands of the genes, which are so becoming dysfunctional and can influence formation of CSC. Methyl group is on the wrong site: on cytosine instead of thymine.

2.14 What Do We Need to Do with the Sum of Information?

Computer is a great help in organizing information of this kind and here is a conceptual proposal of the work on sorting of information which would help prediction, and planning of the work on detection and discoveries of cancerous lesions [49]. Computational modeling is the key factor in the understanding of biological systems. The interplay of computational modeling and experimental data already exist for the plants. Some effort is necessary to elevate the knowledge on animal SC to that extant in order to be able to answer deep questions such as: which signals are involved in the maintaining of stem cell network organization, how are asymmetric stem cell division and renewal achieved, which are underlying molecular mechanisms related to stem cell progeny differentiation?

2.15 How Was the Concept of CSC Therapy Designed?

Analyzing research during past few years, it is estimated that situation is mature enough for establishment of new concept, e.g., targeted cancer stem cell therapy [28–49]. While many investigators in the field of tumor therapy continue to upgrade the existing models of chemotherapy and radiation, in an effort to improve their efficacy by increasing their specificity, a particular cadar of investigators is taking a new road—directed toward CSC [40–42]. The starting contribution by Dick (1997) has established evident criteria of the CSC concept, by using NOD/SCID mouse model). Dick has successfully transplanted stem cells of acute myeloid leukemia (AML) into NOD/SCID mouse model where the AML human cells were regenerated (1994). Classical experiments of Al Hajj (breast carcinoma) and Li (pancreatic cancer) have supported concept even more [26–28]. Fluorescent labeling has detected clonally propagated, stable markers, and high tumorigenicity with resistance to therapy—typical signs of functionality of those, otherwise, rare cells (0.1% total population). Rarity of CSCs—requires development of therapeutic strategies different than conventional [49].

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Chapter 3
Essential Characteristics of Stem Cells: Self-renewal, and Plasticity

Science is a way of thinking much more than it is a body of knowledge.

Carl Sagan

Abstract This chapter is clarifying essential characteristics of stem cells which otherwise one cannot find in other types of cells and why they are regarded so extraordinary, particular, and unique. Their characteristic features of self-renewal and plasticity make them necessary for everyday life of the body which is in constant renewal and recovery and must be maintained in perfect homeostasis in order to stay healthy and alive. The other two features, although present in cells other than stem cell: proliferation and differentiation, are the features that enable them to manifest their extraordinary capacity to exert the building blocks of the tissues and organs which is actually, their fundamental role. We have also emphasized heterogeneity of stem cell populations as well as their "hypoxic" nature.

3.1 The Nature of Stem Cells

Normal Stem cells are heterogeneous population, living in hypoxic conditions or specific environment known as a "niche." Niche is a complex microenvironment which is bombarding both types of stem cells (normal and cancer) with different signals and manages to maintain their self-renewal. There is still a controversy whether stemness is a feature of ancestral organisms or it has developed through the evolution [1]. It is mentioned that in most cases the appearance of stemness is associated with the appearance of multicellular organisms [1]. However, at this point, trying to see whether stemness reflects primitivism linked to hypoxia or is it an evolutionary event, is rather of semantic than scientific value. Cancer stem cells are also primitive and express stemness as a hallmark of their entity, and they are rather living in hypoxic conditions, as well [2]. It is the low level of oxygen that

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stem cells tolerate quite well, and manage to accommodate their life and activity, which for them apparently is a comfortable living condition rather than physiological lack of the oxygen. It might be just "physiological" scenario for adult body's organ and tissues to enjoy homeostasis at 4% of oxygen participation. We still do not know why it is so, although there are trials worth of appreciation to explain that through evolutionary context [1] etc. Thus, the populations of normal and cancer stem cells in the niche are heterogeneous and hypoxic in their nature using classical physiologic criteria/terminology. Their mitochondria are smaller in number and different in molecular infrastructure from mitochondria of normal tissues [3]. This can partly explain why these cells have their life compatible with low oxygen body environment.

3.2 Self-renewal and Its Significance for Stem Cells

The ability to go through numerous cycles of cell division while maintaining the undifferentiated state, is defined as a self-renewal [2–5]. More specifically, self-renewal could be considered as a cell division without activation of commitment-differentiation events, meaning as providing daughter cells identical to the mother cell, separate from differentiation potential which allows that after division at least one cell is produced in which the commitment-differentiation events will be triggered in order to produce a cell different from mother cell [1]. Self-renewal is the process by which stem cells divide to make more stem cells, perpetuating the stem cell pool throughout life. More specifically, it is division with maintenance of the undifferentiated state. This requires cell cycle control and often maintenance of multipotency or pluripotency, depending on the type of stem cell. Self-renewal programs involve networks that balance proto-oncogenes (promoting self-renewal), gate-keeping tumor suppressors (limiting self-renewal), and care-taking tumor suppressors (maintaining genomic integrity) [1, 3–8]. These cell-intrinsic mechanisms are regulated by cell-extrinsic signals from the niche, the microenvironment that maintains stem cells and regulates their function in tissues. In response to changing tissue demands, stem cells undergo changes in cell cycle status and developmental potential over time, requiring different self-renewal programs at different stages of life [9, 10]. Reduced stem cell function and tissue regenerative capacity during aging are caused by changes in self-renewal programs that augment tumor suppression. Cancer arises from mutations that inappropriately activate self-renewal programs. On the other hand, this stage of multiplication is significant with respect to energy conservation in the form of ATP, which is necessary for endergonic reactions of dividing and replication of DNA. In that way, stem cells maintain the pool of undifferentiated cells which will later on, during differentiation, be able to give commited progenitors for one or more cell lines. This "stock" of identical cells with high regenerative potential is the building material for organs and systems in the bodies, whether it is injured or not.

3.3 Plasticity/Pluripotency of Animal Stem Cells and Its Mechanisms

Plasticity is a capacity of stem cells to trans-differentiate into multiple cell types and give mature and functional cellular product. In other words, stem cell plasticity refers to the ability of some stem cells to give rise to cell types, formerly considered outside their normal repertoire of differentiation for the location where they are found [1]. There is a lot of controversies about that how does plasticity work, e.g., about what are the mechanisms for this phenomenon [2].

On the basis of mutant analyses and transcriptional profiling it is proposed that at least five distinct mechanisms may be invoked to explain the observations of stem cell plasticity reported to date [1]. The first mechanism involves the fact that multiple tissue-specific stem cells exist in different organs such that the harvesting of cells from one organ could yield two or more stem cell populations [1]. The alternative mechanism explaining stem cell plasticity would have been direct cell fusion of stem cell with a more differentiated one, with a tetraploid cell as a product [1]. Thus, this chimeric cell could be able to express a variety of features of any cell type. The next mechanism is based upon cellular trans-differentiation or "de-differentiation", where stem cell directly morphs into a cell of another tissue type or evolves at the same final mature cell entity by initially de-differentiating into an intermediate precursor cell [1–3]. And the fourth mechanism proposes the existence of true stem cell in multiple tissues in the body, allowing for isolation and detection of phenotype and genetic signature [10–17]. Finally, the possible contamination of one type of stem cell with another as it was proposed for VSELs-contaminating HSCs when mobilized from BM, could contribute to expression of differentiation into particular cell line [4].

3.4 Other Features of Significance

Other significant features are stem cell proliferation and differentiation. Proliferation is the feature of stem cells which brings up their number to such a quantity that their function is possible. Different proliferation pathways are involved in proliferation of different types of stem cells [12, 18].

Until past 5 years it was a dogma that adult somatic stem cells are restricted from pluri to multipotency. In that respect a lot of studies were done on the BM adult stem cells, particularly hematopoietic stem cells (HSC). Many researchers have shown that in both animal models and human patients, HSC have differentiated into myocardial cells [12–18], neural tissue, liver tissue, skeletal muscle, even alveolar epithelial cells of the lung. The low frequency with which different various stem cell populations participated in plasticity events, and lack of clonal evidence of its origin were the objections of the scientific community for acceptance of the "broken

dogma" [20–22]. It seems that we need more of the proofs in order to accept this as reality, but the amount of data is growing from one to another day [12–19].

The role of miRNA in maintaining the position of the functional stem cell niche within a dynamic structure is an important mode of regulation in both plants and animals. Repression of differentiation-promoting transcripts in stem cells by local miRNAs to promote self-renewal is also observed in animal systems, which provides another mechanistic analogy between plant and animal stem cell regulation. We know today that there are more than 250 general types of cells in the human body [2]. The process by which a cell becomes specialized in order to perform a specific function, as in the case of liver cell, blood cell, or neuron, is called differentiation. Differentiation is the process that takes place inside an embryo that determines which genes are expressed and hence what type of cell will result. Therefore, although all human cells have the same set of genes, their activity/expression is different, making the cells that are going through maturation process different between them. The ability of embryonic stem cells to undergo differentiation into any cell in the body is what makes them a focus of modern research.

There are numerous pathways of significant impact upon the proliferation and differentiation [22]. The crucial stem cell signaling pathways/mechanisms will be described in the Chap. 5.

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Chapter 4
Stem Cell Sources and Types of Animal Stem Cells

Science knows no country, because knowledge belongs to humanity, and is the torch which illuminates the world. Science is the highest personification of the nation because that nation will remain the first which carries the furthest the works of thought and intelligence.

Louis Pasteur

Abstract This chapter describes particular stem cell sources and types of these cells. It emphasizes the distinction between ESCs and FSCs versus adult tissue stem cells. It also comments the questions that are not answered yet, despite the application of stem cells from different sources in clinical arena.

4.1 Introduction

Generally, the Stem Cell (SC)—compartment is divided into embryonic and tissue specific or adult SCs [1]. In summary, stem cell sources can be morula, blastula (epiblast), embryo, fetus, umbilical cord, placenta, adult tissues, and any kind of mature cell transferred to induced pluripotent stem cells (iPSC). All stem cells—regardless of their source—have three general properties: they are capable of dividing and renewing themselves for long periods; they are unspecialized; and they can give rise to specialized cell types [1].

4.2 Embryonic SCs (ES or ESC)

Embryonic SCs (ES or ESC) are by definition the "master cells" with the largest spectrum of differentiation potential, e.g., capable of differentiating into every type of cells either in vitro or in vivo. Thanks to the presence of embryonic body, these cells have ability to develop into three primary layers: endoderm, ectoderm, and mesoderm [1–6]. The discovery of SCs inside cell mass of embryos and in adult tissue has revolutionized the medical field by introducing new therapeutic

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dimensions into previously untreatable diseases and injuries [4–6]. Several experimental or preclinical studies have suggested that application of embryonic SC could be promising in the treatment of various diseases and conditions [2–6]. However, recognition of appropriate ethical aspects, regulatory acts and standardization in embryonic SC mediated regenerative medicine is needed as it is still the matter of controversy. Besides, permanent, persistent, and accurate updating of the facts regarding their phenotypic, functional, and immunologic characteristics is an essential requirement for safe clinical application of SCs. Some authors stand that the initial theory that embryonic SCs are ignored by immunocompetent hosts was overlooked. On the contrary, they think that it is even more evident that embryonic SCs could protect themselves actively by several immunomodulatory mechanisms against T lymphocytes and natural killer cells of host, and actively participate in immune-mediated events.

4.3 Fetal Stem Cells (FSC)

Recent isolation of **fetal SCs** from several sources either at the early stages of development or during the later trimesters of gestation, sharing similar growth kinetics and expressing markers of pluripotency, provides strong support to the statement that these cells may be biologically closer to ESCs. In fact, they represent **intermediates between embryonic and adult mesenchymal SCs** with regards to proliferation rates and plasticity features, thus being able to confer an advantage over postnatal mesenchymal SCs derived from conventional adult sources [1, 6]. The sources are fetal organs and tissues, as well as extraembrional tissues including: *umbilical cord blood*, *blood/cells from cord Wharton's jelly*, *umbilical blood vessels*, *amniotic fluid*, *and placenta*. Most of fetal stem cells are either MSCs or HSCs. Some of them are AFCS, VSELs, and epithelial cells of amnion.

4.4 Bone Marrow and Other Adult Stem Cells Sources

BM was the primary source of SCs for transplant [1]. However, peripheral blood and umbilical (cord) blood are also currently used as sources. SCs derived from these sources may have therapeutic potential (without severe adverse effects) only when given to the individual from whom they were derived (autologous transplants) or from an immunologically matched donor (allogeneic transplants) [1].

Despite the fact that the ideal type and source of cells have not yet been defined, immature SCs are capable of colonizing different tissues due to ability of homing and trans-differentiation or lineage-plasticity, in the settings of regenerative medicine. Furthermore, there are several facts suggesting that adult SCs and even differentiated somatic cells, under appropriate microenvironmental cues or signals, are able to be "reprogrammed" and contribute to a much wider spectrum of

differentiated progeny than previously anticipated. This has been demonstrated using tissue-specific SCs—which like embryonic SCs—do not express CD45 as an exclusive hematopoietic marker. Consequently, adult mesenchymal SCs and endothelial precursors seem to be clinically applicable for cell-mediated, regenerative therapy of patients with myocardial, brain, vascular, liver, pancreas, and some other tissue damages.

It is widely accepted that allogeneic transplants are still the most efficient treatment for patients with liver failure and Chronic Myelogenous Leukemia (CML) [1]. However, there is a lack of donors and some alternative therapeutic approaches are therefore needed. Transplantation of mature hepatocytes has been evaluated, but the long-term efficacy remains unclear and the paucity of donor cells makes this strategy quite limited. The use of SC-therapy transplantation is perhaps a more promising alternative approach.

The intensification of myeloablative radiochemotherapy enlarged the use of SC transplants, as well as the introduction of cell-mediated therapeutic approaches in regenerative medicine resulting in increased needs for both specific blood-derived progenitor/cells, and practical operating procedures inducing minimized cellular damages during their collection or processing and storage in frozen state. Therefore, successful performance of SC transplants or cell-mediated therapy requires efficient collection, processing, and (cryo) preservation procedures for obtaining an acceptable cell yield and post-thawing recovery, as well as advantageous clinical outcome. For wound healing in the skin, epidermal stem cells and bone-marrow progenitor cells both contribute. Thus, it is likely that organ-specific progenitors and hematopoietic stem cells are involved in repair, even for other organ repair. It is suggested by Heidstra and Sabatini [1] that human adult stem cells lack the pluripotent population of stem cells and therefore is not able like embryo, amphibia, or plant to regenerate entire organ. Therefore, its role is limited to repair of the living organs and tissues in the human body [1]. It has been also shown that mechanisms of pluripotency are different in mouse, monkey, and humans [2]. Thus, the future challenges will include exploring the mechanism for monkey lineage specification as well as for the maturation of monkey cynomolgus epiblast (cyEPI), and performing more comprehensive analysis for monkey gastrulation [2]. Such investigation will lead to a better strategy for species distinction and controlling the properties of hPSCs and for generating cells of interest from hPSCs.

In summary, stem cells could be described as

- Foundation cells for every organ, tissue, and cell in the body
- A “blank microchip” that can ultimately be programmed to perform any number of specialized tasks
- Undifferentiated “blank” cells that do not yet have a specific function
- Self-sustaining and capable of replicating themselves for long periods of time.

Under proper conditions, they begin to develop into specialized tissues and organs [1].

These unique characteristics make stem cells very promising potential for supplying cells and tissues instead of organs in a spectrum of devastating diseases from diabetes type1 to stroke, spinal cord injuries, and myocardial infarction [1–6]. In the situation when the number of people needing organ and tissue transplants exceed the number of donated organs and tissues, this is the promise and hope, which deserves a deep and serious consideration. However, despite rapidly growing knowledge on adult stem cell sources, features and use, there are still some fundamental remaining questions regarding them that include: Does only one common type of stem cell migrate to different organs and repair tissue or are there multiple types of stem cells? Does every organ have stem cells (some of which have not yet been discovered)? Are the stem cells programmed to divide a finite number of times or do they have unlimited cell proliferation capacity?

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Chapter 5
Stemness and Stem Cell Markers

There are no shortcuts in evolution.

Louis D. Brandeis

Abstract Stemness is still a contraversive entity and the definition is evolving. It can roughly be defined as the most primitive cell state capable of transdifferentiating into divergent functional cell lines. Different stem cells express different stem cell markers which are hallmarks of these cells together with adequate functionality. Sometimes the cells possess/express the markers (phenotype) but the function is lacking and therefore they cannot be considered stem cells. Markers are protein products of clonal expansion, during self-renewal of stem cells, where the entire energy is invested in their multiplication. They are permanent labels of stemness and different in different stem cell types from different sources. Here we are presenting the examples of stem cell markers known so far.

5.1 Introduction

Mutant analysis and transcriptional profiling experiments determined that stem cell markers are genes and their protein products used in scientific purposes to isolate and identify stem cells, using magnetic bead technology [1, 2]. We now know that many different types of stem cells exist in animal world, but they all are found to participate in very small percentage/populations in the human body. Thus, in some cases one stem cell could be found in 100,000 cells in circulating blood [1]. That is why it is so hard to detect and identify them. As we know, they inhabit the specific parts of the organs known as niche, which is already defined as a hypoxic region of the body under great influence of circulatory, neural, paracrine, endocrine, cytokine, and other factors [3, 4]. Signaling and cross-talk between the elements of niche and stem cells are of critical importance for their perpetuation of stemness. Thus, for example, Paneth cells constitute the niche for Leucine-rich repeat-containing G-protein coupled receptor 5 (Lgr5)—we can find them in intestinal crypts [5, 6]. We also know that BM is the source of three different types of adult stem cells: HSC, MSC, and VSELs. The markers are mostly receptors which can be bound to specific ligands

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causing signaling mechanisms propagation within the cell. Labeled with fluorescent dyes, they can be detected, and isolated/segregated with the help of Flow Cytometry (FC) and Fluorescence Acquired Sorting analysis (FACS) [3–5]. Currently, the marker-based flow cytometry (FCM) technique and magnetic cell sorting (MACS) are the most effective cell isolating methods, and a detailed marker list will help to initially identify, as well as isolate ESCs using these methods [7]. Some functional assays have also been developed for stem cell marker identification and detection [3, 4]. Therefore, we were able to classify stem cells into distinctive categories.

5.2 Human Embryonic Stem Cell Markers and Pluripotency

ESC derives from inner cell mass of the blastocyst of embryo and are totipotent in their full capacity to transdifferentiate into any kind of mature body cell [8]. ESCs retain pluripotency and self-renewing ability due to both their inherent properties and the culture conditions in which they are propagated [7, 8]. The ability to differentiate into all cell lineages in living bodies while maintaining an undifferentiated state during in vitro culture makes ESCs prior to clinical transplantation [7]. Their specific receptors/markers depend on how old the embryo is. However, the crucial markers are: octapeptide4 (**Oct 4**), homeobox protein **Nanog**, **Tra1-60**, sex determining region Y-box 2 **Sox-2/SRY**, and stage-specific embryonic antigen-4 (**SSEA-4**). They can be detected by fluorescently labeled antibodies [7]. The pluripotent status of stem cells can be also characterized by a high level of alkaline phosphatase (AP) expression, along with the expression of multiple pluripotency markers. The epiblast (EPI) is the origin of all somatic and germ cells in mammals, and of pluripotent stem cells in vitro. To explore the ontogeny of human and primate pluripotency, comprehensive single-cell RNA sequencing for pre- and post-implantation EPI development in cynomolgus monkeys (*Macaca fascicularis*) was performed [1]. The group has shown that after specification in the blastocysts, EPI from cynomolgus monkeys (cyEPI) undergoes major transcriptome changes on implantation [1]. Thereafter, while generating gastrulating cells, cyEPI stably maintains its transcriptome over a week, retains a unique set of pluripotency genes, and acquires properties for "neuron differentiation" [1]. Human and monkey pluripotent stem cells have shown the highest similarity to post-implantation late cyEPI, which, despite coexisting with gastrulating cells, bears characteristics of pre-gastrulating mouse EPI and epiblast-like cells in vitro [1]. The authors concluded that these findings not only reveal the divergence and coherence of EPI development, but also identify a developmental coordinate of the spectrum of pluripotency among key species, providing a basis for better regulation of human pluripotency in vitro [1].

In recent years, a wide range of cell surface markers and generic molecular markers have been reported to be indicative of undifferentiated ESCs, especially for human species (Fig. 5.1) [8–10]. Proteins involved in several signal pathways are also known to have important functions in cell fate decision. Lectins and other

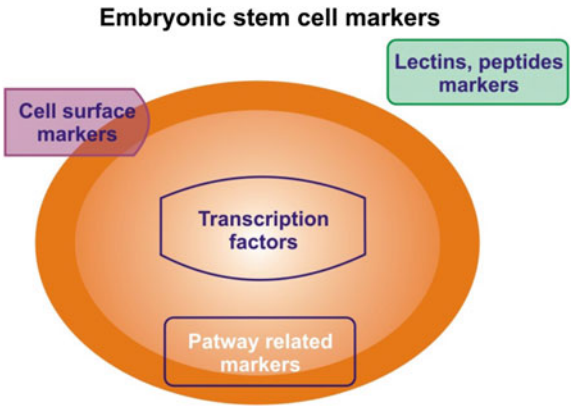

Fig. 5.1 Categories of embryonic stem cell markers

similar peptides have been found to specifically bind to ESCs. Unfortunately, many ESC markers overlap with those of tumor stem cells, making the problems when these markers are used for ESC identification and isolation [8]. In addition, understanding the mechanisms that regulate the pluripotency of human ESCs (hESCs) remains a major challenge, as recent studies have shown that human and mouse ESCs differ in these mechanisms despite their similar embryonic origins [8]. Further knowledge of these markers is critically needed for the proper uses of ESCs and elucidation of the mechanisms governing the pluripotency and self-renewal of ESCs.

5.3 Fetal Stem Cell Markers

FSCs are intermediary stadium between MSCs and ESCs. They originate from different fetal and extraembrional tissues during the fetal life. Growth kinetics, morphology, immunophenotype, potential for differentiation and incorporation in vivo, depend on the origin. They are more primitive and have bigger multipotential from their "adult" analogs (hematopoietic cells of fetal blood-HSCs, and they have bigger proliferative capacity from HSCs from the cord blood and HSCs from bone marrow of adults [11–16]. Certain subpopulations are showing pluripotent potential. These cells show lower immunogenic features and more seldom cause the graft versus host reaction (GvHR), which makes them potentially good cancnicates for transplantation.

Table 5.1 summarizes classification of fetal stem cells, showing that markers are either the markers of HSCs or the markers of MSCs.

Table 5.1 FSCs—classification and distribution of fetal stem cells

Fetal tissues	Extraembrional tissues
Blood (MSCs, HSCs)	Cord Blood (HSCs, MSCs, similar ESCs—CBEs, VSEL, endothelial progenitors, iPSCs)
Liver (MSCs, HSCs)	Tissue of cord Wharton's jelly, (MSCs)
Bone Marrow (MSCs, HSCs)	Umbilical blood vessels (HUVEC)
Lungs (MSCs)	Amniotic Fluid ((MSCs, AFCs, VSELs)
Pancreas (MSCs)	Placenta (Epithelial cells of amnion, MSCs of amnion, MSCs of chorion, and HSCs
Brain cortex	
Mesonephros	

(compilation from different sources)

5.4 Cord Blood Stem Cell Markers

These cells are considered to be multipotent—they can develop into more than one cell type, but are more limited than pluripotent ESCs [17–19]. Cord blood stem cells were reported to be successfully used in the treatment of acute myocardial infarction (AMI) [20]. As well as HSCs they have **CD34+ marker** as the essential for recognition and identification when collected, counted, and prepared for conservation.

5.5 Placental Stem Cell Markers

Given the fact that placenta originates partly from baby and partly from mother, as we can see from Table 5.1. Placental markers are from amnion (markers of epithelial cells of amnion, MSCs of amnion) and chorion and markers of HSCs, mostly CD34+.

5.6 Adult Stem Cell Markers

Adult stem cells typically generate the cell types of the tissue in which they reside. For example, a blood-forming adult stem cell-hematopoietic stem cell (HSC) in the bone marrow (BM) normally gives rise to the many types of blood cells. It is generally accepted that a blood-forming cell in the BM cannot give rise to the cells of a very different tissue, such as nerve cells in the brain. On the other hand, it has been shown that there are stem cells in the brain [19]. Great curiosity of these dividing cells is that they have receptor for Zika virus which explains why the virus

Table 5.2 Markers of different adult stem cells (compilation from many sources)

Cell type	Markers
Hematopoietic Stem Cell (HSC)	CD34, CD45, CXCR4
Endothelial Progenitor Cells (EPC)	CD34, CD73, CD133, CXCR4, KDR, anti-M IgG
Very Small Embryonic Like Cells (VSEL)	CD34, CD133, CXCR4, SSEA4, anti-M IgG
Mesenchymal Stem Cells (MSC)	CD34, CD45, CD90, CD105, CD106, CD44

can cause undeveloped brain or different degree of microcephaly [20]. Experiments over the last several years have purported to show that stem cells from one tissue may give rise to cell types of a completely different tissue. However, we believe that our group has shown that HSC are playing significant role in AMI by trans-differentiating into myocardial cells, which suggests that HSCs can be at least multipotent [21]. Yet, this remains an area of great debate within the research community. There are still scientists who think that pluripotent adult stem cells do not exist in humans at all (as they do in plants) and that it is the reason that they cannot regenerate the whole organism, namely entire organ such as it is the case with amphibia. This controversy demonstrates the challenges of studying adult stem cells and suggests that additional research using adult stem cells is necessary to understand their full potential as future therapies [22].

However, given the fact that many researchers have got the data with adult stem cells, there are three solid candidates with distinct markers that we can consider pluripotent: HSC, MSC, and VSEL stem cells. They have different morphology and different markers presented on Table 5.2. Otherwise, each tissue has its own stem cell markers and they vary with propagation in culture. Additional research on regulation of chromatin factors and genome organization, crucial for stem cell maintenance, and management of pluripotency is needed to consolidate and interpret the data [22–25].

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Chapter 6
Stem Cell Signaling Molecules and Pathways

Science, my lad, is made up of mistakes, but they are mistakes which it is useful to make, because they lead little by little to the truth.

Jules Verne

Abstract Essential stem cell signaling pathways are described and graphically presented for ESC and adult HSC and MSC. Two distinguishing characteristics of embryonic stem cells (ESCs) are totipotency and the ability to self-renew. These traits, which allow ESCs to grow into any cell type in the adult body and divide continuously in the undifferentiated state (self-renewal), are regulated by a number of cell signaling pathways. Adult stem cells such as HSC and MSC have different pathways involved. This can partially explain their restriction of the potency. Up-to-date knowledge on signaling mechanisms and genetic regulation in human stem cells is provided. Transcriptional and posttranscriptional control is described that enable maintaining the boundaries between pluripotent stem cells and differentiating descendants. The involvement of signaling molecules is emphasized with corresponding schematic view. Several key regulators of stem cell maintenance revealed corresponding genetic and humoral regulation which is briefly presented. Similarities at the molecular level between different animal stem cells, in terms of regulation and maintenance, are depicted.

6.1 Introduction

Both animal and plant stem cell niches are specified during embryogenesis, and, post-embryonically. They are located within entire organism, as organized groups of dividing cells that are responsible for most post-embryonic management of the body growth and reparation. Pluripotent stem cells contain open chromatin compared with differentiated cells, meaning less heterochromatin, more loosely bound (or hyperdynamic) architectural chromatin proteins, less methylation, and global

M. Pavlović and K. Radotić, *Animal and Plant Stem Cells*,
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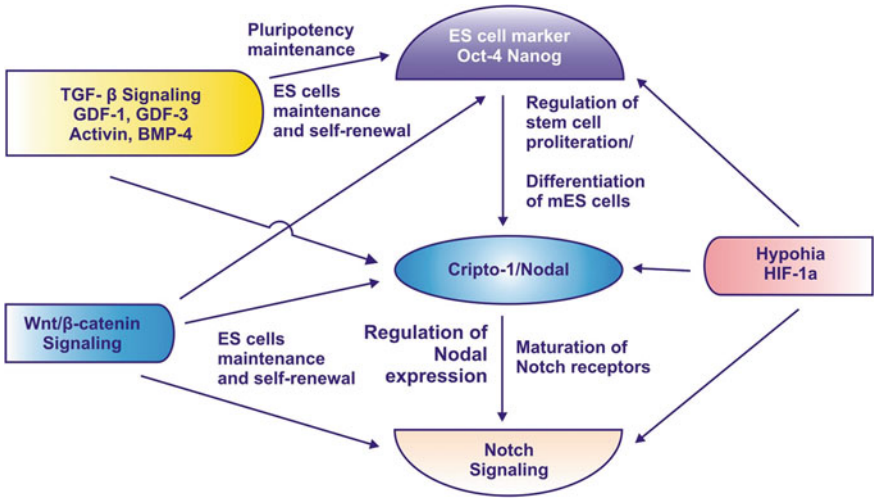

Fig. 6.1 Essential signaling molecules and pathways in ESCs

transcriptional hyperactivity [1–3]. Upon differentiation, the transcriptional program needs to be rapidly switched, which is possibly mediated by the presence of both activating and repressive chromatin marks (so-called bivalent domains) on lineage-specific developmental regulators [3]. This is achieved in a process where these regulators are silenced and at the same time prone to activation. In addition, embryonic stem cells are sensitive to reduced levels of key structural components of chromatin (cohesin and condensing complexes). The regulation pathways of stemness (self-renewal and pluripotency) in animal stem cells depend on whether it is ESC or adult, normal or cancer, human or other animal species (for instance mouse). In humans, ESC are under influence of **Wnt**, **TGF-β**, **BMP**, **Nanog**, **and Notch signaling**, preferably (Fig. 6.1) [1].

Many adult tissue stem cells, such as the cells of the hematopoietic system, gastrointestinal epithelium, brain, epidermis, mammary gland, and lung have now been identified, all of them fulfilling a crucial role in supplying organisms with mature cells during normal homeostasis as well as in times of tissue generation or repair [4]. Two unique features characterize adult stem cells: the ability to generate new pluripotent stem cells (to self-renew) and the ability to give rise to differentiated progeny that has lost its self-renewal capacity, after a while. Mutant analysis experiments and transcriptional profiling have helped significantly understanding that chromatin factors and genome organization factors are crucial for stem cell maintenance and understanding of molecular nature of stem cells in depth. Pluripotent stem cells in both animals and plants contain open chromatin compared with differentiated cells. There is still controversy on the question whether at least some animal adult stem cells are pluripotent. By most of authors who did work with them, HSC, VSELs and MSCs show the features of pluripotency, being able to differentiate in most of diverse cell lines (not only within one lineage), but yet not to

regenerate the entire organism [5, 6]. Our understanding of the mechanisms that determine whether, where and when a stem cell will self-renew or differentiate is still limited, but recent advances have indicated that the stem cell microenvironment, or niche, and their "cross-talk" provide essential cues that direct these cell fate decisions [7–10]. Repression of differentiation-promoting transcripts in stem cells by local miRNAs to promote self-renewal is also observed in animal systems, which provides another mechanistic analogy between plant and animal stem cell regulation [11–18]. Moreover, loss of control over these cell fate decisions might lead to cellular transformation and cancer. Stemness signaling pathways are dysregulated in CSC which contributes to chemo and radioresistency as well as metastasis in reoccurrence of cancer [19–23].

6.2 ESC-Wnt Signaling

Wnts were discovered 30 years ago [1]. Wnt signaling is a master regulator of development, and also of cell polarization. Recently, Wnt has been closely linked to other signaling pathways, such as Hippo, that orchestrate proliferation and apoptosis to control organ size [1]. It is conformed that mechanotransduction, Hippo, Wnt, and TGF-beta have something in common: YAP and TAZ are key orchestrating molecules [1]. The Wnt family of secreted growth factors regulates the developmental processes of cell fate and polarity, as well as general cell maintenance processes such as homeostasis and cell cycle regulation. There are 19 Wnt ligands in humans, which bind to the Frizzled (FZD) family of receptors and the co-receptors LRP5 and LRP6.

Wnt signaling comprises three pathways: the canonical pathway and two non-canonical pathways, planar cell polarity (PCP) and a calcium ion-dependent pathway. The Wnt ligands bind to frizzled receptor family members and activate one of three Wnt pathways: the canonical pathway, PCP, or a calcium ion-dependent pathway. The well-studied canonical Wnt pathway signals through β-catenin and regulates cell cycle, growth, and proliferation. The PCP pathway regulates cytoskeletal dynamics and cell motility, and the Wnt/calcium pathway promotes NFAT transcription. Both pathways operate independently of β-catenin signaling.

The Wnt family of secreted growth factors regulates **the developmental processes of cell fate and polarity**, as well as **general cell maintenance processes such as homeostasis and cell cycle regulation**.

6.2.1 Significant Discoveries in Wnt

The receptor for the Wnt-agonistic R-spondins Lgr5, the Wnt-agonistic R-spondins, marks stem cells in multiple adult organs of mice and humans.

Single Lgr5 stem cells derived from the intestine can be cultured to build epithelial structures that retain hallmarks of the in vivo epithelium [3]. Breakdown of cell polarity complexes upon anoikis sensitivity through the Hippo, Wnt and TGF-beta pathways, emphasizing points of cross-regulation, has been reviewed in J. Cell Sci., January 2013 [3]. The Wnt pathway also promotes pluripotency, although this may occur through a non-canonical mechanism involving a balance between the transcriptional activator, TCF1, and the repressor, TCF3. Signaling through these pathways supports the pluripotent state, which relies predominantly upon three key transcription factors: Oct-4, Sox2, and Nanog [1–4]. The role of these transcription factors is in activation of gene expression of ESC-specific genes, regulation of their own expression, suppression of genes involved in differentiation, and a role as hESCs markers. Other markers used to identify hESCs are the cell surface glycolipid SSEA3/4, and glycoproteins TRA-1-60 and TRA-1-81. In vitro, hESCs can be coaxed into derivatives of the three primary germ layers, endoderm, mesoderm, or ectoderm, as well as primordial germ cell-like cells. One of the primary signaling pathways responsible for this process is the BMP pathway, which uses Smad1/5/9 to promote differentiation by both inhibiting expression of Nanog, as well as activating the expression of differentiation-specific genes. Notch also plays a role in differentiation through the notch intracellular domain (NICD). As differentiation continues, cells from each primary germ layer further differentiate along lineage-specific pathways [5–8].

Wnt signaling is the best studied of all known ESC pathways (Fig. 6.2).

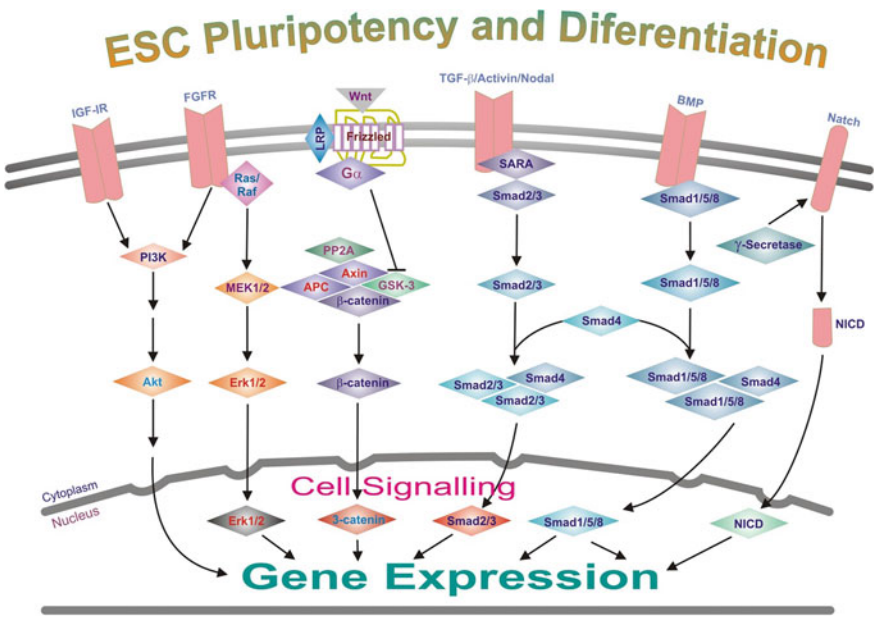

Fig. 6.2 Wnt and other signaling mechanism through ESC

6.3 Notch Signaling

The pathway was originally discovered in *Drosophila melanogaster*, and mammalian homologs were identified later [4]. It is a conserved developmental pathway involved in cell–cell communication, cell fate, apoptosis, and differentiation [4]. Ligands from the Delta and Jagged/Serrate families activate the Notch membrane-bound receptors, inducing cleavage of the NICD. This intracellular domain interacts with the RBPJ family of transcription factors as well as a variety of coactivators and corepressors to regulate target gene transcription. The output of Notch signaling activation is context-dependent, and the proper timing and spatial regulation of its activation is crucial for normal embryonic developmental processes. Notch signaling often cross-talks with two other developmental signaling pathways, Hedgehog, and Wnt [4]. Dysregulation of Notch signaling processes can lead to cancer, while mutations in Notch signaling genes can cause genetic developmental disorders [4]. Analyzing the expression, regulation, and sequence of Notch signaling genes can help determine their relative importance to the biology of the cellular or disease processes under study.

6.4 The Human Hedgehog Signaling

The hedgehog family members, including Sonic hedgehog (Shh), are the most well-known morphogens involved in the developmental pattern formation of various organs, such as the nervous system, muscle, the heart, and the lungs. Hedgehog signaling has also been implicated in the development of several human cancers (Fig. 6.2) [23]. Signaling includes hedgehog family members, hedgehog receptors, and other associated proteins. There are also key genes involved in cell differentiation and multicellular organism development [24–26]. Using real-time PCR, and RT^2 Profiler™ PCR Array profiles, you can detect the expression of 84 key genes involved in the hedgehog signaling pathway [1].

6.5 TGF-Beta Signaling

Which signals through Smad2/3/4, and FGFR, which activates the MAPK and Akt pathways (1) is common signaling pathway in ESCs.

6.6 Signaling Mechanisms in Adult Human Cells (HSC and MSC)

Hypoxia as a possible self-renewal factor (*Oxygen Stem Cell Paradigm*, Ivanovic et al. [27]).

During two-decade period of studying the "hypoxic" nature of hematopoietic stem cells (HSC), in Ivanovic's group, (Bordeaux) an observation came to light: these cells behave as facultative anaerobic single-celled organisms [27, 28]. When they published for the first time (2000), the observation that low O_2 concentration favors HSC self-renewal, they became aware of some analogies in HSC behavior with one of the facultatively anaerobic *protists* [27, 28]. Thus, the link between stem cell physiology and evolution of the first eukaryotes was articulated in their "Oxygen Stem Cell Paradigm" in 2009 [27]. In the meantime, the same features (facultative anaerobiosis related to self-renewal, differentiation in function of oxygen availability, etc.) were evidenced for all categories of stem cells according to Ivanovic et al. [27]. The evolution of stem cell entity in *metazoa* was better explored and documented, and some key features of stemness were recognized in the life cycle of single-cell eukaryotes. A very detailed review on the available data and the cues given in order to build an "evolutionary stem cell paradigm" based on the relationship between anaerobiosis and stemness, could be found in the book which we recommend for further consideration [28]. However, it seems quite natural/logical that stem cells as a sign of their primitivism with very small number of mitochondria, live in their hypoxic niches as a facultative anaerobs and that this status/level of stemness is tightly coupled to self-renewal capability (Fig. 6.3). What kind of molecular pathways and cross-talks with the niches are performed in that context is to be determined and proven. At present, little is known of the molecular determinants that regulate stem cell self-renewal, but this property clearly distinguishes stem cells from other cells.

Thus, although there are groups that would rather classify HSCs, VSELs, and MSCs at least as pluripotent [5, 6], most of researchers consider them rather multipotent due to inability to regenerate complete organism, a feature that is monopolized by ESCs [21].

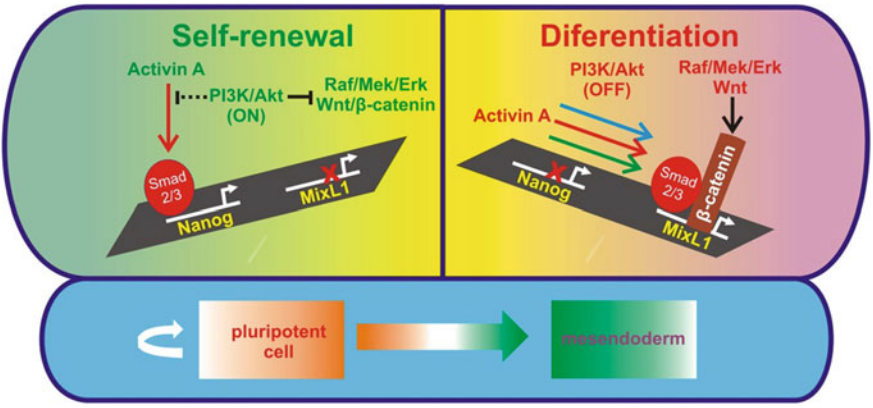

Fig. 6.3 Signaling network cross-talk in human pluripotent cells

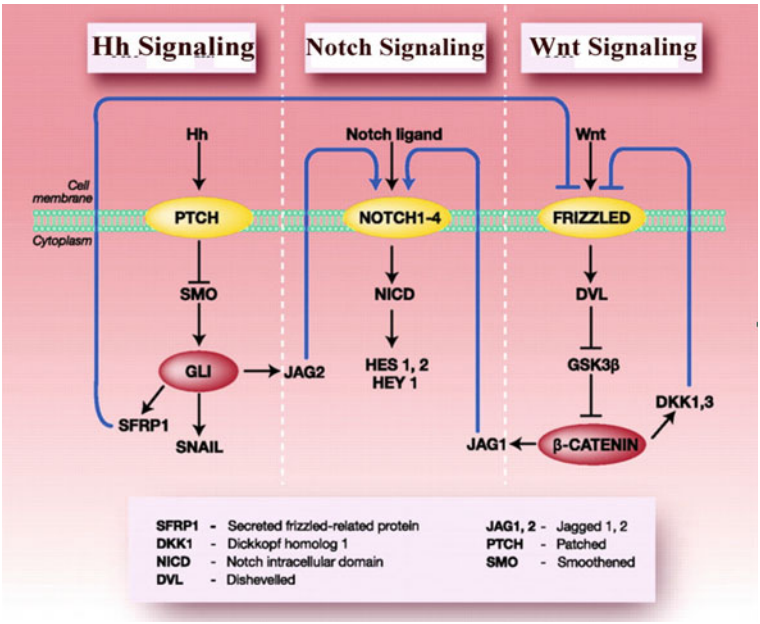

Fig. 6.4 Signaling pathways for self-renewal and pluripotency in HSC (compilation 29, 30, 31)

6.7 Pathways in Adult HSC

Hematopoietic stem cells (HSCs) are self-renewing, tissue-specific stem cells that give rise to all mature blood cell types [17]. The capacity of HSCs to reconstitute the entire adult hematopoietic system after transplantation makes it crucial to precisely characterize the mechanisms of cell signaling events that occur in vivo to form functional HSCs [17, 29, 30]. It can be invaluable for the treatment of various blood disorders.

Signaling pathways for self-renewal and pluripotency (Hh, Notch and Wnt) are presented in Fig. 6.4. However, in adult stem cells they seem to be dispensable to some extent. Members of the cellular polarity network may display a novel class of signaling molecules associated with self-renewal capacity in development and maintenance of HSCs [29]. Current research is going in this direction.

6.8 Pathways in Adult Mesenchymal Stem Cells

Mesenchymal stem cells (MSC) have emerged as a reliable stem cell source for cellular based treatment modality and are currently being tested in numerous ongoing clinical trials. Unfortunately, the fervor over MSC is mitigated by several lines of evidence suggesting that their efficacy is limited by natural aging [18].

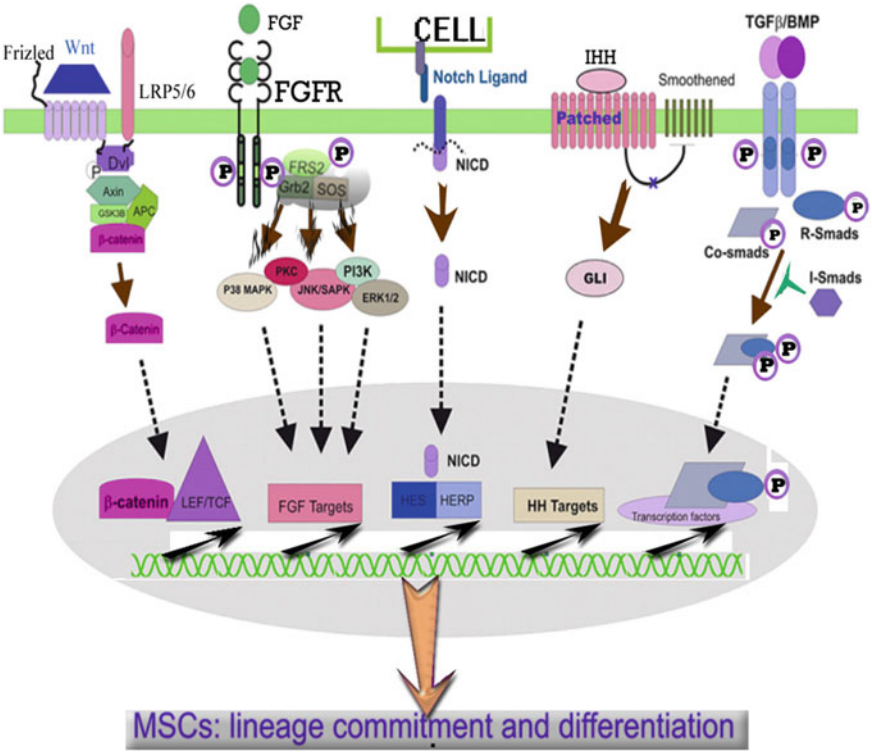

Fig. 6.5 Signaling pathways in MSC

Indeed, the authors have found that MSCs lose their regenerative potential as a result of natural aging [18]. They concluded that perspective is that the aging niche and consequent deleterious changes that occur in this microenvironment might be the main culprit in MSC aging [18]. The key is, therefore, to overcoming the aging issue in MSC-mediated cell therapy to unlocking and furthering the current understanding of the specific microenvironmental factors that compromise MSCs over time [18].

Pathways in MSCs are schematically presented in Fig. 6.5. Additional studies are necessary to prove theoretical considerations.

6.9 Pathways in CSC

There is still a lack of evidence over the complete regulatory control of the pathways network in the CSCs. Not all the pathways for entire spectrum of tumors are covered. However, the JAK/STAT signal mechanisms seem to be operating in some tumors [24, 26].

6.10 The JAK (Janus Kinase)/STAT3 (Signal Transducer and Activator of Transcription) Signal Mechanism

LIF (leukemia inhibitory factor) is a key cytokine for maintaining self-renewal and pluripotency of mESCs (mouse embryonic stem cells). Upon binding to the LIF receptor, LIF activates three major intracellular signaling pathways: the JAK (Janus kinase)/STAT3 (signal transducer and activator of transcription 3), PI3K (phosphoinositide 3-kinase)/AKT and SHP2 [SH2 (Src homology 2) domain-containing tyrosine phosphatase 2)/MAPK (mitogen-activated protein kinase) pathways (Fig. 6.6). These pathways converge to orchestrate the gene expression pattern specific to mESCs. Among the many signaling events downstream of the LIF receptor, activation and DNA binding of the transcription factor STAT3 plays a central role in transducing LIF's functions. The fundamental role of LIF for pluripotency was highlighted further by the discovery that LIF accelerates the conversion of epiblast-derived stem cells into a more fully pluripotent state. We provide an overview of the three major LIF signaling pathways, the molecules that interact with STAT3 and the current interpretations of the roles of LIF in pluripotency. This pathway is activated after binding of leukocyte inhibiting factor (LIF) for its receptor and thus works in both ESCs and Leukemia cells. It activates also transcription factors for self-renewal.

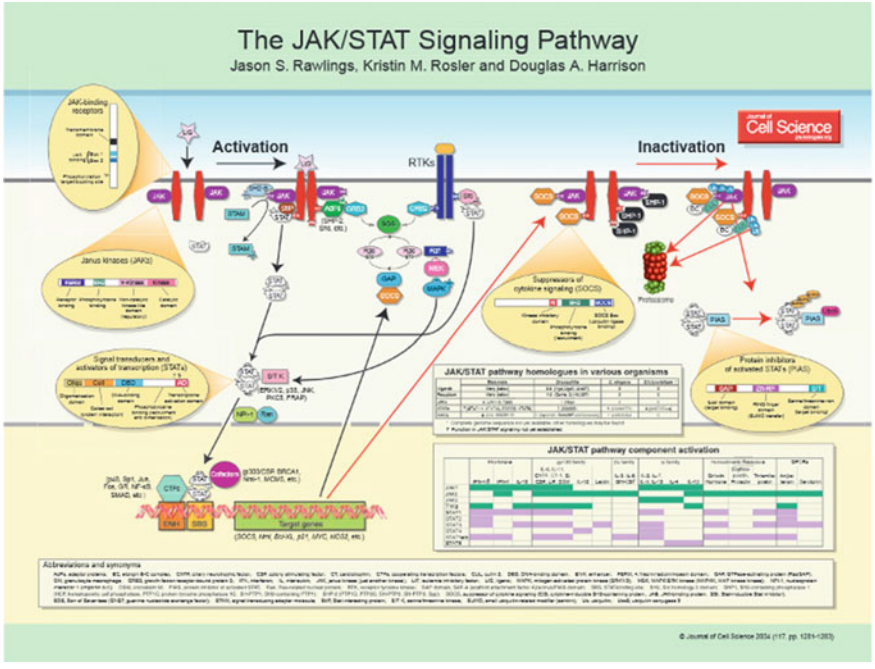

Fig. 6.6 JAK/STAT signal mechanism

- You can see more at: http://www.cellsignal.com/contents/science-pathway-research-stem-cell-markers/esc-pluripotency-and-differentiation-signaling-pathway/pathways-esc#sthash.JixRZEUs.dpuf.

It is widely accepted that signaling pathways in cancer stem cells should be the targets for cancer therapy, knowing that key stemness signaling pathways involved in the induction and maintenance of stemness in CSCs include [5, 6]:

JAK/STAT
Wnt/β-catenin
Hedgehog
Notch
Nanog [19].

We have described their molecular contacts/connections and function in the normal stem cells. However, targeting these signaling pathways may disrupt aberrant signaling in CSCs, potentially reducing cancer recurrence and metastasis [19–23]. Some ongoing studies in advanced cancers emphasize inhibition of cancer stem cell pathways [31]. This is opening the door for further development of the concept.

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Chapter 7
Expansion of Stem Cells: Propagation of Animal Stem Cells Ex Vivo (In Culture)

Science and technology revolutionize our lives, but memory, tradition and myth frame our response.
Arthur M. Schlesinger

Abstract The chapter describes the ways to expand the insufficient number of stem cells into desired, for clinical application. At first, it was regarded that for example, cord blood stem cells should not be stored at all, since their total yield was low. However, with the time, the methods for expansion have been developed and strongly supported the use of stem cells from different sources in clinical arena. Several approaches to expansion mainly through propagation in culture, are briefly described.

7.1 What Is Expansion and What Do We Expect from Expansion?

Finding a bone marrow donor match is challenging and the number of bone marrow cells from a single harvest procedure are often not sufficient for a transplant. Same is with cord blood obtained for transplantation purposes. Additional rounds of bone marrow harvest and clinical applications to mobilize blood stem cells are often required. However, an expansion of healthy HSCs in the lab would mean that fewer stem cells need to be retrieved from donors. It also suggests that adult blood stem cells could be frozen and banked for future expansion and used if it is not currently possible [1–3].

The basic formula stating that equation:

Fold expansion = Number of harvested cells/Number of input cells, explains the essential feature of expansion to increase original stem cell population number.

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7.2 Basic Concept—Ex Vivo Expansion of Hematopoietic Cells Today

It is notorious thing, that although labeled with specific markers that associate them with "stem cells" entities, the human CD34+ cell population is extremely heterogeneous in functional properties [1–4]. In order to expand stem cells, the research today has to offer several approaches that could be classified such as: molecules, distinct stimuli, media, and different optimal protocols [1–11].

7.2.1 Molecules

The capacity for sustained self-renewal—the generation of daughter cells having the same regenerative properties as the parent cell—is the defining feature of hematopoietic stem cells (HSCs). Strong evidence exists that self-renewal of HSC is under extrinsic biological control in vivo [1]. A variety of cytokines, morphogenic ligands and associated signaling components influence self renewal in culture and in vivo [11].

So far most efficient are proteins HOX/CUL4 [10, 11]. Specific **homeobox transcription factors** acts as powerful intrinsic agonists of HSC self-renewal in vitro and in vivo when supplied either as transduced cDNAs or as externally delivered proteins. These findings provide tools for deepening our known—edge of levels of HSC expansion.

During examination of why HOXB4 protein doesn't last long in HSCs, once these cells are removed from the protective stem cell niche that they nest quietly in, it was found that HOXB4 is targeted for degradation so that stem cells can start differentiating, meaning turn into different kinds of adult blood cells [10, 11]. HOXB4 prevents blood stem cells from differentiating, while, at the same time, allows them to renew themselves. The researchers also found that a protein, CUL4, is tasked with recognizing HOXB4 and tagging it for destruction by the cell's protein destruction apparatus [15, 16]. They discovered that CUL4 recognizes HOXB4 because it "sees" a set of four amino acids on the protein. HOXB4 carries a destruction signal that CUL4 recognizes and acts on. This inspired the research team to engineer a synthetic HOXB4 protein with a scrambled destruction signal. They produced large quantities of the protein in bacteria, and then delivered the protein into human blood stem cells in the laboratory. When CUL4 degradation signal was masked, HOXB4's half-life expanded for up to 10 h. Thus, the engineered protein HOXB4 can potentially be administered every 10 h or so to make the quantity of blood stem cells necessary for patient transplant and for banking.

The engineered HOXB4 did its job to expand the stem cell, while keeping all its stem cell properties intact. As a result, cells receiving the engineered HOXB4

demonstrated superior expansion capacity than those given natural HOXB4 protein. Animal studies demonstrated that the transplanted engineered human stem cells can retain their stem cell-like qualities in mouse bone marrow.

7.2.2 Distinct Stimuli

Self-renewal can be driven intrinsically by gene expression and can be regulated by extrinsic factors from environment. Cell-intrinsic regulation of HSC fate includes interplay between specific transcription factors, RNA/DNA-binding proteins, and chromatin-associated factors. That network can be modulated by cell-extrinsic cues such as cytokines, developmental/growth factors, and chemical compounds. These distinct stimuli create a complex matrix of interactions that defines the result of HSC fate, suggesting that combination of distinct stimuli could be required for effective stimulation of self-renewal divisions and stem cell expansion [7–16].

New STEMdiff mixture of different molecules, the first of which is leaving the cells in the state of self-renewal, is still the secret of the company.

7.2.3 Media for Expansion

Ex vivo HSC expansion protocols available today cannot efficiently support symmetrical self-renewal divisions of HSCs, resulting (optimistically) in a maintenance or minor amplification of stem cells and expansion of differentiated progeny. It remains unclear whether stem cells from any source benefit from up-to-date expansion protocols, resulting in a lack of standardized on-demand expanded product.

The challenge remains to expand undifferentiated HSCs (via stimulation of symmetrical self-renewal divisions) in numbers sufficient for therapy of adult patients. This would allow development of clinically relevant and quality-controlled HSPC-expanded products that can be supplied upon demand [17–22].

7.2.4 Hypoxia

Maybe the most efficient factor of CD34+ HSC expansion as well as CB CD34+ cell expansion is a natural stimulus that exists in the niche: hypoxia. Cord (placental) blood represents a source of stem and progenitor cells for engraftment [1, 21]. These cells are, in general, more primitive with respect to those mobilized in peripheral blood. For example, the CD34+ population of cord blood cells is for 1 to 2 log richer in stem cells capable to engraft the immunodeficient mice (Scid Repopulating Cells—SRC). The cells of cord blood did not respond the same way

to the cytokines, and their amplification kinetic ex vivo is different from the one of peripheral blood cells. The transplantation of cord blood cells is limited by a low number of cells in one cord blood unit. In addition, given that these cells are more primitive, the time for mature cells production is rather long. This point has an important consequence: a very slow blood reconstitution after transplantation (agranulocytosis period is about one month). Due to this inconvenience, the transplantation of cord blood cells was limited to children and adults of low body weight. This problem is reduced by the practice of simultaneous transplantation of 2 cord blood units. However, even with this approach, the time of post transplant neutropenia is rarely below 2 weeks. So there is an evident interest for ex vivo expansion of cord blood cells in order to:

- Amplify the number of total cells
- Differentiate several subpopulations of stem cells and progenitors and amplify these populations in order to get a shortage or even abrogation of post-transplant agranulocytosis period.

In the same time the absolute imperative is to:

- Maintain or even amplify the primitive stem cells in order not to jeopardize the capacity of long term maintenance of hematopoiesis.

This third point would allow to consider the ex vivo amplification and consecutive transplantation of the whole cord blood unit without saving a non manipulated part. At the moment, the expansion of hematopoietic cells from cord blood is aimed to the allogenic transplantation, although its use in autologous situations cannot be excluded.

Ex vivo expansion (amplification) of stem and progenitors cells is a concept aimed to resolve the problem of insufficient number of cells for engraftment and/or to accelerate hematopoietic reconstitution after transplantation, if we are using HSCs [21–25]. After a long period, during which this approach failed to demonstrate its clinical utility, the first successful clinical trials were achieved. This breakthrough, mainly resulting from recent understanding of some fundamental properties of stem cell as its anaerobic metabolic character, as Ivanovic et al. suggested [1, 18, 23].

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Chapter 8
Stem Cell Pool: What Are the Best Patterns for Cellular Therapy?

Science investigates; religion interprets. Science gives man knowledge which is power; religion gives man wisdom which is control.

Martin Luther King, Jr.

Abstract This chapter will discuss origin, classification, features of stem cells and fundaments of stem cell therapy as the segment of cellular-based therapy. Generally, the Stem Cell (SC)—compartment is divided into embryonic and tissue specific or adult SCs. Paul Niehans, M.D., (1882–1971), the originator of cell therapy, wrote: "Cellular therapy is a method of treating the whole organism on a biological basis, capable of revitalizing the human organism with its trillions of cells by bringing to it those embryonic or young cells which it needs. Cells from all organs are at our disposal; the doctor's art is to choose the right cells. Selective cellular therapy offers new life to the ailing or diseased organism." The concept of very small embryonic-like stem cells (VSELs) and their phenotypic and functional characteristics are discussed in the light of recent conflicting data. The differences between two adult stem cell compartments (hematopoietic and non-hematopoietic) within the adult bone marrow, (BM) and distant organs are emphasized. The crucial criteria for distinction between these two different pools of stem cells {hematopoietic stem cells (HSCs)}, and VSELs, are presented "hallmarking" VSELs as a separate entity. A possible explanation for the presence of these cells in the adult bone marrow of humans and them impacting stem cell regenerative purposes are summarized, as they are also found in the cord blood (CB). Certain organs/tissues involvement in the VSEL generation and/or storage is also discussed. The experimental approach to this area is analysed, followed by brief description of separation, purification and identification of this cell population in mice and humans. The critical controversies in findings regarding VSELs within the overall stem cell concept/stemness are analysed in depth. The functional role and perspectives of stem cell therapy in the clinical arena using this existing stem cell primitive ancestor are envisioned with regard to their fundamental traits as a great challenge and inspiration for future studies.

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8.1 Introduction

The first use of stem cells in humans was done by physicians who were tempted to use them in trying to treat hematological disorders. Stem cell transplantation was pioneered using bone-marrow-derived stem cells by a team at the Fred Hutchinson Cancer Research Center from the 1950s through the 1970s led by **Edward Donnall Thomas**, whose work was later recognized with a Nobel Prize in Physiology or Medicine [1]. Thomas' work showed that bone marrow cells infused intravenously could repopulate the bone marrow and produce new blood cells. His work also reduced the likelihood of developing a life-threatening complication called graft-versus-host disease. The first physician to perform a successful human bone marrow transplant was **Robert A**. **Good** at the University of Minnesota in 1968 [1]. With the availability of the stem cell growth factors (GM–CSF and G–CSF), most hematopoietic stem cell transplantation procedures are now performed using stem cells collected from the peripheral blood, rather than from the bone marrow. Collecting peripheral blood stem cells provides a bigger graft, does not require the donor to be subjected to general anesthesia in order to collect the graft, results in a shorter time to engraftment, and may provide for a lower long-term relapse rate.

Breakthroughs: Edward Donall Thomas (1920–2012), Robert A. Good (1922–2003), pioneers of stem cells transplantation, Paul Niehans (1882–1971) pioneer of stem cell therapy. Dr. Edward Donall Thomas is an American physician and a Nobel Laureate in Physiology or Medicine 1990. He was awarded the Nobel Prize for his work on the development of cell and organ transplantation. Dr. Thomas shared the award with Joseph Murray.

The first recorded attempt at cellular therapy occurred in 1912 when German physicians attempted to treat hypothyroid children with thyroid cells. Cellular therapy, as practiced today, was developed in the early 1930s by **Paul Niehans**, M.D. (1882–1971), a Swiss physician who became known as "the father of cell therapy." It soon became popular with celebrities as a means of rejuvenation. A 1990 article in *In Health* magazine described Niehans as a "public relations genius" and stated that the Clinique La Prairie, which he had founded in Clarens-Montreaux, Switzerland, had attracted 65,000 patients. Its 1999 one-week "revitalization program" costed about $8000 [1].

Generally, the Stem Cell (SC)—compartment is divided into embryonic and tissue specific or adult SCs [1]. Embryonic SCs (ES or ESC) are by definition the

"master cells" with the largest spectrum of differentiation potential, e.g., capable of differentiating into every type of cells either in vitro or in vivo. Thanks to the presence of embryonic body, these cells have ability to develop into three primary layers: endoderm, ectoderm, and mesoderm [1]. The discovery of SCs inside cell mass of embryos and in adult tissue has revolutionized the medical field by introducing new therapeutic dimensions into previously untreatable diseases and injuries. Several experimental or preclinical studies have suggested that application of embryonic SC could be promising in the treatment of various diseases [2–6]. However, recognition of appropriate ethical aspects, regulatory acts, and standardization in embryonic SC-mediated regenerative medicine is needed as it is still the matter of controversy. Besides, permanent, persistent and accurate updating of the facts regarding their phenotypic, functional, and immunologic characteristics is an essential requirement for safe clinical application of SCs. Some authors stand that the initial theory that embryonic SCs are ignored by immunocompetent hosts was overlooked. On the contrary, they think that it is even more evident that embryonic SCs could protect themselves actively by several immunomodulatory mechanisms against T lymphocytes and natural killer cells of host, and actively participate in immune-mediated events. Recent isolation of fetal SCs from several sources either at the early stages of development or during the later trimesters of gestation, sharing similar growth kinetics and expressing markers of pluripotency, provides strong support to the statement that these cells may be biologically closer to embryonic SCs. In fact, they represent intermediates between embryonic and adult mesenchymal SCs with regards to proliferation rates and plasticity features, thus being able to confer an advantage over postnatal mesenchymal SCs derived from conventional adult sources.

Historically, bone marrow was the primary source of SCs for transplant [1]. However, peripheral blood and umbilical (cord) blood are also currently used as sources. SCs derived from these sources may have therapeutic potential (without severe adverse effects) only when given to the individual from whom they were derived (autologous transplants) or from an immunologically matched donor (allogeneic transplants) [1].

Despite the fact that the ideal type and source of cells have not yet been defined, immature SCs are capable of colonizing different tissues due to ability of homing and transdifferentiation or lineage–plasticity, in the settings of regenerative medicine. Furthermore, there are several facts suggesting that adult SCs and even differentiated somatic cells, under appropriate microenvironmental cues or signals, are able to be "reprogrammed" and contribute to a much wider spectrum of differentiated progeny than previously anticipated. This has been demonstrated by using tissue-specific SCs—which like embryonic SCs—do not express CD45 as an exclusive hematopoietic marker. Consequently, adult mesenchymal SCs and endothelial precursors seem to be clinically applicable for cell-mediated, regenerative therapy of patients with myocardial, brain, vascular, liver, pancreas, and some other tissue damages.

It is widely accepted that allogeneic transplants are still the most efficient treatment for patients with liver failure and Chronic Myelogenes Leukemia

(CML) [1]. However, there is a lack of donors and some alternative therapeutic approaches are therefore needed. Transplantation of mature hepatocytes has been evaluated, but the long-term efficacy remains unclear and the paucity of donor cells makes this strategy quite limited. The use of SC therapy transplantation is perhaps a more promising alternative approach.

The intensification of myeloablative radiochemotherapy enlarged the use of SC transplantats, as well as the introduction of cell-mediated therapeutic approaches in regenerative medicine resulting in increased needs for both specific blood-derived progenitor/cells, and practical operating procedures inducing minimized cellular damages during their collection or processing and storage in frozen state. Therefore, successful performance of SC transplantats or cell-mediated therapy requires efficient collection, processing, and (cryo) preservation procedures for obtaining an acceptable cell yield and post-thawing recovery, as well as advantageous clinical outcome. For wound healing in the skin, epidermal stem cells and bone marrow progenitor cells both contribute. Thus, it is likely that organ-specific progenitors and hematopoietic stem cells are involved in repair, even for other organ repair. In summary, stem cells could be described as:

- Foundation cells for every organ, tissue and cell in the body
- A "blank microchip" that can ultimately be programmed to perform any number of specialized tasks
- Undifferentiated "blank" cells that do not yet have a specific function
- Self-sustaining and capable of replicating themselves for long periods of time
- Under proper conditions, begin to develop into specialized tissues and organs [1].

These unique characteristics make stem cells very promising potential for supplying cells and tissues instead of organs in a spectrum of devastating diseases from diabetes type1 to stroke, spinal cord injuries, and myocardial infarction [1–7]. In the situation when the number of people needing organ and tissue transplants exceed the number of donated organs and tissues, this is the promise and hope, which deserves a deep and serious consideration. However, despite rapidly growing knowledge on adult stem cell sources, features and use, there are still some fundamental remaining questions regarding them that include: Does only **one common type of stem cell migrate to different organs** and repair tissue or are **there multiple types of stem cells**? Does every organ have stem cells (some of which have not yet been discovered)? Are the stem cells programmed to divide a finite number of times or do they have unlimited cell proliferation capacity? [8–16].

8.2 Organogenesis from Adult Stem Cells and Problems with Different Tissues

How do a small number of stem cells give rise to a complex three dimensional tissue with different types of mature cells in different locations? This is the most fundamental question in organogenesis. The hematopoietic and nervous systems

employ very different strategies for generating diversity from stem cells. The hematopoietic system assiduously avoids regional specialization by stem cells. Hematopoietic stem cells are distributed in different hematopoietic compartments throughout the body during fetal and adult life, and yet these spatially distinct stem cells do not exhibit intrinsic differences in the types of cells they generate. This contrasts with the nervous system, where even small differences in position are associated with the acquisition of different fates by stem cells.

While local environmental differences play an important role in this generation of "neural diversity", we must accept that intrinsic differences between stem cells are also critical. Part of the reason why different types of cells are generated in different regions of the nervous system is that intrinsically different types of stem cells are present in different regions of the nervous system. To understand the molecular basis for the regional patterning of neural stem cell function, we are now studying how these differences are encoded.

8.3 Therapeutic Implications for TCSCs as a New Concept

To prove the stem cells derived from bone marrow (BM) and peripheral blood, including hematopoietic stem cells, are indeed transformed into solid-organ-specific cells, several conditions must be met:

1. The origin of the exogenous cell integrated into solid-organ time must be documented by cell marking, preferably at the single-cell level.
2. Cell should be processed with a minimum of "ex vivo" manipulation (e.g., culturing) which may make them more susceptible to crossing lineages.
3. The exogenous cells must be shown to have become an integral morphologic part of the newly acquired tissue.
4. Transformed cells must be shown to have acquired the function of the particular organ into which it has been integrated both by expressing organ-specific proteins and by showing specific organ function.

Organ/Tissue specific niche (like in BM, liver, etc.)—exists as a deposit (storage) of the adult stem cells in a specific location. These cells are circulating in a very low number in the blood [18]. Accumulating evidence suggests that stem cells may also actively migrate/circulate in the postnatal period of life. Stem cell trafficking/circulation may be one of the crucial mechanisms that maintains the pool of stem cells dispersed in stem cell niches of the same tissue, that are spread throughout different anatomical areas of the body. This phenomenon is very well described for HSC, but other, already tissue committed stem cells (TCSC) (for example, endothelial, skeletal muscle, skeletal or neural stem cells) are probably circulating as well [18].

BM is the home of migrating stem cells with not only hematopoietic stem cells within their niches, but also a small number of TCSC, which might be the reason

why many authors think that HSC may transdifferentiate, although we do not have a direct proof for that. They might have plasticity, but not necessarily the "transdifferentional" potential [18]. What is differentiated in the tissue of injection might be TCSC characteristic for that tissue. It has been shown that number of these cells is decreased with aging (long living and short living mice and humans). It would be interesting to identify genes that are responsible for tissue distribution/expansion of TCSC. These genes could be involved in controlling the life span of the mammals. Therefore, BM stem cells are a heterogeneous population of cells with HSC and TCSC, the morphological and functional characteristics of which are different from HSC. Their number among BM MNC is very low (1 cell per 1000–10,000 BM MNC) within young mammals and might play a role in small injuries [1]. In severe injuries like hart infarct or stroke they have no possibility to reveal their full therapeutic potential. The allocation of these cells to the damaged areas depends on homing signals that maybe inefficient in the presence of some other cytokines or proteolytic enzymes that are released from damaged tissue-associated leukocytes and macrophages [17]. We can envision, for example that metalloproteinases released from inflammatory cells may degrade SDF-1 locally, and thus perturb homing of CXCR4 + TCSC. There is possibility that these cells while "trapped" in BM are still in: "dormant" stage-not fully functional, and need the appropriate activation signals by unknown factors [18]. These cells also, at least in some cases could be attracted to the inflammatory areas, and if not properly incorporated into the damaged tissue they may transform and initiate tumor growth. In summary, between the pools of tissue committed stem cells, there are probably those already committed to transdifferentiate into neural cells, or cells of tissues and organs other than neural, but we still do not have the control over their tracking, homing and finally regenerative capacity in the given tissue, which is a fundamental prerequisite for successful regenerative therapy [12–17].

8.4 The Concept of VSEL

In a discovery that has the potential to change the face of stem cell research, a University of Louisville scientist has identified cells in the adult body that seem to behave like embryonic stem cells [18–29]. The cells, drawn from adult bone marrow, look like embryonic stem cells and appear to mimic their ability to multiply and develop into other kinds of cells. The finding, presented the first time at the 47th Annual Meeting of the American Society of Hematology (ASH) in Atlanta, was announced December 12 at the society's news conference. A study by Ratajczak's team published in 2005 year in the journal "Leukemia" was the first to identify a type of stem cell in adult bone marrow that acts differently than other marrow stem cells (18). The newly identified cells, called "very small embryonic-like" (VSEL) stem cells, have the same ultrastructure and protein markers as embryonic stem cells [31–40]. Ratajczak and several other researchers

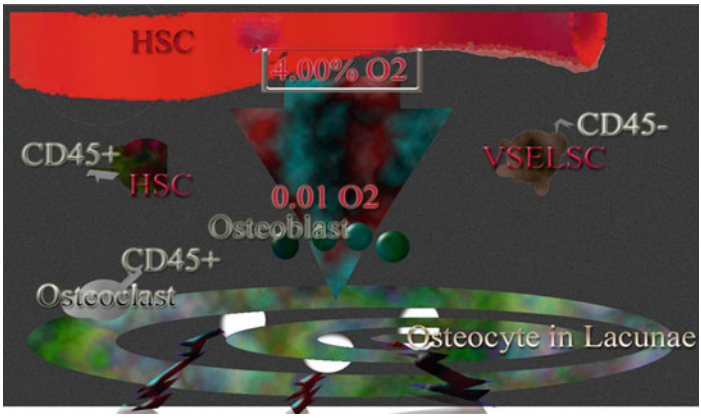


Fig. 8.1 Differences in phenotypes (external and internal markers) between HSC and VSEL from mouse bone marrow

from University of Louisville in the presentation at the ASH meeting showed that VSEL stem cells mobilize into the bloodstream **to help repair damaged tissue following a stroke** [30]. In further research advance, Ratajczak's team also has grown VSEL cells in a lab and has stimulated them to change into nerve, heart and pancreas cells [30]. The difference in markers between HSC and VSELs in mouse are shown in (Figs. 8.1 and 8.3), while the differences in ultrastructure are shown in (Fig. 8.2).

Along with this new concept, there is a premise that in regenerative therapy done before, with hematopoietic stem cells (considered to have plasticity and multipotency) the VSELs were "contaminants" that actually contributed to positive regenerative clinical outcome, since they have those capabilities [18]. This is an interesting concept which should be seriously considered in humans.

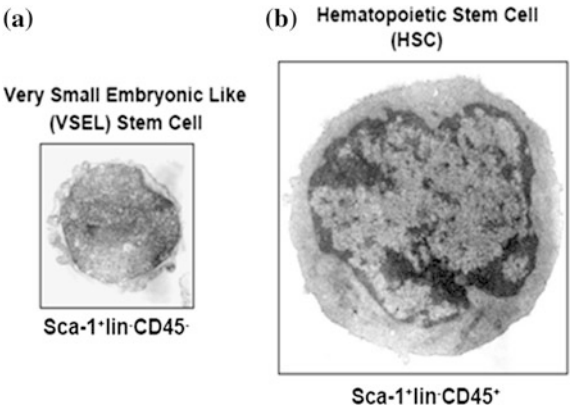


Fig. 8.2 TEM comparing morphological features of VSEL and HSC. With kindness of Dr. Ratajczak

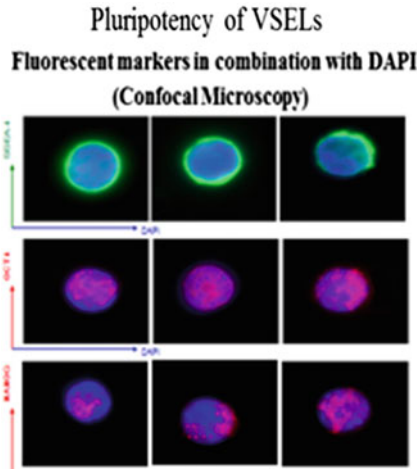


Fig. 8.3 Fluorescent label of Hallmarks of pluripotency in human VSELs intranuclear region. With kindness of Dr. Ratajczak

Thus, since VSELs have been found in human cord blood and bone marrow, they seem to be of a critical importance for consideration of stem cell transplant choice based upon the phenotype and number of stem cells aimed to be transplanted within a given clinical scenario. Despite conflicting data about this population [43–45], they are getting more confirmation in scientific community [31–40]. These cells have a great potential and like induced stem cells, can potentially eliminate the need for embryonic stem cells given that in adult organism they have all necessary components (parameters) that embryonic cells have, with a highest potency for lineage differentiation [41, 42].

1. Morphological studies have discovered that VSELs are unusually small (3–4 μm) eukaryotic cells which do possess several features of embryonic cells. Thus, the strategy based on FACS sorting of these cells should consider whether other adult tissues have those primitive little cells bigger than thrombocytes but smaller than erythrocytes [40–45].
2. These cells also express high nucleo/cytoplazmatic ratio and smaller cytoplazmatic region compared to HSCs and mature granulocytes. Beside the fact that it has confirmed the features such as: size, confocal microscopy has also confirmed that VSELs express Oct-4, a hallmark of pluripotency of embryonic stem cells. In sum, morphological studies have discovered that VSELs are unusually small eucariotic cells with several fundamental features of embryonic stem cells except tumorigenicity (pluripotency, sphere formation, embryonic bodies and small size) [28].

3. These cells in a suitable medium perpetuate self-renewal longer, without "jumping" into differentiation, while on the other side they are capable of differentiating into bigger number of cell types in a suitable/conditioned medium into most of the cell (pancreatic cells, neural cells, cells of heart muscle and liver) which makes them suitable for expansion and reparative and regenerative purposes [28–30].
4. VSEL cells are, accordingly, a unique and distinguished entity rather than state with the features of plasticity, that questions plasticity of HSCs, suggesting strongly that that particular feature of BM stem cells could be in essence artifact caused by contamination of VSELs. Finally, the discovery of VSELs in the CB, PB and BM of humans indicate their significance with respect to other features. Some other researchers before Ratajczak have not succeeded to completely isolate this fraction [46–48], probably due to bad technique of isolation and timing. More extensive and deeper studies in the future will show what is true and possible.
5. Key advantages associated with VSELs, seem to be that they avoid the ethical or moral dilemmas associated with the use of embryonic and fetal cells, the potential negative biological effects associated with ESCLs such as their propensity for tumor formation, and the use of autologous stem cells to avoid immune rejection.
6. The studies on mouse model suggest necessity for the human studies on VSELs since it would be of great interest to check if these intriguing population of stem cells are also involved in caloric intake, longevity and regenerative features of this distinctive stem cell entity [37–39]. While this paper was prepared for print a recent report from Ratajczak's group in the form of Editorial, explained many aspects of conflicting data in VSELs history in a very professional way strongly suggesting that VSELs are rather detectible entity than the state of stem cell (Table 8.1) [46–53].

Table 8.1 VSELs: pros and cons with respect to different findings

Parameters of VSELs Necessary to detect in order to be able to consider their pluripotent function	Authors: Cons Dulak, May 2013 Weissman, August, 2013	Authors: Pros Kassmer et al. [31-33], Bhartya et al. [34-39], Wang and Guo et al. [40, 41], Wojakowski [41], Chang et al. [42], Havens et al. [69]
DNA amount	Little	Abundant
Formation of spheres	No	Yes
Octapeptide-4 expression	No	Yes
Differentiation into other lineages/blood cells	No	Into epithelial cells and cardiac cells, multipotent tissue progenitors in vitro and in vivo

8.5 The Concept of Mesenchymal Stem Cell (MSC) with Dental Pulp Cells (DPSCs) as an Example

Many human tissues are the source of stem cells responsible for tissue development and regeneration. Beside BM (***b**one **m**arrow **s**tromal **s**tem **c**ells*, BMSCs), currently it is considered that dental pulp is practically the most approachable and the most important source of adult mesenchymal stem cells [49–54]. Within the last eight years, several populations of stem cells from dental pulp were isolated and characterized: (1) (***d**ental **p**ulp **s**tem **c**ells*-DPSCs), (2) (*stem cells from **h**uman **e**xfoliated **d**ecidual teeth*, SHEDs) and (3) (***i**mmature **d**ental **p**ulp **c**ells*, IDPCs) [51–54]. These cells are of the ectomesenchymal origin, located in perivascular niche, highly proliferative, clonogenic, multipotent and similar to BMSCs.

In in vitro conditions, they can differentiate with certain intercellular differences toward odontoblasts, hondrocytes, osteoblasts, adipocytes, neurons/glial cells, smooth and skeletal muscle cells. In in vivo conditions, after implantation, they show different potential for dentine formation, as well as osteogenesis; after transplantation in mouse with compromised immune system, they make good grafts in different tissues and are capable of migrating into the brain, where they survive a certain time while reaching neurogenic phenotype. DPSCs have immunomodulatory effect, as they can be involved into immune response during infection of dental pulpe by NF-kB activation, and by inhibiting T-lymphocyte proliferation, suggesting their immunosuppressive effect [51–54]. The future research should give us the complex data on the molecular and functional characteristics of dental pulp stem cells, as well as differences between different populations of these cells. Such research would fundamentally contribute to the better knowledge on the dental pulp stem cells, which is necessary due to their potential clinical application in in vivo cell transplantation, tissue engineering, and gene therapy (in vivo and ex vivo). Actually, by the isolation of IDPCs, which are the most primitive, but also the most plastic, (similar to embryonic stem cells), they are opening the new perspectives in a potential therapeutic application of these cells not only in regeneration of dentine, but also the regeneration of periodontal tissue and bone-junctional tissue of craniofacial region, as well as in the therapy of neurotrauma, myocardial infarction and connective tissue damage (Figs. 8.4 and 8.5).

However, the shift of the logic and turning of the sense, entitling phenotypically defined populations as stem cells (although only some of them within that “cluster” are stem cells indeed), have introduced so much confusion into this discipline, that it is very difficult to perform corrections nowadays. It is at the same time the reason why many discoveries that enable stem cell therapy on the rodents do not work on humans. One has to be very critical with respect to stem cell markers and its functional properties in order not to make a mistake in stem cell therapy (Fig. 8.6).

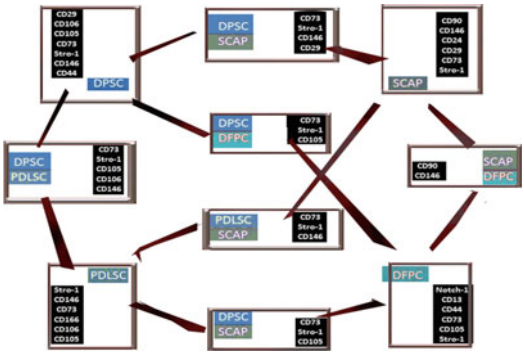
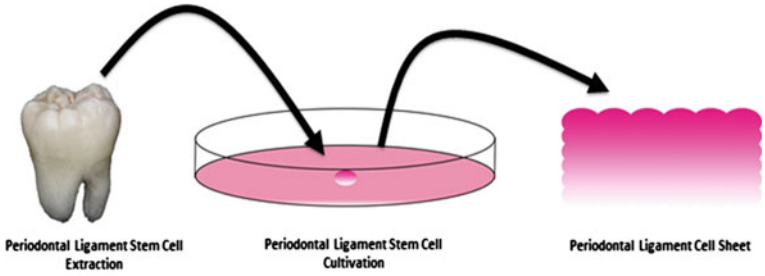


Fig. 8.4 The most important superficial cellular markers of dental pulp stem cell according to DPSC—Dental Pulp Stem Cell; DFPC—Dental Follicular Precursor Cell; SCAP—Stem Cell of Apical Papilla; PDLSC—Periodontal Ligament Stem Cell, Morsczeck et al., Clin Oral Invest 2008; 12:113–118 (19)



DPSC-Dental Pulp Stem Cell; DFPC-Dental Follicular Precursor Cell; SCAP-Stem Cell of Apical Papilla; PDLSC-Periodontal Ligament Stem Cell

Fig. 25. The most important superficial cellular markers of dental pulp stem cell *according to Morsczeck et al., Clin Oral Invest 2008; 12:113-118 (19)*

Fig. 8.5 The most important superficial cellular markers of dental pulp stem cell according to DPSC—Dental Pulp Stem Cell; DFPC—Dental Follicular Precursor Cell; SCAP—Stem Cell of Apical Papilla; PDLSC—Periodontal Ligament Stem Cell, Morsczeck et al., Clin Oral Invest 2008; 12:113–118 (19)

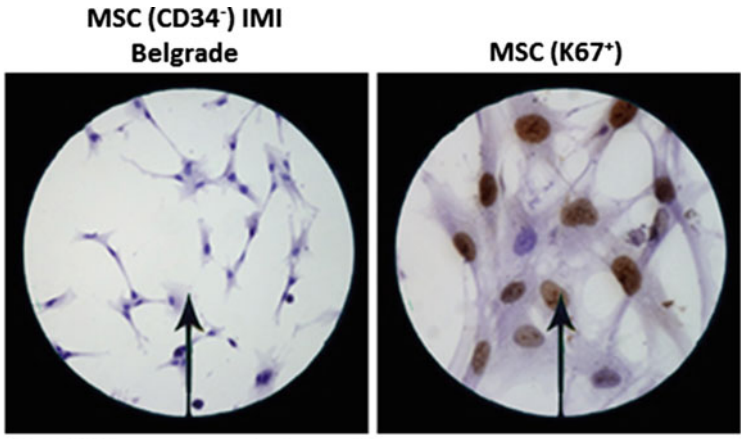


Fig. 8.6 Mesenchymal stem cells (MSC)

8.6 Mobilization as a New Noninvasive Therapeutic Concept

The classification of patients into "good" or "poor" mobilizers is based on CD34+ cell count in their peripheral blood (PB) after granulocyte-colony-stimulating factor (G-CSF) injection. CD34+ cells mobilized into peripheral blood (PB) are considered a more convenient source of hematopoietic stem and progenitor cells than their bone marrow (BM) counterparts, in autologous transplantation protocols. Besides going through a less invasive collection procedure than BM aspiration, leukapheresed CD34+ cell collections ensure a rapid hematologic recovery as a function of transplanted dose of these cells, and their cell cycle status. Patients unable to mobilize sufficient number of CD34+ cells for efficient transplantation procedure are designated as poor mobilizers. Whereas numerous studies were dedicated to defining predictive factors for successful mobilization, 3 only a few characterized the phenotype of mobilized CD34+ in good versus poor mobilizers 4,5 and none explored the functional and metabolic properties of mobilized cells in these two groups of patients. Thus, Ivanovic et al. (2009) hypothesized that, apart from their mobilization from marrow to the blood, the response to G-CSF of CD34+ cells also includes activation of proliferation, metabolic activity, and proliferative capacity. In this study, mobilized PB CD34+ cells purified from samples obtained by cytapheresis of multiple myeloma or non-Hodgkin's lymphoma patients of both good (>50 CD34+ cells/mL) and poor (50 CD34+ cells/mL) mobilizers, were studied [55]. The initial cell cycle state of CD34+ cells after selection and their kinetics of activation (exit

from G0 phase) during ex vivo culture were analysed. Their proliferative capacity was estimated on the basis of ex vivo generatio of total cells, CD34+ cells, and colony-forming cells (CFCs), in a standardized expansion culture. Indirect insight in metabolic activity was obtained on the basis of their survival (viability and apoptosis follow-up) during the 7-day-long conservation in hypothermia (4 °C) in the air or in atmosphere containing 3% O_2/6% CO_2. The results have shown that CD34+ cells obtained from good mobilizers were in lower proportion in the G0 phase, their activation in a cytokine-stimulated culture was accelerated, and they exhibited a lower ex vivo expansion efficiency than those from poor mobilizers. The resistance to hypothermia of good mobilizers' CD34+ cells is impaired. The inevitable conclusion was that a good response to G-CSF mobilization treatment is associated with a higher degree of proliferative and metabolic activation of mobilized CD34+ cells with a decrease in their expansion capacity [56].

8.7 New Concepts in Adult Stem Cell Research with Development of New Strategies: Personal Experience in the Light of Significance of Growing Information

8.7.1 Background and Significance

Edward Thomas developed bone marrow transplantation as a treatment for leukemia. Initially the process was successful only if the donor was an identical twin of the patient. With the development of immunosuppressant drugs to counter organ rejection now many patients are treated for leukemia, aplastic anemia, sickle cell anemia, hurlers syndrome, severe combined immunodeficiency (SCID) and *Wiskott–Aldrich syndrome* as a result of his development in bone marrow transplantation. Dr. Edward Thomas was also awarded the *National Medal of Science 1990*. The primary role of adult stem cells in a living organism is to maintain and repair the tissue in which they reside. As an adult, stem cell is an undifferentiated cell found among differentiated cells in a tissue or organ. It can renew itself, and differentiate to yield the major specialized cell types of the tissue or organ. Within past ten years tremendous piece of work has been done with regard to development of the concepts of "*stemness*", primitive stem cell patterns used in regenerative purposes, and concept of *cancer stem cells*, with significant impact on the development of *new strategies* for their detection and targeted intervention. Despite deep skepticism and arguments these three concepts have their basis in scientific approaches and facts, researched and detected in order to support them. Results obtained are already empowering them to "step" into clinical arena [57–69].

8.8 Directions and Relevant Studies: We and Others

What is "stemness? Stemness has so far been defined as both phenotypically and functionally recognizable cell pattern capable of self-renewal, proliferation and transdifferentiation through the phenomenon of plasticity [1, 70–74].

One has to be aware of the fact that stem cell category, as an elementary term is assuming the particular functionality. As the entity, or the state, it rationally presents the cell which of its all possible functions possesses at the moment of stemness only those that allow it to survive and sometimes divide: all other functions of this cell are at the potential level. When those possible functions really come up into scenario, that cell is not stem cell anymore. That is why the collections and clusters of different antigens expressed all over the cells in different developmental stages of different tissues (such as kit-receptor, CD117) cannot be the stem cell markers (Fig. 8.7).

The "**stemness**" is the status in which only the oldest, the most primitive part of the genome is activated "with the only purpose to save what is stored in the nucleus of stem cell: genetic information, e.g., potential [1]. The purpose of this event is to save the cell of death and (if it comes to the stimulus for differentiation by asymmetric division) from self-renewal [1]. In that way we are becoming aware that the nature does create the standards that we should rather understand, instead of forcing the nature into our simplified concepts, some of which are very superficial. Tremendous advance which has enabled enrichment of stem cells based upon selection using phenotype as a standard could be appreciated as the advance in this discipline. It has also enabled more direct approach to investigation of stem cells.

However, there are other explanations for this status and one of them was defined by Dov Zipori [70–72]. According to him, this feature is not stem cell specific, given the fact that it is unacquired. Most importantly, according to Zipori,

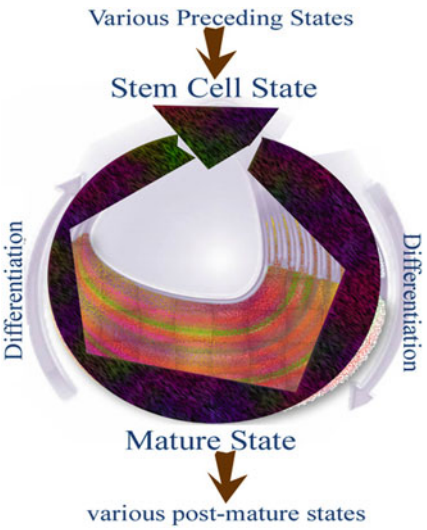


Fig. 8.7 Stem cell maturation according to D. Zipori. Med Sci (Paris) 2011; 27: 303–301

"stemness" is a *transient trait* and cannot be predicted on the basis of momentary gene expression patterns (Fig. 8.7) [73].

We have started optimization of the primitive stem cell pool in the case of acute myocardial infarction with intention to discriminate possible contamination with very small embryonic—like cells (VSELs) within hematopoietic stem cell (HSCs) pool and determine which subpopulation is the best for regenerative purposes.

8.9 Optimization of Primitive Stem Cell Patterns for Regeneration and Repair

Optimization of Primitive Stem Cell Patterns for Regeneration and Repair Has Today at Least Three Strong Candidates

- **HSCs (hematopoietic stem cells)**
- **VSELs (very small embryonic-like stem cells)**
- **MSCs (mesenchymal stem cells)**.

The concept of plasticity have been revised by Ratajczak's group which has recently developed and together with us supported the concept of very small embryonic-like cells (VSELs), shown to be stem cells in bone marrow and other organs in non-hematopoietic compartment, committed to differentiate into some other tissues. These cells can be detected in mobilized bone marrow cells of mice and humans using cell sorter (Fig. 8.8). However, we have also shown that not all the patients must be good mobilizers, which require alternative approach [56]. Therefore, exploring the possibility of using adult stem cells for cell-based therapies has become a very expanding area of investigation [74–83].

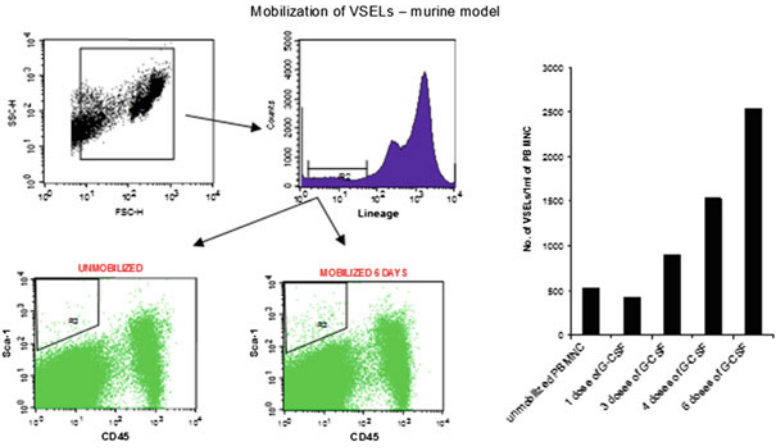


Fig. 8.8 Mobilization of VSELs in murine model by use of G-CSF (Neupogen) in mice and expression of critical markers in mobilized and unmobilized animals. Obtained by kindness of Dr. M. Ratajczak

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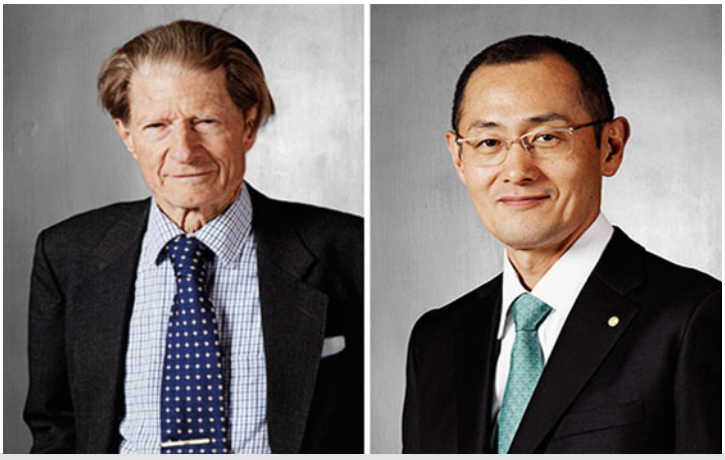
Chapter 9
Induced Pluripotent Stem Cells (iPSCs) and Nuclear Reprogramming

With Alisson Degroat

The saddest aspect of life right now is that science gathers knowledge faster than society gathers wisdom.

Isaac Asimov

Abstract The toti—and pluripotency ascribed to ESC were considered the unique qualities of these cells making them the pattern of choice for regenerative and reparative purposes. However, John Gurdon and Shinya Yamanaka, have discovered that nucleus of somatic adult cells can be reprogrammed to lead the cells into their pluripotency [1–3]. The methods of reprogramming, with focus on the use of nanoparticles, the features of i [1–3] PCs and their significance for clinical application are described and discussed in this chapter.



Breakthrough in stem cell research: John Gurdon (UK) (1933–) and Shinya Yamanaka (Japan) (1963) have won the NP in 2012 for the discovery that mature cells can be reprogrammed to become pluripotent

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9.1 Breakthrough: Induced Pluripotent Stem Cells (iPSC)

The Yamanaka lab identified four factors that, when co-transfected and expressed in mouse adult fibroblast cells, caused those fibroblasts to revert back to a pluripotent like state [1, 2]. One year later, the same four factors were used to successfully reprogram human adult fibroblast cells into induced pluripotent stem cells [1, 2]. These four factors are:

Octamer-4 (Oct-4) encoded by the gene POU5F1 is a transcription factor that is highly expressed in undifferentiated embryonic stem cells compared to other somatic cells. Oct-4 expression in embryonic stem cells is critical to maintain them in an undifferentiated, pluripotent state. In fact, if Oct-4 expression is experimentally knocked out, this causes embryonic stem cells to spontaneously differentiate [1, 2].

SOX2 is a transcription factor critical for the maintenance of pluripotency in embryonic stem cells. SOX2 and Oct-4 work in parallel to co-regulate expression of target genes involved in the maintenance of pluripotency [1, 2].

c-Myc is a well known proto–oncogene. The c-Myc gene codes for a transcription factor that regulates the expression of many genes involved in the control of cell proliferation, growth, differentiation and apoptosis. Aberrant expression of c-Myc on the other hand is associated with tumor formation and cancer. Recent studies have demonstrated that c-Myc is a dispensable reprogramming factor; however, the transcription factor has been shown to greatly improve reprogramming efficiency [1, 4].

Kruppel-like factor 4 (Klf-4) is a transcription factor that is highly expressed in undifferentiated ES cells and is also expressed elsewhere in the adult organism including the gut, testis and lungs and functions to regulate proliferation, differentiation and cell survival [1, 2].

9.1.1 Reprogramming as a Therapeutic Event

John Gurdon shared the NP with YamanaKa since he was already the best known for his pioneering research in nuclear transplantation and cloning [5–7]. Recent data have shown the use of reprogramming technologies to cause cancer cells to lose tumorigenicity in chronic myeloid leukemia cells, melanoma cells, and gastrointestinal cancer cells [8–10]. These results suggest that nuclear reprogramming may be a therapeutic strategy for the treatment of cancer [9, 11–14]. However, these experiments have also revealed that reprogramming technology is not very efficient. Experiments suggest that cancer cells are resistant to reprogramming and this resistance might be related to the role of epigenetic regulations during reprogramming.

The fact that transformation of iPSCs is accomplished by erasing the epigenetic modification similar to those found in early embryos demonstrates the significance of epigenetic changes for successful reprogramming, and thus, its role in carcinogenesis [15, 16].

The field of nanotechnology is rapidly expanding introducing a fundamental breakthrough in bioengineering. On the other hand, a great potential of induced pluripotent stem cells (iPSC) from somatic cells of the patient makes them very attractive for adult stem cell therapy over embryonic stem cell therapy (ESC) with a possibility to completely eliminate the need for ESCs in cellular approaches. However, there is a reasonable concern that this potential therapeutic benefit is questionable due to unwanted features of these cells to cause tumors in some cases. Therefore, there is a need for iPSCs which will be safe in that aspect. What causes tumors in cellular therapy with iPSCs? Are the methods for induction causative factor or the methods that prepare iPSCs for therapeutic event and convey it?

The methodology of EPIGENETIC REPROGRAMMING OF SOMATIC CELLS INTO A PLURIPOTENT STATE is very complex and multidisciplinary oriented [16–20]. There are at least three basic methods to perform this in order to prepare somatic cell to perform induced immature, stem cell functions: *NSCT* (*cloning*, transfer of the nucleus of somatic cell into enucleated ovarian cells, *cell fusion*, *direct reprogramming* induced pluripotency, constitution of iPSCs).

Nuclear Transfer. Diploid nucleus of somatic cell (2n) is transplanted into enucleated oocyte. In the environment of the oocyte's cytoplasm, nucleus of the somatic cell is reprogrammed, so that the cells arising from it will be pluripotent. Actually, from such an oocyte transplanted nucleus, a blastocyst is formed from which is inside cellular mass extracted and laid onto the scaffold and that is how the ESC are formed. If you continue with the development of blastocyst you can get cloned organism.

Cell fusion. In this approach, two different types of cells are combined, with formation of new entity. The result of cell fusion is the development of HETEROCARYON, or HYBRID. If the fused cells proliferate, they will give rise to hybrids, and through their division fused nuclear becoming 4n (doubled number of chromosomes of somatic cells) or more. If the cells for fusion are extracted from the same organism, karyotype of the fused cell can remain euploid (due to the balancing of the set of chromosomes): however if the cells originate from different organisms, they can be aneuploid, due to chromosome loss and their re-arrangement. On the contrary, heterocaryons have a short life and do not divide. Due to that they are multinucleated: –nucleuses from original cells remain intact and specific and therefore it is possible to investigate the influence of one genotype upon the other in stable systems which do not lack chromosomes. If the education is created through mixing of varieties, the genetic product of two cell varieties can be different. Disturbances in nuclear ratio during the fusion, and due to existence of stoichiometric regulation, for which is responsible each type of the cell, heterocaryon is being reprogrammed in a direction of emergence of new type of the cell. However, the conditions of cultivation also have

influence and it is necessary that medium for cultivation has the content which favorizes the emergence of the determined type of cells.

Transfer of *transcription factors*. This method is used to create the induced pluripotent stem (iPSCs), with similar characteristics to ESCs, and can derive from any cell in the body by induction of the expression of activity of 4 genes (Oct4, Sox2, Klf4 and c-Myc) using retroviruses. The pluripotent state is possible to maintain through inheritance and the biggest number of cells designed through this approach is applicable in clinical arena.

9.2 Induced Pluripotent Stem Cells (iPSCs) and Nanotechnology: State of the Art

Induced pluripotent stem cells were created as the demand rose to find a comparable counterpart to the controversial embryonic stem cells. Unlike embryonic stem cells which are derived from the inner cell mass of mammalian blastocysts, iPSCs originate from somatic cells and are driven to express pluripotency through nuclear reprogramming. iPSCs have the capacity to revolutionize personalized regenerative therapies by offering many of the same benefits as embryonic stems cells but without immune rejection the ethical stigma. Just as embryonic stem cells have the capabilities to proliferate indefinitely under appropriate in vitro conditions and to differentiate into any cell type of all three germ layers, iPSCS possess these same qualities. Since this technology exploded onto the scene in 2006 with its discovery by Yamanaka and Takahashi, there has been steady progress to improve upon the efficacy and safety of the reprogramming techniques [20]. The original reprogramming protocol comprised of a set of four key transcription factors Oct4, Sox2, Klf4 and c-Myc (OSKM). However, this technique and many others for iPSC generation create harmful mutations by integrating viral vectors into the genome and can lead to the transcription of unwanted genes. This undesirable side effect greatly increases the risk of tumors. To help abate this effect, the expression of the four transgenes must be silenced after reprogramming. c-Myc, a tumor promoting gene, especially must be silenced after programming or the risk of tumor development becomes too great for clinical use. Additionally, reprogramming has been attempted with microRNAs, environmental stress stimulation, small molecule compounds and reprogramming proteins. Although iPSCs can be obtained through nonintegrative adenoviral delivery of OSKM, the efficiency of reprogramming is extremely low. Here we will report the successful creation of iPSCs utilizing different nanoparticle based techniques, which serve as nonviral transfection vectors. The last review on this matter (2014) has elucidated the actual use of nanoparticles in iPSC driven tissue engineering and regenerative medicine but failed to give a detailed account of their use in reprogramming and their mechanism of action [21]. Therefore, we are focused in this particular review on that aspect of iPSCs.

9.3 Nanoparticles for Genetic Reprogramming of Somatic Cells to iPSCs

The era of nanoparticle based therapies is underway, and it's potential to carve innovative pathways in the health care arena appears limitless. Currently, nanoparticles have applications in drug delivery and therapies against cancer, neurodegenerative diseases, and many others [22–29]. In the heart of nanotechnology are geometry and minimization. In Nanotechnology, a "nanoparticle" is any material that consists of discrete entities with one, two or three dimensions of the order of 100 nm or less [23]. There is a diverse population of nanoparticles that have been discovered, including polymeric nanoparticles, lipid-based nanoparticles and mesoporous silica nanoparticles. Nanoparticles possess several unique properties that make them a particularly valuable tool in regenerative medicine research. They are inert with lower toxicity and have high surface area to volume ratio due to their nanoscale. Nanoparticles are valuable for in vivo and in vitro biomedical applications because their size is similar to most biological molecules such as lipids, proteins, nucleic acids, hormones, metabolites etc. Although nanoparticles have been extensively investigated in drug delivery systems, this technology is still relatively overlooked in the field of stem cell biology and cell reprogramming. A recent breakthrough in the use of nanoparticles in the study of iPSCs has spurred interest in this new application.

Poly(amidoamine) (PAMAM) dendrimers are a promising tool for nonviral targeted gene delivery due to their exceptional biocompatibility, non-immunogenicity, water-solubility, unique multivalency and well-defined radial and symmetrical structure [29]. This class of nanoparticles has primary surface amines, which promote the attachment of many types of negatively charged molecules, including gene segments. These terminal amine groups facilitate cell penetration, gene loading, and endosomal escape during transfection, resulting in successful gene transfection [24]. Arginine residues are added to the nanoparticle to develop a cost efficient, low generation PAMAM that still retains the high membrane penetration efficiency as its high generation counterpart. Consequently, it has been shown that vectors complexed with arginine-rich motifs enhanced cellular uptake and gene delivery [19]. Recently, Zhu et al. [24] created a nonviral gene delivery system in which a G4Arg nanoparticle was developed for delivery of a single plasmid construct carrying OSKM (pOSKM) into mouse embryonic fibroblasts (MEFs) to induce pluripotency. G4Arg is an arginine-terminated generation 4 poly(amidoamine) (PAMAM) nanoparticle which was found to effectively deliver pOSKM into MEFs. Using their transfection conditions, G4Arg nanoparticle transfected pOSKMG at an efficiency of $14.25 \pm 2.11\%$, which was much higher than that of the commercial transfection vectors Lipofectamine 2000 ($5.81 \pm 0.84\%$) and FuGENE HD ($8.02 \pm 0.71\%$) ($P < 0.05$). It is assumed that one of the mechanisms associated with cellular uptake of nanosized particles is endocytosis that is limited to a positive

zeta potential, and size ranges of approximately 20–200 nm in diameter. Thus, G4Arg-DNA complexes exhibited a suitable zeta potential (26.4 ± 1.2 mV) and particle size (168.4 ± 20.1 nm). These properties can greatly increase the possibility of endocytosis-mediated cellular uptake, leading to relatively higher transfection efficiency. They also reported that G4Arg nanoparticle-based transfection resulted in low toxicity (>80% cell viability). Additionally, it was observed that iPSCs were capable of differentiating into derivatives of the three germ layers in immunodeficient mice. Zhu concluded that G4Arg nanoparticles are a safe and effective delivery system for pOSKM and provide great potential for generating virus-free iPSCs. This protocol serves as an effective and biocompatible approach to deliver reprogramming transcription factors without the time consuming, labor intensive and potentially harmful factors of viral systems.

9.3.1 Poly(Beta-Amino Ester) Nanoparticle-Based Non-viral Protocol

Poly(beta-amino esters) (PBAEs) are a novel class of polymeric nanoparticles that can bind DNA, promote cellular uptake, facilitate endosomal escape, and allow DNA to enter the cytoplasm and subsequently the nucleus [25]. PBAEs are synthesized via Michael addition reactions between compounds containing diacrylates and primary or secondary amines [30, 31]. Bhise et al. [25] investigated the use of a biodegradable poly(beta-amino ester) nanoparticles for reprogramming human fibroblasts to iPSCs. They compared their approach with an electroporation-based method to deliver episomal plasmids encoding reprogramming factors to induce pluripotency in human fibroblasts. The study determined that nanoparticles formed by self-assembly of 1-(3-aminopropyl)-4-methylpiperazine end-terminated poly (1,4-butanediol diacrylate-co-4-amino-1-butanol) polymer (B4S4E7) with episomal plasmid DNA are more effective than Lipofectamine 2000, FuGENE HD, and 25 kDa branched polyethylenimine (all leading commercial reagents) for nonviral gene transfer to IMR-90 human primary fibroblasts and dermal fibroblasts derived from a patient with retinitis pigmentosa. However, they concluded that certain nonviral reprogramming methods may not necessarily be safer than viral approaches. They observed that although iPSCs derived from both methods stained positively for the pluripotency markers Tra-1-60, SSEA4, and Oct4, the nanoparticle based reprogramming method took longer and generated EP2-iPSC like cells with gross karyotypic abnormalities, whereas the electroporation method generated EP1-iPSC-like cells with a normal karyotype. Therefore, it was determined that there may be modifications to their approach that will be more amenable to successful generation of human iPSCs.

9.3.2 Calcium Phosphate Nanoparticle Based Non-viral Protocol

Recently, there has been an emergence of studies exploring nano-structured calcium phosphates in a multitude of applications, including bioactive coating for implants, biomimetic remineralization, fluorescent labels and non-viral vectors for gene and drug delivery [32]. Calcium phosphate nanoparticles are one of the most commonly used inorganic materials in biotechnology due to several innate properties which drive gene delivery [33]. Calcium phosphate has excellent biocompatibility as it is already ubiquitous in the body in the form of amorphous calcium phosphate and crystalline hydroxyapatite. It can be found in high concentrations in all vertebrates yet remain benign and nontoxic. Additionally, calcium phosphate solubility in the body is variable due to kidney regulation. Its natural occurrence in the human body qualifies it as a superior source over other synthetic delivery systems. The calcium phosphate transfection system utilizes divalent metal cations, such as Ca^{2+}, Mn^{2+}, Mg^{2+}, forming ionic complexes with the phosphates attached to DNA. Cao et al. [26] generated iPSCs from human umbilical cord mesenchymal stem cells (HUMSCSs) by co-delivery of the four plasmids OSKM with calcium phosphate nanoparticles. Utilizing the calcium phosphate nanoparticle system they were able to achieve a reprogramming efficiency of 0.049%. In addition to expressing positive pluripotency markers, the iPSCs were capable of differentiating into cells from all three germ layers in vitro. Also, immunocompromised mice that received subcutaneous injection of the iPSCs revealed the formation of teratomas containing a multitude of tissues from all three germ layers. Cao and company proved that plasmid-encapsulated calcium phosphate nanoparticles can indeed serve as a safe, simple and efficient alternative to the viral-based iPSC generation protocol.

9.3.3 Polyketal Nanoparticle-Mediated Non-viral, Non-genetic Approach for Delivery of Mature MicroRNAs

Nanoparticles fabricated form polyketals present an exciting transfection platform as they degrade into the neutral, well-tolerated compounds acetone and cyclohexanedimethanol which avoids the inflammatory problems linked to polyester-based materials. Formulating polyketals into nanoparticles allows for the delivery of molecules of a range of sizes. Sohn et al. [27] induced pluripotency in bone marrow mononuclear cells via polyketal nanoparticle-mediated delivery of mature microRNAs. Although many non-viral approaches including microRNA, mRNA, small molecule transduction and protein have been developed to generate iPSCs from human and mouse fibroblasts, there were no techniques which utilized bone

marrow (BM)-derived hematopoietic cells. It is extremely desirable to reprogram from hematopoietic-lineage cells as these can be from patients and are easily accessible. This group created microRNA-loaded nanoparticles using the acid sensitive, rapidly hydrolyzing polyketal polymer PK3 (PK3-miR) and delivered them to somatic cells. These particles were made by ion-pairing the miRNA with the positively-charged carrier DOTAP. Ion-pairing with this technique was efficient and the conjugate was easily extracted to the organic layer for use in a hydrophobic nanoparticle. Encapsulation of miRNAs was efficient and the particles contained high levels of miRNAs for transduction. The miRNA was rapidly released from acidic pH of 5.0, but not in the neutral 7.0. It was confirmed that macrophages rapidly internalize the nanoparticles and release their contents within the cells. After 6 days isolated colonies expressed substantial positive pluripotency markers including Oct4, Sox2 and Nanog. Furthermore, colonies that were transferred to feeder layers also stained positive for pluripotency markers, including SSEA-1. Sohn successfully demonstrated the activation of pluripotency-associated genes in mouse BM-mononuclear cells utilizing embryonic stem cell (ESM)-specific microRNAs encapsulated in the acid sensitive polyketal PK3. They proved that reprogramming somatic cells to iPSCs without permanent genetic manipulation in an efficient manner is possible through a polyketal-microRNA delivery vehicle.

9.3.4 Non-viral Magnetic Nanoparticle Based Transfection

Magnet-based nanofection (magnetofection) is an innovative and highly effective transfection method centered on the use of magnetic nanoparticles and a magnet [34]. Magnetofection employs a magnetic field to concentrate particles containing nucleic acid into the target cell to promote transfection. The cellular uptake of the genetic material is accomplished by endocytosis and pinocytosis. Lee et al. [28] generated iPSC lines from mouse embryonic cells (MEFs) using a non-viral magnetic nanoparticle-based transection method that utilizes biodegradable cationic polymer polyethyleneimine (PEI)-coated super paramagnetic nanoparticles (tsMAG-PEI, PolyMag). The PolyMag nanoparticles have a core size of 10–20 nm with a smooth, spherical morphology and are made of iron oxide, which is fully biodegradable. These nanoparticles were complexed to plasmid DNA using free PEI and the complex was exposed to a magnetic force that guides the gene vectors for all nucleic acid transfection towards the target cells. Lee achieved successful transfection of MEFs cells using the magnetofection method and found that reprogrammed iPSCs have typical embryonic stem cell characteristics of self-renewal and pluripotency. This method obtained a reprogramming efficiency of 0.001–0.003%. Interestingly, they found that more than 60% of the iPSC s produced were not integrated with exogenous plasmid DNAs, which suggests that their method is a simple and efficient way to generate exogenous DNA-free safe iPSC

lines. The general utility of the magnet-based nanofection for iPSC generation is tested on mouse bone marrow stromal cells (MSC). Although they do not establish successfully efficient MSC derived iPSCs, their magnet-based nanofection produced some iPSC like colonies in MSC. Additionally, they confirmed the differentiation potential of their iPSC lines into three germ layers both in vitro and in vivo. They noted however, that it should be further investigated whether or not the iPSCs are as consistent or efficient as embryonic stem cells at becoming brain, pancreas, heart, kidney and other types of cells.

9.3.5 The Impact of iPSC upon the Research and Practical Application

The recent availability of human cardiomyocytes derived from induced pluripotent stem (iPS) cells opens new opportunities to build in vitro **models of cardiac disease**, screening for new drugs, and patient-specific cardiac therapy. **Notably, the use of iPS cells enables studies in the wide pool of genotypes and phenotypes**. The progress in reprogramming of induced pluripotent stem (iPS) cells towards the cardiac lineage/differentiation is going on. The focus is on challenges of cardiac disease modeling using iPS cells and their potential to produce safe, effective and affordable therapies/applications with the emphasis on cardiac tissue engineering. The researchers emphasize implications of human iPS cells to biological research and some of the future needs [17, 35].

iPSCS have the potential to become multipurpose research and clinical tools to understand and model diseases, develop and screen candidate drugs, and deliver cell replacement therapy to support regenerative medicine. Reprogramming technology offers the potential to treat many diseases, including Alzheimer's disease, Parkinson's disease, cardiovascular disease, diabetes and amyotrophic lateral sclerosis (ALS). Theoretically, easily accessible cell types (such as fibroblasts) could be biopsied from a patient and reprogrammed, effectively recapitulating the patient's disease in a culture dish. Such cells could then serve as the basis for autologous cell replacement therapy. Immune rejection of the differentiated derivatives would be obsolete as the source cells originated within the patient. As a result, the need for immunosuppressive drugs to accompany the cell transplant would be lessened and perhaps eliminated altogether. Although progress has been made in understanding tumorigenicity and genomic instability in iPSCs, we still have many obstacles to overcome for clinical development to move forward. The ultimate goal, with advancements of iPSCs in the near future, is that these cells will become less of a precious commodity and more of a standard protocol in daily clinical practice. For the sake of understanding the amazing research efforts in this field, the authors are suggesting the rest of references [36–236].

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Chapter 10
Cancer Stem Cell Concept

Let both sides seek to invoke the wonders of science instead of its terrors. Together let us explore the stars, conquer the deserts, eradicate disease, tap the ocean depths, and encourage the arts and commerce.

John F. Kennedy

Abstract The chapter is describing two CSC concepts that are currently actual: the concept of clonal evolution and the concept of cancer cell. The idea is the CSC is tumorigenic as the result of their stemness, and therefore should be targeted by specific approach.

According to their **functionality**, stem cells can be divided in two categories: normal and cancer stem cells [1].

1. *Normal stem cells* are immature cells that can replicate, or renew them, and are able to differentiate, or mature into all the cells that an organism or particular organ system needs. In other words, they posses a kind of immortality marked as self-renewal because these cells can divide indefinitely to produce more copies of them. Each stem cell is unspecialized, but it can produce progeny that mature into the various cell types of, say, the brain or the immune system. Once this maturation occurs, these adult stem cell heirs may divide rapidly but only a limited number of times [1–7]. The primary purpose of **adult stem cells** is healing [8–11]. Finding out how adult stem cells store information and transform themselves into other cells with different properties is a fascinating topic for exploration [12–14]. Stem cells are so named because cells are derived from a main stem or mother set of cells. This is similar to a tree trunk that provides the stem from which other cells grow and branch out into other types of cells.
2. *Cancer stem cells* Finding cancers' stem cells is a rapidly growing area of research [5, 7–11]. These cancer-causing cells, which make up a tiny fraction of cells within tumors, have properties similar to those of stem cells [5]. Cancer stem cells make up only a tiny number of the total cancer cells in a leukemia

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patient, which makes the cells next to impossible to find. Therefore, it seems that promise of this line of research can only be realized, by studying adult stem cells as well as embryonic stem cells (ES). The latter are still ethical problem and therefore substantially controversial because an early embryo is destroyed when researchers remove stem cells from it. An alternative is to take the stem cells from embryos that carry a genetic defect for specific diseases. Are cancer cells transformed normal stem cells? Researchers have traditionally thought of cancer as a collection of cells, all growing exponentially. According to the new research, conventional cancer therapies do an effective job killing the majority of cells within the tumor, but they may miss cancer stem cells. As a result, cancers often reoccur. Even hematologic and some non-hematologic malignancies treated by autologous stem cell transplant and high dose chemotherapy, have shown that regardless of survival rate of some cancers, the final outcome is death, due to recurrence of cancer. The reason is (among others) in the fact that clinicians are injecting also cancer cells with healthy stem cells during reinfusion after apheresis collection, which accumulate and renew with a time to the critical level causing relapse or death. Ontogeny (development of an organism) and oncology (cancer development) share many common features. From the 1870s, the connection between development and cancer has been reported for various types of cancers [1]. Existence of "cancer stem cells" with aberrant cell division has also been reported more recently [5]. The connection between cancer and development is clearly evident in teratocarcinomas. As early as 1862, Virchow discovered that the germ cell tumor teratocarcinoma is made up of embryonic cells [1]. In 1970, Stevens derived embryonic carcinoma cells from teratocarcinoma (a spontaneous tumor of germ cells that resembles development gone awry) [1]. This tumor may contain several types of epithelia: areas of bone, cartilage, muscle, fat, hair, yolk sac, and placenta. These specialized tissues are often adjacent to an area of rapidly dividing unspecialized cells. The teratocarcinomas are able to differentiate into normal mature cells when transplanted into another animal. This alternation between developmental and tumor cells status demonstrates how closely development and cancer are related. The present-day challenge is to decode the common molecular mechanism and genes involved in self-renewal for cancer cells and stem cells. The very new concept in the field of cancerogenesis is the cancer stem cell (CSC) [5]. Cancer stem cells share many characteristics with normal stem cells, including self-renewal and differentiation. CSC is defined as "a cell within a tumor that possesses the capacity to self-renew and to cause the heterogeneous lineages of cancer cells that comprise the tumor." These cells have functionality allowing them the capability of causing an invasive group of cells (tumorigenic) that create metastasis [7–11]. There are two theories with respect to CSC entity [11]:

A. Stochastic/clonal evolution model	B. Hyerarchic/cancer stem cell model
This model states that all cancer cells hold tumorigenic potential and they are the product of clonal evolution by the acquisition of genetic mutations and epigenetic changes	Tumors show hierarchy, with a subpopulation of cancer cells having a tumorigenic potential much greater than that of other cancer cells

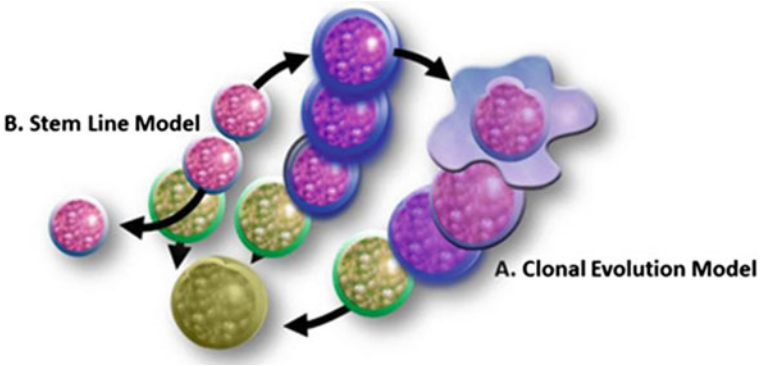

Fig. 10.1 **A**, **B** Two leading theories and cancer stem cell models: **A** Clonal evolution and **B** Cancer stem cell/Hyerarchycal model

There are two well defined yet different models of Cancer Stem Cell (Fig. 10.1a, b) within scientific community none of which completely can describe the features of cancer stem cell:

Table 10.1 Cancer stem cell markers

Cancer	Cancer Stem Cell Markers																		References()
	CD24	CD44	ALDH1	CD90	CD29	CD117	CD133	Integrin	Integrin	CD166	Nanog	ABCC22	CD96	CD34	NCAM1	CD271	CD108	POLEF1	CD38	
Breast		■	■																	Hajj (2003), Ferro de Beca (2012)
Prostate		■					■	■	■											Maeda (2009), Hoogland (2013)
Colon	■	■			■		■			■										Vermeulen (2008)
Brain							■													Zepperick (2008)
Lung							■													Bertolini (2008)
Pancreate	■	■																		Li (2007), Zhu (2012)
Hepatic		■		■			■					■								Zheng (2013), Yen (2008), Shengyong (2007)
Ovarlan						■	■				■									Luo (2011), Zheng (2011), Siu (2013)
AML													■	■					■	Hosen (2007), Horton (2011), Bonnet (1997)
Wilm's tumor			■												■					Shukrun (2013), Pode-Shakked (2012)
Melanoma			■													■				Luo (2012), Civenni (2011)
Gastric	■	■																		Tekaishi (2009), Zheng (2011)
Renal																	■			Sandlund (2006), Busso-leti (2008), Azzi (2011)
Thyroid																		■		Soon-Hyan (2013)

*Upgraded by: Mirjana Pavlovic, Jennifer Tarakmi, John Mayfield and Shimon Knutsen

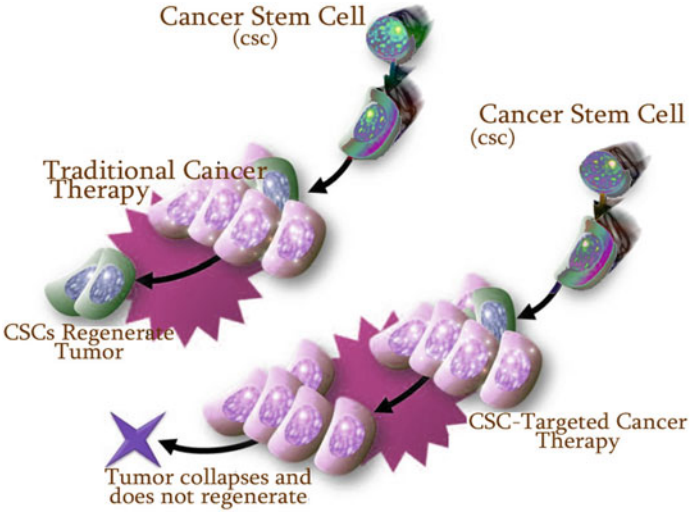

Fig. 10.2 Concept of cancer stem cell targeted therapy

(a) **Stochastic (clonal evolution) model**: This model states that all cancer cells hold tumorigenic potential and they are the product of clonal evolution by the acquisition of genetic mutations and epigenetic changes.
(b) **Hierarchical (cancer stem cell) model**: Tumors show hierarchy, with a subpopulation of cancer cells having a tumorigenic potential much greater than that of other cancer cells. Tumor contains hierarchical organization consisting of stem cells at top, which are cells within a tumor with the capacity to self-renew and generate heterogeneous lineages of cancer cells, progenitors, and differentiated cells which are no longer able to produce tumors.

Unlike cancer cells in a tumor, CSCs are capable of establishing new tumors when xenotransplanted into NOD/SCID animal models [5]. Although it has been shown that cancer cells are able to proliferate at a faster rate than CSCs, they have slight tumor initiating potential.

Therapeutic strategies that focus on targeting CSC markers (Table 10.1) will help address the ineffectiveness of traditional cancer therapies, which would otherwise result in therapy resistance and relapse (Fig. 10.2) [11–14].

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Chapter 11
Metabolic Reprogramming in Cancer and Metabolic Theory of CSC

Cells are small electrical motors.

Nikola Tesla

Abstract This chapter discusses the basics of vital processes in cytosolic and mitochondrial compartments: metabolic events, and their deviation in cancer cells, based upon metabolic reprogramming which is today regarded as the essence of dramatic mechanisms in cancer stem cells. The genetic mechanisms were so much in trend that the work of Warburg, Pasteur, Crabtree, and Racker which was almost forgotten for several decades, emerges now again as inevitability linked to specificities of cancer stem cell metabolism detected in their time. Disturbances in glycolysis and respiration as well as mitochondrial changes such as uncoupling of respiration and oxidative phosphorylation are today given second look. And with new set of information, the cancer stem cell could be looked at from another angle, explained in this chapter. The metabolic theory of CSC and its roots are described.

11.1 Metabolism of CSC: New Explanations and the Old Fundament

Cancer metabolism has long been equated with aerobic glycolysis, seen by early biochemists as primitive and inefficient [1–7]. There is no doubt that four men's contribution to understanding of differences between normal and cancer cells on molecular/metabolic level was evidently critical (Otto Warburg, Luis Pasteur, Herbert Grace Crabtree and Ephraim Racker) [8–13]. Rudolf Virchow is in charge for the very idea of the stem cell as a special cell entity with fundamental function in body cell and tissue renewal ("*omnis cellula e cellula*") [1]. Despite the early beliefs about its inefficiency, the metabolic signatures of cancer cells today are not considered as passive responses to damaged mitochondria, but rather result from oncogene-directed metabolic reprogramming required to support anabolic growth.

Recent evidence suggests that metabolites themselves can be oncogenic by altering cell signaling and blocking cellular differentiation. No longer cancer-associated alterations in metabolism can be viewed as an indirect response to cell

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proliferation and survival signals [2–7]. It is a time for scientific community to contend that altered metabolism has attained the status of a core hallmark of cancer.

11.2 Terminology of Energy Metabolism

(A) Glycolysis

Glycolysis is an essential cytosolic metabolic pathway—the breakdown of glucose by enzymes, releasing energy in the form of ATP and forming pyruvic acid. It brings up to 5% of total ATP synthesis (on ATP-ases of the cell plasma membrane) [14, 15]. It is known also as anaerobic glycolysis since it occurs without presence of oxygen. The other part is aerobic (within Mt) in the presence of O_2.

(B) Respiration (cellular respiration)

Cellular respiration takes place in mitochondria, mostly on the inner mitochondrial membrane [16–40]. It involves the function of respiratory enzymes (known as the enzymes of the respiratory chain), hydrogen, oxygen, and ATP-ases of the inner mitochondrial membrane. It is the process by which cells use oxygen to break down sugar and other metabolites and obtain energy in form of ATP (95% synthesized on the ATP synthase of the inner mitochondrial membrane). The interplay between mitochondria and the differentiation of stem cells, besides respiration, is one of the crucial events. Stem cells undergo a maturation of the mitochondrial network and a metabolic shift during their differentiation toward multiple cell types. Reciprocally, mitochondria and metabolism contribute to the regulation of pluripotency and differentiation. The mitochondrial cristae are connecting stem cells to differentiated cells, to depict the maturation of the mitochondrial network and function observed during the differentiation of stem cells.

Krebs cycle—the sequence of reactions by which most living cells generate energy during the process of aerobic respiration. It takes place in the mitochondrial matrix, consuming oxygen, producing carbon dioxide and endogeneous water as waste products, and converting ADP to energy-rich ATP. It starts and ends with oxaloacetate through the brake of ten aminoacids.

(C) Oxidative phosphorylation (OXPHOS)

- Oxidative phosphorylation (or OXPHOS in short) is the metabolic pathway in which cells use enzymes to oxidize nutrients, thereby releasing energy used to reform ATP. In most eukaryotes, this takes place inside mitochondria. Almost all aerobic organisms carry out oxidative phosphorylation.
- ATP synthase uses the energy stored in the proton gradient to make ATP. That is why the process is called oxidative phosphorylation (because oxygen is the final electron acceptor and the energy released by reducing oxygen to water) and is used to phosphorylate ADP to generate ATP.

It is:

- a biochemical process in cells, the final metabolic pathway of cellular respiration, after glycolysis and the citric acid cycle. 26 out of the total 30 ATP (energy carrier) molecules generated from a single glucose molecule during cellular respiration come from oxidative phosphorylation.
- the process in cell metabolism by which respiratory enzymes in the mitochondria help in synthesis of ATP from ADP and inorganic phosphate during the oxidation of NADH by molecular oxygen.
- the process by which the energy liberated by oxidation of metabolites is used to synthesize the energy-rich molecule ATP.
- the aerobic synthesis of ATP from phosphate and ADP, coupled to electron transport.
- the synthesis of ATP by phosphorylation of ADP for which energy is obtained by electron transport chain, and which takes place in the mitochondria during aerobic respiration (Fig. 11.1).

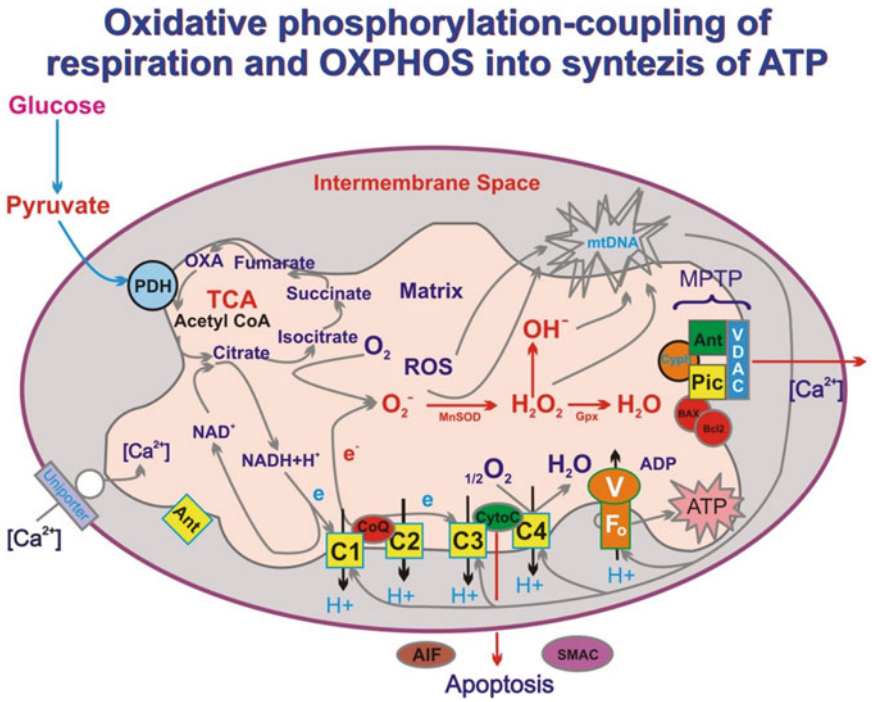

Fig. 11.1 Mechanism of coupling of OXPHOS with respiration (transfer of reducing equivalents)

11.3 Coupling of the Respiration with OXPHOX

Chemiosmotic theory suggests essentially that most ATP synthesis in respiring cells comes from the electrochemical gradient across the inner membranes of mitochondria by using the energy of NADH and FADH2 formed from the breaking down of energy-rich molecules such as glucose and transfer of reducing equivalents into mitochondria. Chemiosmotic coupling is important for ATP production in mitochondria, chloroplasts, and many bacteria and archaea.

11.4 Mechanisms of ATP Synthesis

Chemiosmotic model (Peter Mitchel)—returning of protons through ATP synthase of inner mitochondrial membrane into mitochondrial matrix (without carriers, by chemiosmosis). It is the movement of ions across a selectively permeable membrane down their electrochemical gradient. More specifically, it relates to the generation of ATP by the movement of hydrogen ions across a membrane during cellular respiration (in mitochondria) or photosynthesis (in chloroplasts). Hydrogen ions (protons) will diffuse from an area of high proton concentration to an area of lower proton concentration, and an electrochemical concentration gradient of protons across a membrane can be harnessed to make ATP. This process is related to osmosis, the diffusion of water across a membrane, which is why it is called chemiosmosis; ATP synthase is the enzyme that makes ATP by chemiosmosis. It allows protons to pass through the membrane and uses the kinetic energy to phosphorylate ADP, making ATP. The generation of ATP by chemiosmosis occurs in mitochondria, chloroplasts as well as in most bacteria and archaea.

Mechanism of rotational catalysis (Hans Boyer)—synthesis of ATP on ATP synthase of the inner mitochondrial membrane due to shrinking of ATP units and rotation of one of them for 120 degrees, which brings ADP and Pi close enough to cause their bonding in ATP (Fig. 11.2). This would never happen without that high energy investment of the proton-motive force of the respiratory chain which is built into the ATP molecule as the storage of energy for endergonic reactions. Therefore, ATP is known as the "energy currency of the cell".

11.5 Metabolic Abnormalities in Cancer Cells and Mitochondria

(a) Uncoupling effect.

Coupling between the chemical energy of redox reactions in the respiratory chain and the oxidative phosphorylation catalyzed by the ATP synthase (sometimes called as "mitochondrial mushrooms") is a necessary process for ATP synthesis. If

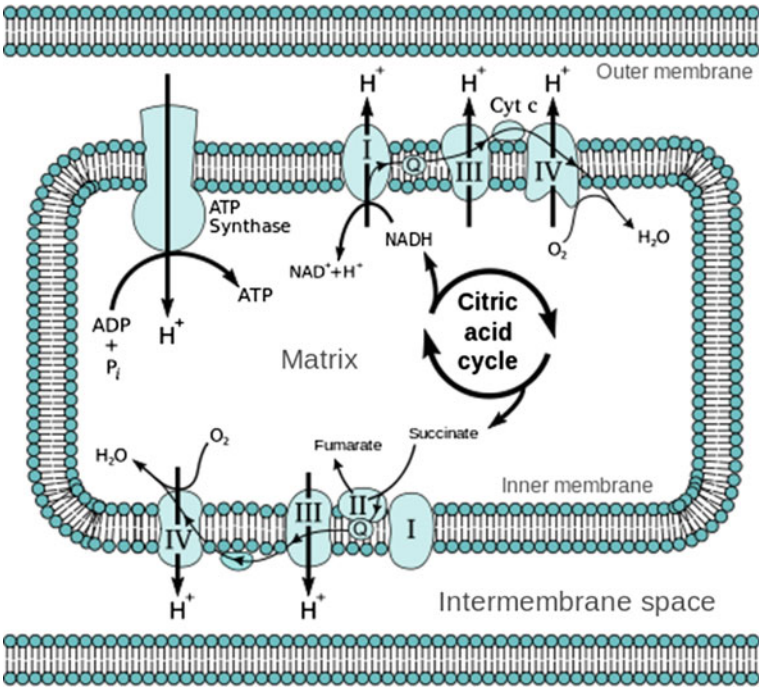

Fig. 11.2 Chemiosmotic model for explanation of mechanism of ATP synthesis

the coupling is missing for any reason, we call that *uncoupling effect.* That means that as a result of uncoupling effect, ATP will not be synthesized on the ATP-ase of the inner mitochondrial membrane.

That is what happens in CSC due to the genetic reprogramming (Fig. 11.3) which orchestrates the synthesis of the uncoupling protein molecules (UTP1 and UTP2) which does not allow the coupling of OXPHOS to respiration and ATP synthesis [15]. Therefore, CSC and any cancerous cell have lower energy level compared to normal.

Likewise, it is interesting that pharmacological inhibition of fatty acid oxidation has been shown to potentiate apoptosis induced by a variety of chemotherapeutics in cancer cell lines, as well as palmitate-induced apoptosis in hematopoietic cells, suggesting a priori that the metabolism of fatty acids in the mitochondria may be linked to cell survival.

(b) Warburg effect: mechanisms and impact.

In oncology, the Warburg effect is the observation that most cancer cells predominantly produce energy by a high rate of glycolysis followed by lactic acid fermentation in the cytosol, rather than by a comparatively low rate of glycolysis followed by oxidation of pyruvate in mitochondria as in most normal cells (suppression of respiration by enormous anaerobic glycolysis).

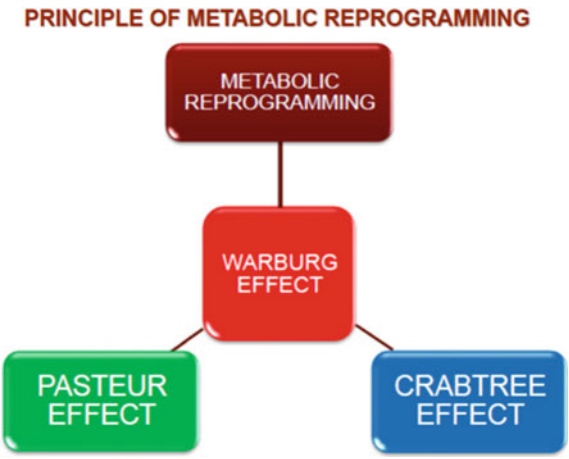

Fig. 11.3 Metabolic reprogramming in cancer cell

Glycolysis in mitochondria is aerobic (uses oxygen). Malignant, rapidly growing tumor cells typically have glycolytic rates up to 200 times higher than those of their normal tissues of origin; this occurs even if oxygen is plentiful.

Otto Warburg postulated that this change in metabolism is the fundamental cause of cancer, a claim now known as the Warburg hypothesis. Today, mutations in oncogenes and tumor suppressor genes are thought to be responsible for malignant transformation, and the Warburg effect is considered to be a result of these mutations rather than a cause.

It is important to point out that in the study by Bonnet et al. solid tumor cell lines were found to display elevated $\Delta\Psi M$ (mitochondrial proton gradient) and this was associated with increased aerobic glycolysis indicating that mitochondrial uncoupling may not be a universal phenomenon by which cancer cells activate the Warburg effect.

In conclusion, recent investigations into the mechanisms that underlie the Warburg effect suggest that:

(1) mitochondrial uncoupling can promote aerobic glycolysis in the absence of permanent and transmissible alterations to the oxidative capacity of cells,
(2) aerobic glycolysis may represent a shift to the oxidative metabolism of non-glucose carbon sources, and
(3) mitochondrial uncoupling may be associated with increased resistance to chemotherapeutic insults.

The above suggest the importance of understanding the mechanisms of mitochondrial uncoupling and their relation to metabolic alterations observed in cancer cells (Fig. 11.4).

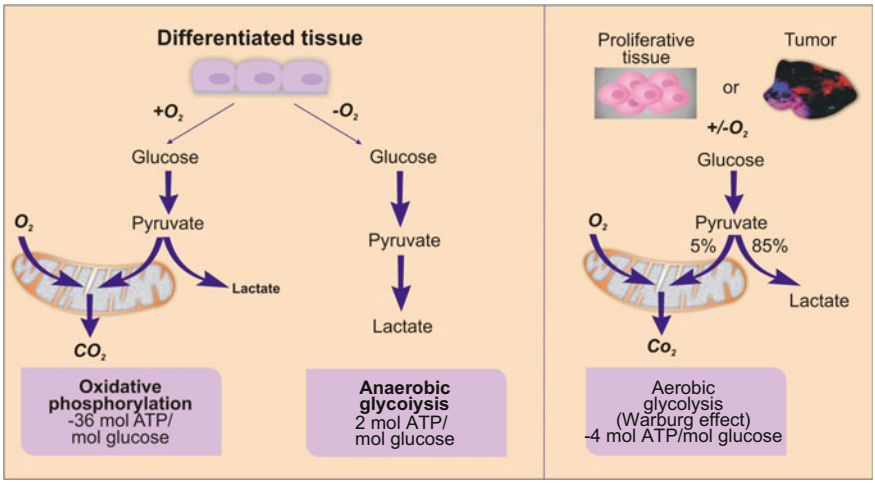

Fig. 11.4 Diagram of normal glycolysis and cancer cell glycolysis: the impact of Warburg's effect

11.6 The Hallmarks of Cancer Cell Energy Metabolism: The Warburg Effect and the Crabtree Effect

Suppression of respiration by glycolysis and vice versa.

Excessive glycolysis in malignant cell and therefore in CSC, leads to the suppression of respiration (by glycolysis), known as Warburg–Folkman effect.

(a) Warburg effect. In order to proliferate, cells must comply with the energy demand imposed by vital processes such as macromolecule biosynthesis, DNA replication, ion gradients generation, and cell structure maintenance. Mitochondria play an important role in energy metabolism as they synthesize most of the cellular ATP through oxidative phosphorylation. However, it was suggested that cancer cells suppress mitochondrial respiration. This effect can be explained as follows: Aerobic glycolysis (Warburg effect) leads to overproduction of lactate which raises pH in tumor and blood, giving negative balance of ATP (−4 mol/mol glucose). CSCs are showing uncoupling effect—the effect of non-coupling of OXPHOS with the proton-motive force of respiratory chain—and due to that reason they do not synthesize sufficient ATP on ATP-asis of the inner mitochondrial membrane. Comparatively, NSCs are showing the coupling effect with positive balance of ATP, which is a fundamental difference in mitochondrial metabolism [1–7].

The early discoveries from O. Warburg pointed out that cancer cells display a decreased respiration along with an enhanced lactate production, suggesting that they depend mainly on fermentative metabolism for ATP generation. In spite of the decrease in energy yield as a consequence of the "glycolytic phenotype", this seems to allow an increase in cell proliferation rate and be applicable to other fast growing cells.

(b) Crabtree effect. Some cancer cells, in spite of possessing functional mitochondria can switch between glycolytic and oxidative metabolism in a reversible fashion (the Crabtree effect). Herbert Crabtree, a contemporary of Warburg, suggested that pathological overgrowths use aerobic glycolysis as a source of energy and glucose uptake and glycolytic activity has a depressive effect on oxygen consumption, which results in uncoupling effect. The Crabtree effect on tumor cells could be eliminated by adding an excess of phosphate (Pi) in vitro and, because of that, it has been proposed as the actual trigger of this metabolic phenomenon. Fermentation is also suppressed by respiration [14].

(c) Pasteur effect—The effect is originally discovered by Luis Pasteur in yeast and later on confirmed by others in cancer cells [41]. Cells can obtain energy through the oxygen-dependent pathway of oxidative phosphorylation (OXPHOS) and through the oxygen-independent pathway of glycolysis. Since OXPHOS is more efficient in generating ATP than glycolysis, it is recognized that the presence of oxygen results in the activation of OXPHOS and the inhibition/suppression of glycolysis (Pasteur effect) by respiration (opposite of Warburg effect). Accumulating evidence suggests that the persistent activation of aerobic glycolysis in tumor cells plays a crucial role in cancer development; the inhibition of the increased glycolytic capacity of malignant cells may therefore represent a key anticancer strategy. Although some important knowledge has been gained in the last few years on this growing field of research, the basis of the Warburg effect still remains poorly understood, and therefore needs more studies.

(d) Crabtree and Pasteur effects. Suppression of glycolysis by respiration in CSC is the consequence of disbalance between one and other fundamental functions of energy metabolism. CSC cannot shunt the excess of glucose through main metabolic pathway and simply "shunts" it into lactic acid through alternative fermentative pathway. (Pasteur has defined fermentation to ethanol in the yeast, while Crabtree discovered it as lactic acid fermentation in isolated malignant carcinoma cell). Human cells cannot produce ethanol, but can lactic acid. All of these were confirmed by the work of Racker [12]. Thus, it took several decades to understand, and provoke a kin interest of scientific community for this phenomenology which despite doubts of some investigators is taking over again with a new insight and inspiration [5].

Thus, there is really the essential question: are Pasteur, Warburg, and Crabtree the three edges of the triangle coin? [5].

In short, with regard to proliferative cells, there are two unifying principles in the three apparently diverging hypotheses of Pasteur, Warburg, and Crabtree, i.e., an inverse relation exists between glucose uptake and oxygen utilization (respiration); while fermenting cells require more glucose, the proliferative cells require both glucose and oxygen.

Pasteur, in addition, points to the requirement of nitrogen as an additional source (albuminoid) for growth of yeast.

11.7 Resume: Energy Metabolism in Cancer Cell Compromised?

What are the striking points in metabolic phenomenology of cancer stem cell?

Respiration suppressed by glycolysis (Warburg).

Glycolysis suppressed by respiration (Pasteur) → fermentation → acid (lactate) in animal and humans, ethanol in the yeast.

Uncoupling effect respiration does not couple to oxidative phosphorylation and ATP synthesis lowers.

In all the cases energy level is diminished in cancer cell (ATP synthesis low). What does it mean? Possible reprogramming in metabolic domain?

11.8 The Overview on CSC Metabolic Activity

Judged by the evidence, we can conclude that both normal and cancer stem cells are hypoxic in their nature, but normal SC is adapted to hypoxia since it lives in those conditions and does respond by normal mechanisms of glycolysis and respiration with adequate synthesis of ATP, sufficient to supply all processes ahead of it: proliferation, differentiation, and mature cell production. CSC is in a need for oxygen due to disturbed intrinsic, crucial mechanism for ATP synthesis, which through the uncoupling of the OXPHOS with respiration and lack of the reaction of rotational catalysis, is decreased, so that energy is mainly used for renewal, proliferation, and incomplete differentiation.

In normal stem cell, glycolysis is going through the pathway of pyruvate formation, which by transition into oxaloacetate becomes transportable for mitochondria where it is a smoking gun for Krebs cycle, the remains of which are CO_2 and endogenous water, which are excreted from the body as the waste.

In CMC due to fundamental disturbance at the level of aerobic glycolysis and respiration (uncoupling effect) glycolysis is mostly anaerobic, going through the lactate production pathway, suppressing even more respiration and channeling the glucose lytic product into lactate which accumulates in the tumor and blood. This is happening due to regulation of reversible conversion of lactate into private under control of LDH enzymatic activity which is usually very high in tumors. As mentioned, Herbert Crabtree observed that in some tumors the high glucose concentrations are “choosing” fermentative pathways and produce lactate rather then suppress respiration with transport of the reducing equivalents into mitochondria.

The metabolic reprogramming as a concept of CSC is getting more proofs, rapidly. Thus, recently, Vittorio Sartorelli and colleagues (2015) reported that proliferating skeletal muscle stem cells (satellite cells) shift from fatty acid oxidation to glycolysis, with downstream effects on epigenetic states and gene expressions undergo metabolic reprogramming [42] and are using different metabolic substrates during differentiation. Analyzed transcriptomes of quiescent and proliferating mouse

satellite cells showed the signs of transcriptional activation of the glycolytic program. The shift to glycolysis was accompanied by a decrease in the levels of NAD^+, a corresponding decrease in the activity of the NAD^+-dependent enzyme SIRT 1 and an increase in H4K16ac, the substrate of SIRT 1-mediated deacetylation. The authors generated a skeletal muscle-specific knockout of Sirt1, which exhibited defects in skeletal muscle development and regeneration following injury. The RNA-seq and ChIP-seq for gene profiling expression and SIRT 1 and H4K16ac localization across the genome were performed in order to determine links between SIRT 1, H4K16ac, and gene expression. This work connects metabolic shifts with epigenetic regulation in tissue stem cells [42].

The other interesting finding is that Rb links reprogramming and cancer. Gene RB1 (retinoblastoma) was the first tumor suppressor gene to be described as often mutated in many human cancers [43]. Its product, Rb protein, is well known for its negative regulation of the cell cycle and, more recently, for its accessory role in chromatin remodeling. Kareta et al. (2015) investigated the function of Rb in reprogramming, leading to new insights into tumorigenesis. They discovered that inactivating Rb facilitates reprogramming of fibroblasts to a pluripotent state. Surprisingly, their data indicate that this does not involve interference with the cell cycle but instead that Rb directly binds to, and represses pluripotency-associated loci such as Oct4 (Pou5f1) and Sox2. Loss of Rb seems to compensate for the omission of Sox2 from the cocktail of reprogramming factors. Furthermore, genetic disruption of Sox2 precludes tumor formation in mice lacking functional Rb protein [43]. This study positions Rb as a repressor of the pluripotency gene regulatory network and suggests that loss of Rb might clear the path for Sox2, or other master regulators of stem cell identity, to induce cancer. The potential role of Rb in other types of in vitro reprogramming is an attractive field of metabolic reprogramming research.

Electromagnetic aspect of our cells, including non-exitable and exitable tissues and also cancer cells, originates from several sources [44–54]. Let us only mention the polarization of the inner mitochondrial membrane during the work of respiratory chain where the separation of hydrogen to proton and electron causes the occurence of proton and electron currents with relatively high frequencies (Fig. 11.5). The

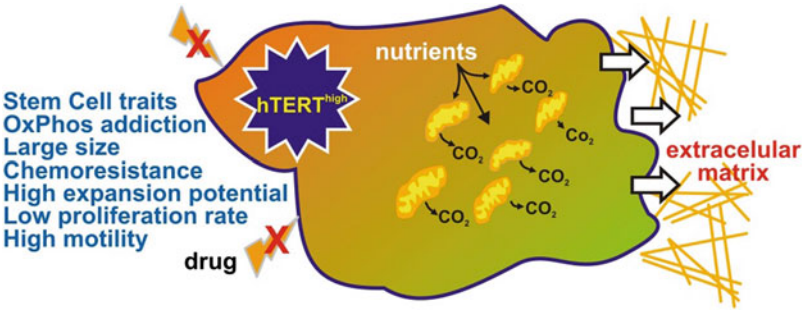

Fig. 11.5 Diagram of human cancer stem cell with mitochondria as a target

other level is polarization of the cell membrane in positive charge outside and negative inside, and the third is biophotonic source from DNA itself, which is of much lower frequence. All of this is placing the cell into situation to respond to the magnetic influences and that response is distinguishable between healthy and sick cell. Thus, NSCs, healthy mature cells and CSCs in electrical and magnetic field, show differences which turned to be very significant and which we shall mention, soon.

11.9 New Cancer Therapy Concept

As we know, decades ago, Otto Warburg observed that cancers ferment glucose in the presence of oxygen, suggesting that defects in mitochondrial respiration may be the underlying cause of cancer. We now know that the genetic events that drive aberrant cancer cell proliferation also alter biochemical metabolism, including promoting aerobic glycolysis, but do not typically impair mitochondrial function [55]. Mitochondria supply energy, provide building blocks for new cells, and control redox homeostasis, oncogenic signaling, innate immunity, and apoptosis. Indeed, mitochondrial biogenesis and quality control are often upregulated in cancers. While some cancers have mutations in nuclear-encoded mitochondrial tricarboxylic acid (TCA) cycle enzymes that produce oncogenic metabolites, there is negative selection for pathogenic mitochondrial genome mutations [56]. Eliminating mtDNA limits tumorigenesis, and rare human tumors with mutant mitochondrial genomes are relatively benign. Thus, mitochondria play a central and multifunctional role in malignant tumor progression, and targeting mitochondria provides therapeutic opportunities [57].

In light of the above, it is intriguing to propose that targeting the mitochondrial metabolism of fatty acids and/or glutamine may hold therapeutic promise for the treatment of human malignancies. Conversely, given the important role of uncoupling proteins in the metabolic shift associated with increased fatty acid and glutamine metabolism in favor of glucose oxidation, it would be of great interest to develop therapeutic strategies that would target these proteins. The new concept on metabolic nature of the cancer is on the horizon seeing the evolution of tumor metabolism as a reflection of carcinogenesis as a reverse evolution process [58].

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Chapter 12
Concept of Targeted Cancer Stem Cell Therapy and New Versions

If you want to find the secrets of the universe, think in terms of energy, frequency and vibration.

Nikola Tesla

Abstract The concept of cancer stem cells has inevitably inspired the concept of targeted CSC therapy. Recent identification of surface markers and understanding of molecular feature associated with CSC phenotype helped with the design of effective treatments. This concept envisions CSC as a unique target that should be destroyed with either physical, pharmacological, immunological, or even combined modalities, which do not affect normal cells. This chapter is a consideration of novel strategies aimed at targeting CSCs. The ideas discussed in this review can be summarized as a set of propositions for novel therapeutic approach.

12.1 The Idea of Targeted Therapy: Before, Now, and in the Future

If CSCs primarily drag the growth of cancer cells and metastases, the efficient anticancer treatment must attack the very CSC [1–11]. Shrinking of cancer/tumor or the reduction of number of leukemic cells in peripheral blood, can lead to temporary releaf, but not to permanent cure if all CSCs are not eliminated. Moreover, if only CSCs are eliminated, the remaining CSCs in the body will be attacked by immune system or they will naturally die after cancer cells which are not stem cells by definition, and do not have the capability to reproduce cancer [18–21]. The most efficient cancer treatments, therefore, will be those which are specifically targeting CSCs [11–13]. Such treatments will very possibly have minimal side effects after they leave other types of cells intact. Innovation of such a treatment will require more knowledge about qualities and behaviors of CSCs [2–7, 13–18, 20, 22, 23].

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12.2 Can We Use NSCs in the Therapy of Cancer?

Yes—we are doing that through decades working on the transplantation of NSCs of the bone marrow and peripheral blood to the patients with hematological malignancies, where we are getting remissions after cytostatic cleaning of the BM from CSCs. As the CSCs are resistant to chemo- and radiotherapies, with the time, relapse will take over, but yet, in most of the patients the life is prolonged [18, 19, 21].

We are doing that also in the situations in which the radiation therapy and cytostatics damage the bone marrow in the case of solid tumors when we apply that as prophylactic/protective measure [19].

12.3 Mitochondria as the Target

Better understanding of the energetic metabolism in CSC lead to the idea that it is necessary to target their mitochondria since they are pretty much the cause of the problem [13, 17]. Thus, the antibiotics were found (meaning, not the poisons as cyanide or MIBG (metaiodobenzylguanidine)) which would, theoretically, be able to attack different localities in diseased mitochondria. We now know for five sorts of drugs/antibiotics meaning nontoxic which are attacking three different molecular targets either in mitochondria or mitochondrial ribosomes which participate in the synthesis of vital mitochondrial proteins—enzymes of respiration [17, 20]. There is essential problem to solve: how to succeed to reach and target only mitochondria of the sick cells without targeting healthy? What kind of vehicle is to be designed and used for that purpose?

12.4 Other Targeting Possibilities

We know that in the past, first targeted therapies of the cancer produced antibody for cancer antigen linked to some toxin (botulinum, etc.) which would then, after binding of the antibody for the cancer cell, destroy cancer cell. These types of therapies, although enthusiastically imagined, did not give results primarily due to the changes of the antigen in the tumor during tumor development leading to loss of the antigen as a target. More modern approaches such as

- Kinases, blocking of signaling pathways, ROS status
- Libraries of functional peptides for killing cancer cells
- Immunological and physical forces
- Nanotechnology.

are later on developed. These approaches were also not quite successful since they targeted also tissues other than cancerous [24, 25].

12.5 New Modalities of Targeted Therapy and Idea of Remote Control

The new approaches of targeted therapy can be roughly separated into two main directions: immunooncological and approaches linked to physical properties of CSC which are per se physical [25–32].

1. In immunological approaches, dependent on the case, T lymphocytes are used based on vaccines with either dendritic cells (DC) or prime effector T cells [32, 33].
2. Physical methods include the application of nanoparticles, magnet, electric current, electromagnetic fields (EMF), laser, insertion of the chip, etc., introducing at the same time a new term of *remote control of drug delivery* into malignant cell [34–38].

12.6 Principles of Targeted Therapy Based on Nanoparticles

In the heart of nanotechnology are geometry and minimization. Many natural as well as man-made nanoparticles are shown to be appropriate for targeted drug delivery [34–38]. More details on nanotherapy will be given in Chap. 14.

12.7 What is the Best Way to Kill CSC: New Methods?

The combined use of traditional therapies with targeted CSC-specific agents may target the whole cancer cells and offer a promising strategy for lasting treatment and even cure [39]. One of the complex models of targeted therapy which is using several levels on units road toward final destruction of CSC: interaction of antibodies, nanoparticles, and NIR laser, in the attack to CSC where NIR in cancer cells is heating the gold nanoparticles assembled through the antibody recognition of the surface CSCs molecules, killing it finally at certain temperature, is the best, but still at the conceptual level [39].

12.8 Different Combinations of Targeted Therapy

We can consider/visualize two scenarios:

Scenario1. Antibody penetrates through the phospholipid bilayer of the CSC membrane bearing little magnets. Irradiated with magnetic waves of the frequency

of only few Hz, the CSC becomes the "victim" of the field since the membrane from outside and inside is distending and breaking up causing the cell death [39].

Scenario 2. Here we can imagine delivery of intrabuccal radiofrequencies (RF) EMF. The patient receives low level of EMF-RF (27.12 Hz) which is minimally absorbed and systematically distributed with the patient's body functioning as antenna. Yellow color obtained indicates the regions of the body which are receiving the strongest irradiation [40]. Distinction between molecular mechanisms accounting for the anticancer effects of tumor-specific modulation frequencies is likely to lead to the discovery of novel pathways in cancer and therefore novel possibilities for treatment with adequate frequencies in given scenario [40].

12.9 The Use of Electromagnetic Fields for Growth Inhibition of CSC

Model of electromagnetic interaction and concept of ultraweak biophotonic emission: how does it function?

Critical significance of CSC is in a new concept of targeted therapy which involves original approaches for precise targeting, resulting in shrinking of CSC and tumor death. These treatments are numerous and involve precise drug delivery (nanoparticles/nanotechnology, biomagnetism, etc.). A special achievement is remote control of drug delivery along with application of wireless manipulation based on microchip or magnet, which enables the drug delivery in real time and its tracking. Using closed electrical fields, during the studies of living cells, today's scientists frequently reject the obligatory existence of magnetic fields with electric fields [41, 42]. Electricity and magnetism cannot be separated, since they are two aspects of one same phenomenon—electromagnetism. It is forgotten fact that by changing magnetic field we are also changing electrical, as well as that by changing electrical field we are changing/producing magnetic, so that electromagnetic character of living cells: human, animal, and plant is not quite well understood on larger scale. Research has shown that alternating currents can disrupt cancer stem cell replication, while TTF can improve the regression of glioblastoma [41, 42].

12.10 Cancer and Electromagnetism

Everything in the Universe is moving, revolving, pushing, and starting from black holes, galaxies, solar systems till the miniature atoms and their particles. This entire constantly organized movement, creates electromagnetic waves, induces and conveys different information through the system. What connect us are the frequencies.

The studies of electrolyte features of proteins/amino acids as the carriers of charges, the effects of enzymes as protein reaction catalyzers, theelectromagnetic effects of all electromagnetic and sound waves, heavy atoms, and paramagnetic and

diamagnetic atoms and molecules, not clean neighborhood of living cells, show theoretically and experimentally that the cancer is caused by increased electromagnetic activity of the cells. This means that for understandable cause, we can create the cure.

Where does the body's electrical field come from? As it is mentioned, macrocosmos is replicated in the body micro-cosmos—the cell in which all body's atoms and electrons during their constant revolutions and movement from one to other location create micro-currents (described earlier), micro-potentials, and EMF which are the basis of body energy field. This energetic field is increasing very much by additional biophotonic radiation originated from DNA, which carries the signals for regulation of life processes. Based upon such understanding, in medicine the new horizons are opening which have the fundamentals in laser light and electromagnetic oscillations are the manifestation of its development [41, 42].

Remote NIR laser stimulation/control

Co-encapsulating Doxorubicin (DOX) with indocyanine green (DI) thermosensitive lyposomes (DI-TSL) were treated with NIR laser (808 nm, 0.5 W/cm^2, 5 min) after injection, achieving the killing of cancer cells in different ways.

(1) Intracellularly stimulating DOX release from endosomes.
(2) Extracellularly by releasing DOX through swelling and explosion of the cells related to diffusion into tumor cells on the basis on the high concentration gradient. It is concluded that DI-TSL may provide a smart strategy to release drugs on demand for combinatorial cancer therapy (remote control of drug delivery).

12.11 Different Principles: Drug Delivery Based upon Nanoparticles of Ferrous Oxide

Nanoparticles of ferrous oxide are especially appropriate for medical application. Their inside is hollowed so that different drugs can get into it [43]. Thus, filled with drug, nanoparticles reach the target and gradually release the drug through molecular valves or different deformations of the drug—polymer, which is "looking for its road" through the holes [43].

12.12 3D–Printed Tumor Model

Printing of tumors and their 3D growth is one of the new approaches in cancer and CSC research, since the solid tumor is not only CSC—it is a complex structure which besides cells involves preferably vascular and neural component, surrounded by specific microenvironment. Therefore, Gordana Vunjak-Novaković and Alessandro Vilassante propose a "*minimal functional unit*" which offers limited, but

sufficient level of complexity, for studying specific tumor aspects [44–47]. It again, requires integration of several disciplines. Tumor modeling is still using cultivated tumor aggregates (meaning, not clear, isolated CSCs) with NSCs. Using CSCs in these models has very much facilitated the confirmation of the CSC concepts and theories and tested their validity with high priceseness and correctness.

12.13 Computerized 3D Model for Analysis of Aggregation of Tumorigenic Cells Is Revealing Specialized Behavior and Unique Cell Types Which Accelerate the Aggregation Process

It has been shown that when the cells from tumorigenic lines and cells cultivated from fresh tumor tissues are seeded into 3D Matrigel matrix, they multiply in order to form individual clonal islands or primary aggregates which are afterward fused in order to form huge aggregates [48]. Eventually, most of the primary aggregates in the territory of Matrigel are becoming incorporated into one massive aggregate, which, in some cell lines forms in time, "empty"—hollowed sphere of differentiated architecture. Despite that, cells from nontumorigenic and very slightly tumorigenic lines, and fresh noncancerous tissue seeded on the Matrigel are forming clonal aggregates, through cellular multiplication, but these aggregates do not fuse. Such an alternative scenario can be observed in cell preps made of different tumorigenic cellular lines and cells from fresh tumor tissue [48].

12.14 Possible Mechanisms of Tumor Cellular Heterogeneity: Mediated Coalescence?

Mediated coalescence/fusion today is considered as one of the possible mechanisms by which heterogeneity of tumors could be explained. Some cells are more active in aggregation than others [48]. The advantages are in specificity and decrease in toxicity and disadvantages in harder manipulation of such a small population of CSCs and need for better definition of markers of CSCs which is also being changed by mutations and therefore is changing the tolerance to the therapy.

12.15 Summary

By summarizing targeted therapy of CSCs we can say that it represents alternative/second option for physicians comparing to surgery, radiation, and chemotherapy and can give better quality of life to the patient since it does not target healthy

tissues and does not contribute to cytotoxicity. So, what do we have now? Is that genetic engineering with reprogramming of NSCs in terms of cytotoxicty and make them attack the CSCs?

12.16 Conclusions

Studies so far have shown that not all cancer cells are the same. Within malignant tumor or between CTC of solid tumors and leukemias there is a variety of cell types. CSC theory proposed that among cancerous cells several of them function as stem cells with self-renewal capabilities and support cancerous state, like almost normal stem cells which are normally self-renewing and by that maintain our tissues and organs. According to this viewpoint, cancer cells which are not CSCs can cause problem, but are not capable of supporting the attack to the body for a longer time.

The idea that cancer primarily occurs dragged by some small population of stem cells, have important implications. For instance, many new anticancer therapies were evaluated on the basis of their capability to shrink the tumors, but if that does not kill CSC, tumor will grow again (very often with smaller resistance compared to the very first therapy).

The other important implication is that CSC is the one which is supporting metastases (when the cancer travels from one place to another) and can also function as a reservoir of cancer cells which can cause relapse after surgery, radiation, or chemotherapy, which have eradicated any misting sign of the cancer.

One of the components of the CSC theory deals with that of how do the cancers occur? In order for cell to become cancerous it has to get through significant number of essential changes in DNA sequences which regulate the cell function. Conventional theory of cancer says that any cell in the body can get through these changes and become cancerous. However, researchers Ludwig Center (CA) have observed that our NSCs are the only ones which have the feature of self-renewal, and therefore long enough around in order to accumulate all necessary changes which are producing cancer. Therefore, we are with the theory that CSC emerges from NSC, or it is a precursor cell as the product of NSC.

Thus, the idea that CSCs are close relatives to NSCs and therefore will share many behaviors and features just of those, NSCs is very attractive. Other cancer cells are produced by CSC and should follow many rules observed in the behavior of daughter cells in normal tissues. Some researchers say that cancer cells are something like caricatures of NSCs: they are showing many of the characteristics of normal tissues but in one: “twisted” way. If it is so, then we can use our knowledge about normal stem cells for identification and attack against CSCs, and all of malignant cells produced by them. One recent success which is illustrating such approach is research in anti CD47 therapy.

12.17 A Look Ahead; What Does the Future Say?

Bioengineering is the fantastic movement of science to get integrated and thus help by all strength and prevent extreme reactions with looking for the solution in the context of biological complexity, with the possible best solutions. We did not talk about the application *in extenso*—but have emphasized the novel therapeutic approaches indicating even the combination of the treatments with the goal of establishment of the previous balance in the attacked tissues. Not only targeted, but the therapy will also be individual/personal. In summary: there is a multiple/divergent approach to the CSC therapy summarized in Fig. 12.1, where we can see that many active molecules and pathways and even organelle could be the efficient target or at least adjuvas in eradicating malignancy from the organism.

Till that moment we have to answer a lot of questions that are awaiting for the answers in the future. Where are the failures? What cannot be treated and do we know why? What is still necessary to know? Does it mean, that the new, physical methods will be a big concurrent to biochemical approaches and pharmacotherapy—not to mention organic chemistry? Does the CSC originate from NSC? How does it really occur? Which theory is "right"? Or maybe: all? Cancer is very broad spectrum of diseases with about 100 different manifestations within. Everything is going

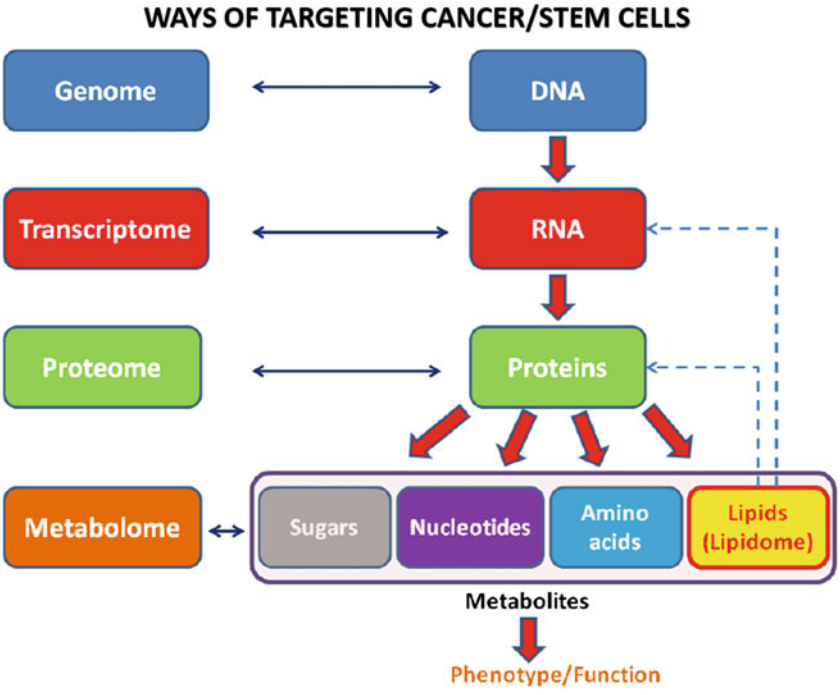

Fig. 12.1 Multiple ways of targeting CSCs (conceptual and applied)

toward individual targeted therapy. In some 6–7 decades, all of the therapies will be targeted. What will be then, with stem cell?

Is the stem cell answer to all questions?

It would be wonderful to say: yes! But, it is not and we know that. There will not be neither in the near nor the distant future. It is awaiting for us. A huge work is in front of us, but the guidelines are somewhat more clear than 50 years ago due to accumulation of knowledge from all directions, and the integral application of that knowledge in Bioengineering. It is a huge challenge which will elevate the solutions in the cancer domain (and not only there) on quite different level.

The aging of tissue-specific stem cell and progenitor cell compartments is believed to be central to the decline of tissue and organ integrity and function in the elderly. In order to completely succeed in curing sick cells, we need to examine evidence linking stem cell dysfunction to the pathophysiological conditions accompanying aging, focusing on the mechanisms underlying stem cell decline and their contribution to disease pathogenesis.

It is quite obvious that how N. Tesla's work and ideas are applicable today. His great scientific intuition was looking and sensing the dimensions of life within Universe and linking that to it [40–42].

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Chapter 13
HLA Typization Choice of Donors: Match or Match Me Not

We live in a society exquisitely dependent on science and technology, in which hardly anyone knows anything about science and technology.

Carl Sagan

Abstract This chapter will deal with fundamentals of typization for the choice of donor and essential principles unclufing immunological genetic basis for that. The role of MSH and HLA systems in diversification of these mechanisms is described and clarified. The significance of good match for successful engraftment is also emphasized. Some aspects of the matter are illustrated.

13.1 Distinction Between HLA and MSH Genes Molecules and Functions

13.1.1 HLA (H-Histocompatibility Locus Antigen)

In humans, these genes are called Human Leukocyte Antigen or HLA genes, as they were first discovered through antigenic differences between white blood cells from different individuals; in mouse they are known as the H-2 genes [1, 2].

13.1.2 MHC (Major Histocompatibility Complex)

The function of MHC molecules is to bind peptide fragments derived from pathogens and display them on the cell surface for recognition by the appropriate T cells. The consequences are almost always deleterious to the pathogen—virus-infected cells are killed, macrophages are activated to kill bacteria living in their intracellular vesicles, and B cells are activated to produce antibodies that eliminate or neutralize extracellular pathogens [1–6]. Thus, there is strong selective pressure in favor of any pathogen that has mutated in such a way that it escapes presentation by an MHC molecule.

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DOI 10.1007/978-3-319-47763-3_13

13.1.3 HLA Genes

In humans, these genes are called Human Leukocyte Antigen or HLA genes, as they were first discovered through antigenic differences between white blood cells from different individuals; in mouse they are known as the H-2 genes [3–5].

13.1.4 MHC Genes

The major histocompatibility gene complex is located on chromosome 6 in humans and chromosome 17 in the mouse and extends over some 4 centimorgans of DNA, about 4×10^6 base pairs. In humans it contains more than 200 genes. As work continues to define the genes within and around the MHC, both its extent and the number of genes are likely to grow; in fact, recent studies suggest that the MHC may span at least 7×10^6 base pairs.

The genes encoding the α chains of MHC class I molecules and the α and β chains of MHC class II molecules are linked within the complex; the genes for β2-microglobulin and the invariant chain are on different chromosomes (chromosomes 15 and 5, respectively, in humans and chromosomes 2 and 18 in the mouse). There are three regions of MHC genetic locus (Ag) presented in Fig. 13.1.

13.1.5 Polygeny and Polymorhysm of MHC

Two separate properties of the MHC make it difficult for pathogens to evade immune responses in this way. First, the MHC is *polygenic*: it contains several different MHC class I and MHC class II genes, so that every individual possesses a set of MHC molecules with different ranges of peptide-binding specificities [8]. Because of the polygeny of the MHC, every person will express at least three different antigen-presenting MHC class I molecules and three (or sometimes four) MHC class II molecules on his or her cells. In fact, the number of different MHC molecules expressed on the cells of most people is greater because of the extreme

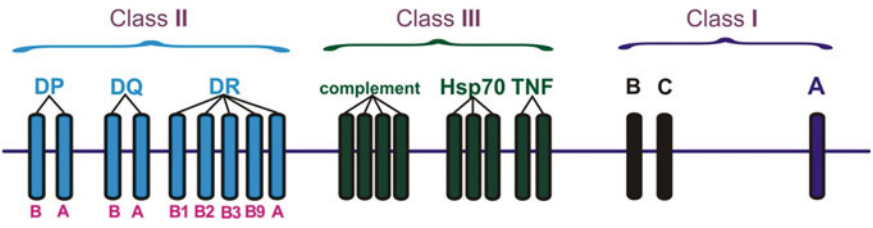

Fig. 13.1 Regions of MHC genetic loci

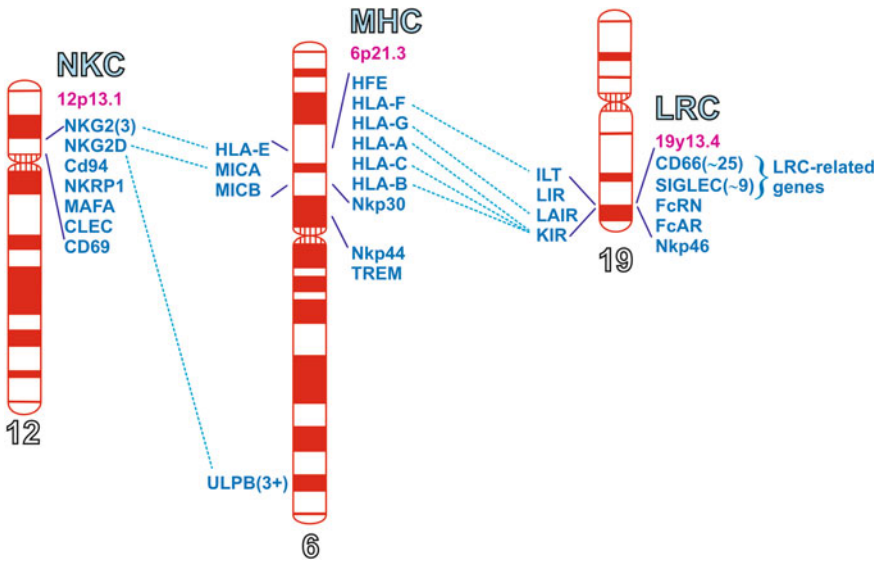

Fig. 13.2 MHC with HLA genes (antigens and pseudo-antigenes)

polymorphism of the MHC and the codominant expression of MHC gene products [6, 7].

Second, the MHC is highly *polymorphic*; that is, there are multiple variants of each gene within the population as a whole [9] (Fig. 13.2). The MHC genes are, in fact, the most polymorphic genes known. In this section, we will describe the organization of the genes in the MHC and discuss how the variation in MHC molecules can affect engraftment and cause the graft versus host reaction (GvHR) [10].

We can also see how the effect of polygeny and polymorphism on the range of peptides that can be bound, contributes to the ability of the immune system to respond to the multitude of different and rapidly evolving pathogens.

The term *polymorphism* comes from the Greek poly, meaning many, and morphe, meaning shape or structure. As used here, it means within-species variation at a gene locus, and thus in its protein product; the variant genes that can occupy the locus are termed alleles. There are more than 200 alleles of some human MHC class I and class II genes, each allele being present at a relatively high frequency in the population [11–14].

So there is only a small chance that the corresponding MHC locus on both of homologous chromosomes of an individual will have the same allele; most individuals will be heterozygous at MHC loci. The particular combination of MHC alleles found on a single chromosome is known as an MHC haplotype. Expression of MHC alleles is codominant, with the protein products of both the alleles at a locus being expressed in the cell, and both gene products being able to present antigens to T cells. The extensive polymorphism at each locus thus has the potential

to double the number of different MHC molecules expressed in an individual and thereby increases the diversity already available through polygeny.

13.1.6 The Mechanism of Gene Rearrangement Is Contributing to and Allowing for Antigen Diversity

As we look inside the gene-arrangement performance discovered by Susumu Tonegawa, the matching can be influenced also by high diversity in antibody response to an antigen through both gene rearrangement of B (antibodies) and T (T-cell receptors), lymphocytes. This table will explain how many combinations of V, D, J segments on three different gene loci can allow for antibody diversity through this gene combining mechanism from different chromosomes (saving space) (Table 13.1).

13.1.7 Transplantation Needs Matching to Support Engraftment

The SC transplant is potentially curative therapy for variety of diseases. HLA and ABO antigens have a central role in survival of the transplant. Matching of donor and recipient for HLA antigen is essential to the success of SC transplant. The ABO blood group is the most significant blood factor in clinical applications involving blood transfusions [15–21]. ABO incompatibility is not contraindication for SCs transplant but it is an important element for survival of transplanted cells and development of immediate posttransplant or delayed complications, such as anemia due to immune-mediated hemolysis [22–27]. However, HLA matching through haplotyping is more demanding criterion. Technically, PGD for HLA typing is a difficult procedure due to the extreme polymorphism of the HLA region. Taking into account also the complexity of the region (presence of a large number of loci and alleles) the use of a direct HLA typing approach would require standardization

Table 13.1 Combinatorial diversity of antiobodies based on S. Tonegawa

Combinatorial diversity in human Ig genes			
Segment	Light chain		Heavy chain
	K	λ	H
Variable (V)	40	31	51
Diversity (D)	0	0	25
Joining (J)	5	4	6
Max no. combinations	200	124	16218
No. of possible HL combinations ~5.2 Million			

of a PCR protocol specific for each family, presenting different HLA allele combinations, making it time-consuming and unfeasible. The use of a preimplantation HLA matching protocol irrespective of the specific genotypes involved, facilitates notably the procedure.

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Chapter 14
Engraftment: Homing and Use of Genetic Markers

Scientific advancement should aim to affirm and to improve human life.

Nathan Deal

Abstract This chapter is focused to engraftment and use of genetic markers in cellular therapy. It is concisely explaining conditions for homing in recipient and the significance of both in Tissue Engineering and cellular therapy.

14.1 What is Engraftment/Homing?

Homing refers to the stem cells' innate ability to travel to the right place in the body—the bone marrow–suited for making blood. The term "engraftment" means that the stem cells have begun their work; they are functioning properly within the marrow by producing various kinds of blood cells. Not only that bone marrow is recruited with fresh pool of concentrated stem cells, but it is also being gradually repopulated by those cells that emerge through differentiation of transplanted stem cells. Experimental evidence suggests that manipulated stem cells may lose some of their homing and engraftment abilities [1, 2]. If this evidence is true for humans as well, a troubling paradox may arise: The very success of an umbilical cord blood transplant could be undermined by the manipulations performed on stem cells—manipulations intended to increase their healing properties, not decrease or eliminate them. Research needs to clarify this. Work of this kind, at the University of Minnesota, is crucial to the success of stem cell expansion [1].

14.2 Genetic Markers

As in gene therapy, stem cells that are being genetically marked are activated to accept new genes. But instead of receiving genes that change their behavior, they receive genes that serve as "ags" or markers that are reproduced and expressed in

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every generation of subsequent cells. The markers can then be used by researchers to keep track of stem cell activity in the body after transplantation. For example, genetically marked stem cells are being used in a new experimental protocol at the University of Minnesota [1]. In this study, one-third of the stem cells the patient receives has been expanded and genetically marked outside the body in a lab [2]. Definitely, this could have a very significant impact on development of Tissue Engineering which is based upon the integration of stem cells, scaffolds, and active molecules that are enabling and facilitating the three-dimensional (3D) growth of tissue cells.

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Chapter 15
Nanotechnology in Stem Cell Research

Stem cell research can revolutionize medicine, more than anything since antibiotics.

Ron Reagan

Abstract There is a tight bond between nanotechnology and stem cell research reflected in many directions. This chapter is a brief description of direct and indirect relationships operating in nuclear reprograming, magnetic bead technology, drug delivery and targeted drug delivery, cancer stem cell therapy, reduction of drug toxicity, remote control, etc.

15.1 Nanotechnology/Nanoparticles

Nanoparticles are entities between 1 and 100 nm in size [1–5]. In nanotechnology, a particle is defined as a small object that behaves as a whole unit with respect to its transport and properties. Particles are further classified according to diameter [4].

Currently many substances are under investigation for drug delivery and more specifically for cancer therapy [5]. Interestingly, pharmaceutical sciences are using nanoparticles to reduce toxicity and side effects of drugs and until recently did not realize that carrier systems themselves may impose risks to the patient. For nanoparticles, the knowledge on particle toxicity as obtained in inhalation toxicity shows the way how to investigate the potential hazards of nanoparticles. The toxicology of particulate matter differs from toxicology of substances as the composing chemical(s) may or may not be soluble in biological matrices, thus influencing greatly the potential exposure of various internal organs [5]. This may vary from a rather high local exposure in the lungs and a low or neglectable exposure for other organ systems after inhalation [1–5]. However, absorbed species may also influence the potential toxicity of the inhaled particles. For nanoparticles the situation is different as their size opens the potential for crossing the various biological barriers within the body.

From a positive viewpoint, especially the potential to cross the blood–brain barrier may open new ways for drug delivery into the brain. In addition, the

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nanosize also allows for access into the cell and various cellular compartments including the nucleus. A multitude of substances are currently under investigation for the preparation of nanoparticles for drug delivery, varying from biological substances like albumin, gelatine and phospholipids for liposomes, and more substances of a chemical nature like various polymers and solid metal containing nanoparticles [5–14]. It is obvious that the potential interaction with tissues and cells, and the potential toxicity, greatly depends on the actual composition of the nanoparticle formulation.

This paragraph in the chapter provides an overview on some of the currently used systems for nanoparticle drug delivery in cancer and cancer stem cell research [5–14]. Interesting ideas are coming from the work of Dr. Andrew Koehl who indicates an amazing fact that there are compounds on your breath that indicate illness, that has been shown through a number of studies and we can detect those with for that purpose designed nanosensors [6]. There have already been a number of research papers published suggesting we can detect cancer, tuberculosis, asthma [6].

Anticancer drugs can often shrink tumors but do not kill CSCs. Although CSCs might only make up a small part of a tumor, their resistance to drugs allows them to persist. They can then cause a tumor to regrow or spread cancerous cells throughout the body. Xiaoming He and colleagues aiming at development of a nanoparticle system to overcome these cells' defenses by placing anticancer drug doxorubicin into nanoparticles coated with chitosan, a natural polysaccharide that can specifically target CSCs have got positive experimental results [7]. Once in the acidic environment of the tumor, the nanoparticles degraded and released the drug. Tests on tiny, tissue-like clumps of both normal and cancer stem cells in vitro and on human breast tumors grown in mice showed that the therapy successfully killed CSCs and destroyed tumors. The mice showed no obvious side effects. The group has shown that chitosan binds with a receptor CD44 on cancer stem-like cells, enabling the nanoparticles to target the malignant stem-like cells in a tumor [7]. The magnetic beads for extraction of the CSCs are already designed. This will enable the extraction of each particular CSCs and tests for targeted therapy in vitro. Some of nanoparticles have been used in the induction of iPSC. All other nanoparticles will probably be more precisely tested and used as targeted or eliminated [15–17]. Development of AFM is very instrumental in examination of both surfaces and inner part of nanoparticles as well as their physical changes in contact with different polymers, which is the developing field per se for future applications in medical and other purposes.

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Chapter 16
Stem Cell Therapy: Optimization, Regeneration, Reprogramming, Expansion, Tissue Engineering

Everything is theoretically impossible, until it is done.
Robert A. Heinlein

Abstract A very brief overview on five important aspects of stem cell therapy are given in this chapter. The chapter is dealing with criteria for optimization of the pattern for cell-based therapies, extent of regeneration in animals and humans, nuclear reprogramming as a possible modality for treatment with pluripotent adult cells, significance of expansion, and essential role in Tissue Engineering.

16.1 Optimization of the Pattern for Stem Cell Treatment

It is an inevitable fact that the number of stem cells for the treatment is critical for the success of application [1]. The contraversive data from clinical arena are the results of still not synchronized criteria for different type of treatments as well as the fact that the stem cells are still heterogeneous group of cells due to their specific position and dynamics within that [2–11]. Further work is necessary to rectify these discrepancies. However, at least the choice of cells is pretty much limited to HSCs, MSCs, and probably in the future iPSCs. Most of the improvements in different diseases are coming from use of these candidates with so far the best reputation. The criteria for their pluripotency should be completely precise and evident in each particular case.

16.2 Regeneration in Animals and Humans

It is quite clear that different mechanisms are governing plant and lower animal organisms in comparison to higher vertebrates. While plants and amphibia can regenerate part or entire body, humans are extremely limited after their fetal life. There is a significant movement in that direction caused by research of Steven Badilack, who used pigs' intercellular matrix molecules in order to regenerate the

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tip of the finger (not a joint). The lower animals activate their stem cell pool known as *blastema* at the place of lost limb and renew that without the problem. The humans react with accumulation of fibrocytes and fibrous tissue in order to heal with scar. The plant has unlimited growth and can reproduce the leaf. Are the mechanisms for regeneration "forgotten" in humans?

16.3 Nuclear Reprogramming as One of the Methodological Modalities to Get iPSC

It was unimaginable till recently that one can revert the process of matured somatic cell into embryonic stage, but after Gudron and Yamanaka did it, humanity is in front of great possibilities. The research is in advance to determine whether the iPSCs produce tumors, in order to exclude that possibility and use them without hesitation in therapeutic purposes.

16.4 Significance of Expansion

A great achievement in cellular therapy is also a possibility for expansion with different active molecules, media, hypoxia, etc. since it opens the door for rational use of cord blood, amniotic fluid, placental stem cells instead of any other source if necessary and inevitable. This work will expand with understanding of nanoparticles and other active molecules within the concept of stemness and renewal [12–22].

16.5 Stem Cell as a Part of TE Triangle

It is generally accepted that stem cells are the part of Tissue Engineering (TE) triangle which includes

1. Stem cells
2. Molecules of extracellular matrix
3. Scaffolds.

This is the breakthrough in stem cell research with capital discovery of 3D printing and new approaches in Tissue Engineering. The field of scaffolds gave us the new insight into materials and their use in natural or artificial form for TE purposes. This is expanding area with a lot of new discoveries which are going to change the future very soon [14, 15, 21].

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Chapter 17
What Are Positive Results of Stem Cell Therapies?

With Joseph Levy

Science is the great antidote to the poison of enthusiasm and superstition.

Adam Smith

Abstract The chapter is dealing with expectations and results related to stem cell therapy. It summarizes pros and cons for stem cell therapy, use of particular patterns, and final output in different diseases. The controversive debate about adult stem cell pluripotency is underlined. The encouraging results are presented and critical remarks stated.

17.1 An Overview of Some Disease States in Which Stem Cells Could Help

This author has already published from this field therefore, we recommend the books for more detailed reading [1, 2]. Due to the plasticity of at least ES cells, there appears to be virtually no limit to the number of disease states which these cells could help improve, although there is still a lot of controversies dependent on the model used, cell types applied, and other criteria critical for engraftment [3–9]. The majority of conditions which are being examined for study with both embryonic and adult stem cells have the similar nature of being degenerative in some manner [10–13]. Whether it is through injury or deterioration, these states show a decrease/end of tissue function due to the loss of cells in a specific region. Despite apparently successful and helpful intervention with some adult stem cells, there is a category of scientists which are very skeptical with respect to adult stem cell plasticity and/or pluripotency [14, 15].

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17.2 Heart Attack

In an event such as a heart attack, a large number of cells within the heart are killed due to the lack of nutrients flowing to them. This can often lead to a significant weakening of the heart muscle and functionality. Currently there are no available solutions for repairing such damage to the heart, and the body is typically unable to cope with the large degree of damage done in these instances. Through stem cell therapy though, there is evidence that the damage done can be corrected. By introducing large volumes of stem cells to the affected region, it has been shown that the cells will not only differentiate into cardiac muscle, but also restore functionality to the entire tissue [16–22]. As the heart tissue is so unique in its properties, it is very significant that these cells are able to recreate the structure and functionality.

17.2.1 Optimization of the Stem Cell Source for Intracoronary Grafting

Post-acute myocardial infarction (AMI)-personal experience with Dr. Bela Balint
As mentioned, plasticity is very desirable, but still disputable feature of stem cells. Effort has been invested toward identification of primitive populations of Hematopoietic Stem Cells (HSCs), the ones that would have had high level of plasticity as intended in a proper bioengineered scenario [23–25]. It is also noticed that one low-dose of Neupogen (3–5 μg/kg BM) can activate Bone Marrow (BM) with consecutive in situ multiplication and activation of HSCs in the niches [25]. This contributes to more efficient collection of primitive populations of HSCs for conventional transplantation and regenerative medicine purposes. Valgimigli et al. [26] used the granulocyte-colony stimulating factor during acute myocardial infarction to enhance bone marrow stem cell mobilization in humans: and gave clear clinical and angiographic safety profile. Again, in our more than a decade long clinical work crucial question was: can optimized, most primitive HSCs transdifferentiate into myocardio-myocite lineage in in vivo conditions after AMI and can it be proved and checked in clinical arena? For that study younger than 71 years old patients, with large AMI of the front left ventricular wall which 5 days after AMI have had ejection fraction of left ventricle less than 41% but more than 19%, were studied. They were without any known disease or status of influence upon surveillance or heart function, and without significant damage of heart valve functions. Stem cell mobilization and harvesting were done in a standard manner including apheresis and positive cell selection with emphasis on $CD34^{+}$ subtype [22]. Autologous, pluripotent progenitor adult HSC aspirate from BM have been used beside apheresis and both have shown to cause AMI improvement measured by clinical parameters of ventricular ejection and PET during decade of work of

Bela Balint's group [1, 2]. Application was done into coronary arteries, and myocardium. Results could be summarized as following:

- Primitive HSCs express plasticity proven through recovery of left ventricle ejection fraction within clinical follow-up showing.
- Preliminary results indicate positive influence upon reduction of negative remodeling of the left ventricle.
- Future studies require larger patient cohort to define statistical relevance.

17.3 Physically Induced Injury of Neural Tissues

Spinal Rupture

One such example of a physical injury resulting in a loss of tissue function is the breaking of a nerve pathway resulting in the loss of function of both, the tissue excited by that nerve, but also any others located down its pathway. These physical ruptures are typically caused by a significant and sudden stress placed upon the neural tissue or by a structure striking the tissue and severing it. While not as common, an increasing number of instances where the neural structure is crushed due to an external trauma have also been shown to cause loss of functionality of the neural pathway. In these cases, the neural tissue is left disconnected or too damaged to function along its length and due to its own properties is unable to properly or completely repair itself. With current technology, a person suffering from such an injury would be highly unlikely to heal completely, and would most likely lose some function of their self due to this injury.

Current technology can still provide some help with these such injuries, but within our own healing abilities, the damage done often results in tissue formation which is not concurrent with that of the original [22]. The result of this different tissue being introduced at this junction can result in poor conduction through the nerve, if conduction through is still even possible [27].

Through the use of stem cells, it has been shown that nerve cells can in fact be regrown in the locations of these breaks [22, 28]. When introduced to these locations and induced, it has been shown that the new structure is not only functional, but capable of restoring complete functionality of the tissue. Though there are still many limitation of this process, the ability of stem cells to function in this way show the promise of method.

Stroke

Similar to the events which lead up to a heart attack, a stroke is often the result of a large cell die off within the brain due to an obstruction within the vasculature leading to the brain. When an instance like this occurs, the individual will often not only lose tissue, but functionality as well. In this case though, functionality goes

further than just the organ itself. Since the brain controls many of the functions of the body as well as having been trained to many further functions, these loses can be much more significant. With notable loses in both motor control as well as memory, the damage done by a stroke is one of great issues within the body. Even with time and therapy, there is no current technology which can fully repair the damage done by one of these events. Currently all that can be done is to reteach the body how to perform the functions it had "forgot" due to the damage done by the event.

Stem cell therapies applied to stroke victims have been met with limited success due to the nature of the damage done. Although stem cells have been able to be isolated and implanted or mobilized to the locations of damage, they are often not able to restore all that is lost due to the nature of the brain. Priller [29] has shown by immunohistochemistry that adult bone marrow stem cells populate the brain. The treatments though have been shown to have significant influence on functionality which is not associated with some of the higher functionality feature of the brain. Functions such as balance and communication within the brain have been shown to be significantly improved through the introduction of stem cells after these events [30–33]. These abilities are some of the basic functions of the brain and work on a lower cognitive level when compared to movement, intellect, or reception of the senses. There is a spectrum of clinical and experimental approaches to the stroke with different type of stem cells (MSC, HSC, human BM stromal cells) which all show that adult stem cells from blood and BM can turn into different types of neurons [34–42]. And although the results do require to be further explored, in order to get significant improvement in function after the stroke, they are encouraging.

17.4 Degenerative Injuries

Autoimmune Diseases (AID)
In general, the existence of AID reflects the broken tolerance of immune system which is perpetuating the flares, and it is impossible to permanently cure the AD by trying to replace sick immune cells with healthy stem cells. However, longer remissions are possible unless we do not figure out how to treat these devastating diseases. This, stem cell therapy in specific diseases, may play the role of adjuvant therapy in concert with other medication.

Amyotrophic Lateral Sclerosis (ALS)
Amyotrophic lateral sclerosis (ALS), commonly referred to as Lou Gehrig's disease, is a progressive disease which is characterized by the loss of motor neurons in the spinal cord and brain stem. This loss is associated with the atrophy of muscles

within the body typically resulting in paralysis and death [43–46]. Even though this disease has been shown to cause such significant harm, very little is known about it, nor has any effective treatment or suppressant been found.

Due to these concerns, stem cells have been targeted as the possible "magic bullet" for ALS. In studies performed by Letizia Mazzini and colleagues there have been significant advances made in the quest for a treatment, if not a cure [43]. In their study, neurons were grown ex vivo and then implanted into ALS patients. Thanks to these grown neurons, the muscles not only showed a slowing of muscle atrophy, but in some cases even increases in muscle strength after three months [43].

Multiple Sclerosis (MS)

Another progressive disease, multiple sclerosis (MS) targets the myelin of the nervous system, in specific regions of the CNS for the optic nerve, spinal cord brain stem and cerebellum [19, 47]. The deterioration of these tissues comes as a result of a few factors including plaques attaching and degrading the myelin as well as local inflammatory responses of the body [19]. Even with these loses; the nerve itself appears to remain whole, with little to no damage occurring on the axon, body or dendrites of the neuron. The results of this degeneration are a general weakening of limbs eventually leading to limb paralysis, gait ataxia, and brain stem symptoms [19]. This degeneration typically takes place over many years, with symptoms presenting themselves before subsiding leaving the individual in a more inferior state. Even though myelin does eventually regenerate around the axon of a nerve, it is often much thinner than what it was previously. Also, the rate at which myelin regenerates is much too slow and inefficient to completely help recover the damaged tissue. Even with thorough study of this disease, the scientific community is still unsure of what the cause of MS is; only noting a few cues as to what may lead someone to be susceptible.

Current treatments for MS focus on mitigating flare-ups, trying to keep localized swelling down as well as preventing an immune response in the area. It has been noted that the immune system and response plays a significant role in the degeneration of the myelin. With stem cells appearing to be a source for better myelin generation, they have been a steady source of interest [19, 47]. Works in other studies have shown that stem cells are able to help re-myelinate nerve cells in order to improve signal transmission due to spinal cord injuries [48, 49]. The notable difference between myelin regenerated typically in the body and that generated through stem cell therapy is the thickness of the myelin. Stem cell generated myelin typically appears to be much thicker than that regenerated by the body, this results in better signal conduction through the nervous system [49]. An additional benefit to stem cell therapy for MS is its ability to lower the amount of inflammation in an area. This secondary benefit should not be overlooked, as the inflammatory response of the body appears to have almost as much of a deleterious effect on myelin as the plaques generated by the disease.

17.5 Persistent Disease States

Alzheimer's Disease (AD)
Alzheimer's disease (AD) is a type of dementia, another progressive disease state, associated with the formation of amyloid plaques within the brain [50]. Due to the presence of these plaques, the brain tissue degenerates over time, causing the individual to lose learned skills, until eventually the brain is deteriorated to the point of losing the capacity for basic life functions. Currently there are no treatments for this disease, with the scientific community unsure of how to prevent the plaques from forming or damaging brain tissue.

Current research into treatments for AD has led investigators to injecting neural stem cells into an injured brain [50]. Although only early in the research phase, trials on mice have shown that injected stem cells have been able to regenerate damaged brain cells [50]. In addition to direct injection, researchers are attempting to allow stem cells to migrate to the regions of damage and use secondary treatments to increase the rate of cell proliferation [50]. These treatments have been shown to not only increase the number of stem cells present within the brain tissue, but also reverse the effect of AD in mice. This new treatment option has also been proved to lower the amount to the amyloid plaques within the brain tissue [37]. A feat not copied in any of the current treatment regimens available.

Parkinson's Disease (PD)
Parkinson's disease (PD) is a neurodegenerative disease affecting the dopamine producing cells found within the brain. PD is characterized by the death of at least 80% of the dopamine producing cells found within the substantia nigra. The death of these cells not only causes tissue loss in the brain, but lowers the brains ability to produce dopamine; it is this by-product of PD which causes the problems associated with this disease. Dopamine within the brain is responsible for allowing the body to have smooth and coordinated movement [51]. Current treatments for this disease focus on providing the body with a dopamine substitute, typically the drug Levodopa (L-dopa), which the brain can then use to help compensate for its lower production. This treatment is not a permanent solution, as the effectiveness of L-dopa decreases over time, to a point where it is no longer effective and the side effects of the drug become too great (1).

Current work on treatments for this disease using stem cells has been mixed with various methods and approaches taken. One of the most successful has been the use of adult stem cells, and in particular neural stem cells, to grow neurons in vitro. The grown neurons were then implanted into the patient where they were able to help improve the condition of the patient. The neuronal stem cells used had the added benefit of not causing an immune response within the patient due to their level of differentiation unlike their embryonic counterparts. Trial with mesenecaphali tissue reach in postmitotic dopaminergic neurons has provided a proof of principle that neuronal replacement can work in human brain [1]. This study alone should not be

taken as the end of this disease as a threat, but a good stepping point in the hopeful treatment of this serious and debilitating disease.

Huntington's Disease (HD)
Huntington's disease (HD) is another progressive neurodegenerative disease with few treatments currently available [52–65]. HD is characterized by the presence of a specific sequence of repeating subunits within the huntingtin gene [52]. The disease has been shown to cause the loss of medium spiny projection neurons within the striatum of the brain. This degeneration appears to be a result of a misfolding of a protein associated with the huntingtin gene. As the body ages and repairs damage done to the gene from oxidation, repeats of the extra segment found within the huntingtin gene expand and result in the malformed protein [66].

Treatments for this disease using stem cells focus on the production of the spiny projection neurons of the striatum. These grown neurons are then implanted into the striatum in an effort to replace the lost tissue [64, 65]. Unfortunately, results of this treatment has been less successful than other avenues as the number of cells needed to provide relief have been hard to generate in the lab. There is also the issue of the continuing production and accumulation of the abnormal proteins which cause the loss of the neurons to begin with. This is not the end for stem cell treatment for HD as many still believe that stem cells are a necessary component for any cure as a way to replace the lost neurons within the brain once the issue of the degenerative protein has been solved.

17.6 Concerns with Stem Cells

Stem cells do pose many problems along with the benefits they have shown. One of the most recent issues being addressed is the concern over cell growth and replication associated with cancer. New studies suggest that cancerous masses have some stem cells present within them and could be a cause for the expansion of the mass [67]. This finding raises concerns with the treatment model of many cancers, as during many of the treatment cycles; stem cells are also introduced to the patient. This can have the unwanted effect of allowing these stem cells to be added to or changed by the cancerous mass and allow the reformation of the cancer in the body at a later time. In addition, the presence of stem cells with cancers has pushed forward the idea that these cell growths are not as different from normal cell replication as once thought [68, 69]. Due to this, many are rethinking their approaches to cancer treatments and methods of approach.

Another subject of concern with stem cells is the ability to direct their differentiation. This issue comes in no small part from the issue of cell numbers. Since stem cells are so few in number within the body, it is important to be able not only to direct their development into the cells of interest, but also grow their numbers in a culture. One of the issues found with the culture is the differentiation of the cells

[1, 2]. Since the growth medium has an effect on the stem cells due to its components, the cells begin to differentiate. This may result in cells further down a developmental line which could cause them to be unable to differentiate into the cell line of original interest.

There is also the issue of mobilizing stem cells form certain areas within the body [70]. When larger volumes of stem cells are needed for a task, it is beneficial to allow some stem cells from other areas of the body to diffuse to the region of interest. The concern here is how effective the process is with targeting the region which the stem cells should go to, and how many cells need to be mobilized to create the necessary number at that location.

17.7 The Recovery of the Five Senses with Stem Cells: Are the Concepts Realistic?

As we know, the five main senses of the human body are: hearing, vision, smell, taste, and touch [71–89]. Sensory loss can occur due to nerve damage, head injuries, and ineffective nerve receptors from birth or infections [72, 73]. However, sensory loss for the specific senses listed above come in the form of: deafness, blindness, anosmia, ageusia, and somatosensory loss (usually through paralysis). Various types of stem cells are being used to repair or recover these damaged cells, with the most notable successes in vision and hearing repair [71, 89].

Hearing

How do we hear things? The ear has many parts that allow us to hear. It has the capability of processing sound waves. The most important is Cohlear part located in the inner ear with cells containing hairs that react to sound waves (receptors for the sound). There are 11,000 of these hairs cells that convert sound waves into electrical signals which are extremely important in the hearing process. Damage is often irreversible, and it cannot be fixed naturally. The solution is still conceptual.

Stem Cells in the Cochlea: A concept

Using induced pluripotency (iPSC), skin cells can be reprogrammed into stem cells. These cells can be implanted into the cochlea. This may allow the hairs inside of the cochlea to regrow. Using stem cell therapy, this is a viable way to improve hearing in patients with hearing loss. No prosthesis is required, this uses the patient's own cells. No risk of rejection of the organ by the patient is expected [71].

Nerve Cells

Directly take the signals produced in the cochlea to the brain. Scientists have made neuron connections in vitro with induced stem cells [71]. Experiments involving animals showed hearing improvement [71]. This may be crucial in improving human hearing. What is the future of hearing? The research and science is still new.

The process of using stem cells to cure hearing loss will take time to develop. Research is being done today, leading to advancements for a better tomorrow.

Vision: Macular Degeneration

Macular Degeneration is an eye disease that leads to vision loss. It is caused when the central portion of the retina (macula) begins to deteriorate. The condition begins with a decline in detailed vision, and slowly worsens until central vision is completely lost.

Vision: Macular Degeneration (Receptors). Macular degeneration is said to be the biggest candidate for stem cell therapy since it involves the loss of cones, rather than multiple types of cells. Stem cells that have been used in research are: embryonic, fetal, umbilical cord, and bone marrow. The most success has been seen with embryonic stem cells due to their high potency. "ARMD" is a common eye problem caused by the loss of cones. Bernier's team has developed a highly effective in vitro technique for producing light-sensitive retina cells from human embryonic stem cells. "Our method has the capacity to differentiate 80% of the stem cells into pure cones," Professor Gilbert explained. "Within 45 days, the cones that we allowed to grow towards confluence spontaneously formed organized retinal tissue that was 150 microns thick." (G. Bernier, Universite de Montreal) [83].

Vision-the optic nerve

Vision loss due to damage of the optic nerve is hard to treat due to the loss of functioning neurons and cells such as oligodendrocytes [83–86]. Oligodendrocytes are cells that produce myelin, a fatty substance full of proteins, which surround neurons. Optical nerve atrophy is a new and upcoming topic of research in the field of stem cell therapy. We recommend

– https://www.youtube.com/watch?v=qPQinGzSGFU

What is the future of vision? Stem cell therapy towards correcting vision has a lot of potential. There are many studies that are directed towards treating the loss of vision. Scientists are researching ways to "switch on" dormant light-sensitive cells in the retina to cure degeneration. The use of embryonic stem cells is seen as unethical, so researchers are now looking into using menstrual blood [89]. It was discovered that menstrual blood has similar stem cell properties to the ones found in the umbilical due to their immaturity [89].

Smell

Humans perceive smells when odor molecules bind to cilia (hair-like receptors in the nose), which trigger neurons at the top of the nasal passage and send an electrical pattern to the brain. In the brain, the pattern is interpreted as a specific odor by the olfactory bulb. Old smells are linked to memory, which allows people to recognize and distinguish between different scents [77–81].

Anosmia

Anosmia: inability or greatly lessened ability to perceive odor. Some 3–5% of Americans suffer from anosmia. Current treatment for anosmia is mostly limited to steroids and antibiotics, although there are surgical options for serious cases. Anosmia also affects the way food tastes. Causes: nasal sinus disease, head trauma, exposure to toxic chemicals, and most commonly, viral infection [80].

Nasal SCs are multipotent: they can turn into neurons or supporting cells of nasal tissue. Research center called Monell is currently researching SC therapy for viral anosmia. Researchers are attempting to identify and isolate nasal stem cells from healthy volunteers and successfully develop them into olfactory receptor cells in vitro. Then, the fully functional cells will be transplanted to an anosmia.

Successful Attempts: University of Michigan successful repaired the cilia of mice with SCs. Use of umbilical cord blood to regrow olfactory cells problems: Each subset of neurons can only detect specific smells. It will be difficult to make sure that the engineered cells send the right signal. We will work on humans within a decade or so.

Taste

Ageusia is the loss the sense of taste [87, 88]. There are many potential stem cell treatments to reverse the effects of ageusia. Taste stem cells have recently been discovered on the surface of the tongue. The discovery of these cells could help restore the taste of patients who have undergone chemotherapy, and the elderly.

– https://www.sciencedaily.com/releases/2013/02/130204094520.html

The taste stem cells have a marker known as Lgr5. The marker indicated two types of cells [88]. The first were strong stem cells found on the surface of the tongue, and the other type lies a layer beneath. Future studies involving the taste stem cells and Lgr5, will be focused on differentiating the cells into different taste cell types.

The Importance of Touch

The sense of touch is a very important sense that allows the brain to be alerted to close proximity objects that may be harmful for the body. Discomfort, pain, and irregularities near the body may need immediate action by the brain; touch allows the brain to react accordingly. Touch is also important in the psychological development of children. It is also critical in social situations, allowing for trust to be built between people such as hand shakes, hugs, etc.

How does it work? The sensation of touch is activated for a certain body part when it comes into contact with another object. Nerve endings that are located in the lowest layer of the skin, the Dermis, send signals to the brain and spinal cord when a sensation is triggered.

We understand that sensation as touch.

How touch can be damaged? As nearly the entire body can perceive touch, the sense is very vulnerable to damages. Burns and deep cuts can damage the nerves

that are responsible for touch. Even though skin transplants are available, the sense of touch may be compromised when using skin therapy that does not include nerve reconstruction.

A Japanese team has been able to create skin using induced pluripotent stem cells [75]. They claim that the skin functions normally, able to touch, sweat, produce oil, and grow hair. The researchers tested the new skin on mice, and saw results within 14 days of the experiment. This technology can be used on humans, allowing for patients with large wounds to recover and preserve tactile sensory in the area that has been harmed. In addition to the actual skin, the nerves that transfer the information will also need to be repaired.

Spinal cord injuries also cause sensory malfunctions in patients, and other symptoms including paralysis and chronic pains. By implanting stem cells into the patient's spinal cord, which cannot naturally regrow, researchers were able to return sensory to patients [75].

Touch for bionic limbs

Researchers are developing technologies that can allow bionic limbs to "feel" touch, heat, and vibrations, similar to human limbs. Still early in development, the technology can prove revolutionary for people who wish to have sensory in their bionic limbs. The technology uses microchips that mimic human neuron charges, allowing it to give sensory signals via nerve cells.

17.8 Conclusions

Stem cells are a rapidly growing and important part of science and technology of the day. They are being used and researched for many of the diseases which we face today and have been shown to be an important, if not necessary component of most treatments and cures. This use and belief holds true even with our limited knowledge of their full function and potential. It will be critical in the future to further explore these crucial cells for not only their functionality within the body, but how we might be able to modify them for our own purposes. This will require a large commitment to the study of these cells as well as a greater partnership with research and clinical testing.

In the future, research with stem cells will need to develop not only better methods of obtaining these cells, but also improved ways of increasing their numbers. As stated earlier, the ability to keep stem cells at the high level of plasticity in vitro has been a challenge, and one, which if solved, can be one of the greatest leaps in the field. The other point to contend with going forward is where we think stem cells should lead us. Many feel uncertain if stem cells should be used to develop entire cellular systems, as they feel that concerns with cloning could be brought forth.

It is essential to keep in mind the importance and promise these cells have. Even with our limited understanding of these cells, we have been able not only to treat, but also to cure many issues which were once thought to be incurable (even if this is still currently at the research and testing level). These giant leaps forward in science and health are too great to overlook and should be kept in the forefront of research and given as much support as possible for the promises they hold and have even already delivered upon.

Overall, stem cells are a very viable option to repair sensory loss. There have been successful attempts of hearing and vision recovery on humans. There have been successful attempts of smell and touch recovery on mice, and progress is being made to use the techniques on humans. This research can be monumental for someone who has lost or is currently losing a sense. Does it mean that the future of stem cell research is bright?

For those, who would like to gain more precise knowledge, there is extended list of very classical and valuable references on this matter [89–211].

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Chapter 18
Topic Novelties in Animal Stem Cell Research

Nothing has such power to broaden the mind as the ability to investigate systematically and truly all that comes under thy observation in life.

Marcus Aurelius

Abstract Physical aspects of targeted therapy approaches especially of CSCs are the topic of the cancer stem cell research. Whether they will appear to be better then classical chemo and radiotherapy, or as additional treatment, is still a matter of investigation. This is a brief summary with respect to possibilities with emphases on biophotones.

18.1 Biomagnetism, Electromagnetism, and Biophotonics

In the Chap. 12 we have mentioned the possibilities that the cancer is caused by increased electromagnetic activity of the cells. This means that for understandable cause, we can create the cure.

The entire world is moving, constantly waving and what connects us, are the frequencies [1, 2].

Where does the body's electrical field come from? As it is mentioned, macrocosmos is replicated in the body micro-cosmos—the cell in which all body's atoms and electrons during their constant revolutions and movement from one to other location create micro-currents (described earlier) micro-potentials and electromagnetic fields which are the basis of body energy field. This energetic field is increasing very much by additional biophotonic radiation originated from DNA which carries the signals for regulation of life processes [3, 4]. Based upon such understanding in medicine, the new horizons are opening, which have the fundament in laser light, and electromagnetic oscillations are the manifestation of its development. The stem cell research in future will rely vary much on that.

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Chapter 19
Resume

Science, like art, religion, commerce, warfare, and even sleep, is based on presuppositions.

Gregory Bateson

Abstract A brief summary on entire matter of animal stem cells is given. The outlines of future research directions are roughly drawn.

19.1 The Directions in Animal Stem Cell Research Development Require Further Work

Stem cell cancer concept had reached its spike this year. Further work is necessary in basic research on:

- More firm and precise definition of stemness
- Optimization of best candidate for different tissue engineering manipulations in clinical arena and optimization of scaffolds for particular tissue engineered patterns
- Expanded work on cancer stem cells in order to discriminate origin, underlying causes and mechanisms of the sort of malignancy and particular, selective, targeted therapeutic approach.

It is clear nowadays that the presence of multipotent stem cells in the adult might open up new therapeutic opportunities on the basis of tissue and organ replacement. Therefore, the exact definition of stem cells and the ability to isolate them are matters of supreme importance. However, despite the efforts of many investigators who strive to determine their nature, a definitive stem cell "portrait" is lacking. Yet, quite recently, two independent studies claimed to have identified a stem cell-specific group of genes that form a "stem cell signature." In fact, these studies have defined two different and unrelated groups of genes; the conclusion that these signatures characterize stem cells is therefore premature. Experimental and/or technical reasons might explain the disparity of the results from these independent studies, and alternative approaches that might lead to identification of the "correct"

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gene-expression profile of stem cells were suggested. But should one expect to find a stem cell-specific signature using an approach based on the analysis of gene-expression? Due to the complexity of the problem, the solution to determining the molecular configurations that dictate a stem cell state should, therefore, come from an overall genomic and proteomic analysis, coupled with mathematical modeling.

Hematopoietic stem cell transplantation remains a risky procedure with many possible complications. It has traditionally been reserved for patients with life-threatening diseases, such as malignancies. While occasionally used experimentally in nonmalignant and nonhematologic indications, such as severe disabling autoimmune and cardiovascular diseases, the risk of fatal complications appears too high to gain wider acceptance. Yet, this is the most well known and the most developed stem cell regenerative approach, given that if successfully engrafted, it repopulates and later on recruits the new, healthy bone marrow cells in circulation.

Embryonic stem cell research is still the matter if controversies at a very stratified levels, although many researchers agree that it might be the source of stem cells with the highest differentiation potential.

The experimental and clinical trials have shown both in animal models and humans the neovascularization and myocardial tissue repair through transdifferentiation into myocardiocytes, or some other mechanism. Repair of damaged organ/tissue (myocardial, neuronal, liver, cartilage, bone, etc.) is shown mostly in animal models, although very good data are coming from the Belgrade group in treatment of AMI (Balint et al., already mentioned). Maybe the most illustrative of all is the bunch of experimental data suggesting the great potential for stem cell differentiation and homing into damaged tissues either when mobilized or injected into the tissue of interest after apheresis or BM puncture, with or without cryopreservation. Although the adult stem cell regenerative therapy after BM aspiration and apheresis injection into coronary arteries is becoming more and more successful, the most evident success of mesenchymal stem cell treatment at regenerative therapy level in clinical arena is seen so far in children with osteogenesis imperfecta where the results with diseased children dramatically visible and easily reproducible. Yet, due to the obstacles already mentioned above, this is not the case with nervous system regenerative treatment, especially in humans.

The key for managing diseases and cancers with personalized medicine may lie with iPSCs. The progress in this field has made tremendous strides since iPSCs were discovered nearly a decade ago. However, to proceed with human clinical trials, it is crucial we discover the safest, most efficient method for reprogramming cells to a pluripotent state. Once this critical step is accomplished, the therapeutic applications of iPSCs could be limitless.

Apparently, basic adult stem cell research is still evolving, and is the matter of ever changing issues. Due to our extensive studies, but yet limited knowledge on their behavior and potentials, it is not yet easy to determine how to act in clinical arena. It is obvious that each approach to any particular disease or damage has to be optimized within team work and by bridging the gap between fundamental and clinical studies. Knowing molecular level in depth, will help clinicians to

orchestrate the team work and overcome critical obstacles in each particular scenario. There is no doubt that adult stem cell therapy (and probably embryonic as well) belong to the future, but we have to act as that we shall belong to the future, as well. Continuous efforts in both molecular and clinical directions will lead to the unique and optimal plan for each particular regenerative treatment. How far away we are from that goal it will inevitably show up in a near future.

19.2 Quo Vadis?

It is not easy to comment briefly the overwhelming field of stem cells [1–3]. There is tremendous piece of work beneath, which started decades ago. It was going parallel with the development of instrumentation, methodological approaches, protocols for application and continuous need for more information in all areas of science. This book reflects the effort to show the intellectual investment of countless people from research, clinic, and application of this knowledge on stem cells in multiple purposes. This effort will for sure result in more comfortable life and more precise therapeutic treatments. The molecular level and level of electromagnetic waves as well as biophotonic frequencies will advance in detection and application [1–3]. It will open new avenues in discoveries and therapy. We have accomplished just a part of that long road to be traveled [4–16].

There are two worlds that we consider alive on this planet: animal and plant. They have their similarities and differences. This interaction can tell us a lot about the past as well as the future. Let us enter now in the other world-the world of plants and their stem cell phenomenology. What is similar? What is different? What is so particular?

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Part II
Plant Stem Cells and Bioengineering

Ksenija Radotić

Introduction

Plant growth is a phenomenon different from animal growth. Animals exert characteristic determinate growth.

- After fertilization, the zygote cells are rapidly dividing, through self-renewal and resulting undifferentiated cells.
- After a certain critical stage, the cells differentiate, mature, and form tissues. In this stage their development is finished. There are exceptions to this (i.e., stem cells in bone marrow). What is the difference?
- In most animals body plan is preprogrammed, and therefore their body is predictable in shape and structure (most humans have two hands, two legs, 10 fingers and toes, two eyes, a heart with four chambers, etc.).
- Most animals grow until a certain age.

Plants have a characteristic growth pattern called indeterminate growth.

- Such growth pattern is possible due to existence of areas of rapidly dividing, undifferentiated cells that remain throughout the life of the plant. These areas are called meristems.
- Meristematic tissue continues to rapidly divide producing undifferentiated cells which may eventually differentiate to form the tissue and cell types.
- Plants are more unpredictable; they do not have a preprogrammed body plan. There are constants like leaf shape and branching patterns (opposite, alternate, etc.) but it is impossible to predict where a new branch will appear on a tree.
- Plants continue to grow throughout their life.

Chapter 20
Stem Cells in Plants. Meristems

Each problem that I solved became a rule, which served afterwards to solve other problems.

Rene Descartes

Abstract This chapter gives definition of plant stem cells, which are located in the organized structures called meristems. Definition, description, and function of different types of meristems (primary and secondary meristems), and their location in a plant are provided.

Plants that are several hundred years old and yet produce new organs are one of the impressive examples of plant developmental capacity. The origin of this capacity is in the continually dividing cells in meristems, which function like stem cells in animals. Plant growth is concentrated in these localized regions of cell division, called apical meristems. Nearly all nuclear divisions (mitosis) and cell divisions (cytokinesis) occur in these meristematic regions.

Apical meristems are located on the tip at each side of the plant body—elongation of both root and shoot takes place as a result of repeated cell divisions and subsequent elongation of the cells produced by the shoot apical meristem and the root apical meristem (Fig. 20.1). Growth in this direction is known as primary growth. Primary growth is found in herbaceous and woody plants, and by the other classification in monocots and dicots. The root and shoot apical meristems are called primary meristems. After germination, the activity of these primary meristems produces the primary tissues and organs that constitute the primary plant body.

In most plants there are also various secondary meristems, developing during postembryonic development. The structure of secondary meristems can be similar to that of primary meristems, but some secondary meristems are quite different. Such are inflorescence meristems, floral meristems, intercalary meristems, and lateral meristems (the vascular cambium and cork cambium). Vegetative meristems may be converted directly into floral meristems when the plant is induced to flower. Floral meristems differ from vegetative meristems in that instead of leaves they produce floral organs: sepals, petals, stamens, and carpels. Besides, floral meristems are determinate, meaning that all meristematic activity stops after the last floral organs

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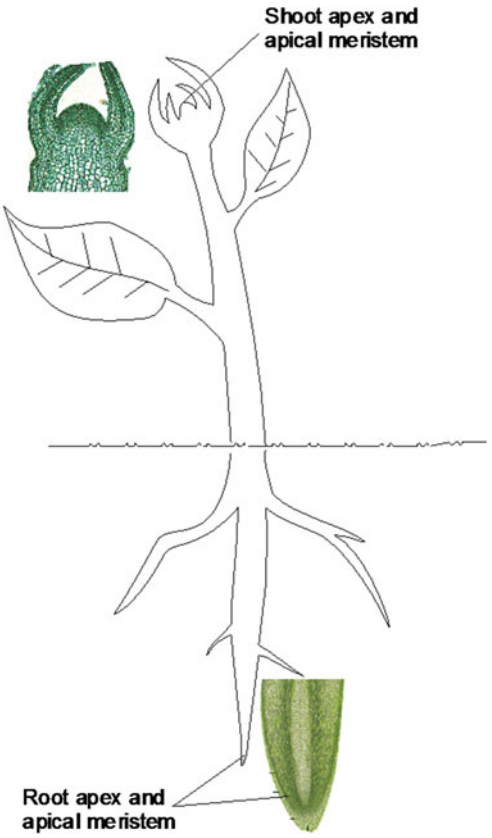

Fig. 20.1 Schematic representation of the position of plant apical meristems

are produced. In many cases, vegetative meristems are not directly converted to floral meristems, but they are first transformed into an inflorescence meristem. An inflorescence meristem produces different types of lateral organs comparing to the types produced by a floral meristem. The inflorescence meristem produces bracts and floral meristems in the axils of the bracts, while floral meristems produce sepals, petals, stamens, and ovules. Inflorescence meristems may be determinate or indeterminate, depending on the species. Intercalary meristems are formed within organs, often near their bases. The intercalary meristems of grass, leaves, and stems enable them to continue to grow despite moving or grazing by cows [1, 2]. The lateral meristems in the stems are most prominent in vascular plants (most trees and shrubs), called vascular cambium—located near the periphery of the plant, usually in a cylinder, and producing an increase in girth. Growth in this direction is known as secondary growth. Lateral meristems and secondary growth is found in all woody and some herbaceous plants, and by the other classification only in dicots [1, 3].

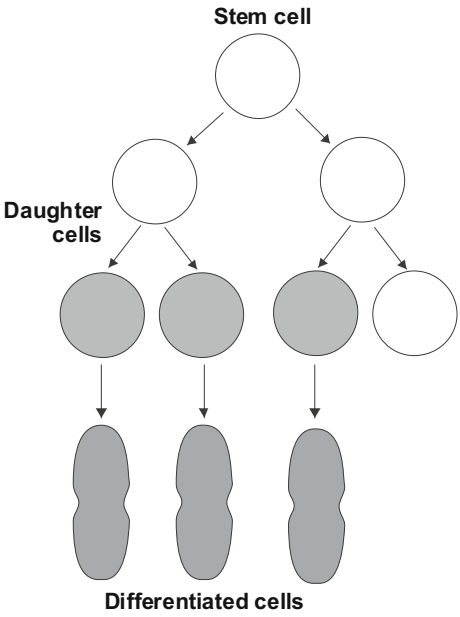

Fig. 20.2 Schematic presentation of generation of daughter cells by division of stem cells. Some of daughter cells retain the properties of stem cells, while the others differentiate

Meristems are populations of small, isodiametric (having equal dimensions on all sides) cells with embryonic characteristics. Vegetative meristems are self-perpetuating. They produce the tissues that will form the body of the root or stem, and they also continuously regenerate themselves. A meristem can retain its embryonic character indefinitely, possibly even for thousands of years in the case of trees. The reason for this ability is that some meristematic cells do not enter differentiation phase, and they retain the capacity for cell division, as long as the meristem remains vegetative. Undifferentiated cells that retain the capacity for cell division indefinitely are said to be stem cells. Although historically called *initial cells* in plants, in function they are very similar, if not identical, to animal stem cells [4]. When stem cells divide, on average one of the daughter cells retains the identity of the stem cell, while the other enters a particular developmental pathway (Fig. 20.2).

Throughout the plant's life, the meristem retains its size and shape, despite cell division and cell differentiation. There is a balance between cell differentiation and cell division. If cell differentiation were restricted, then the meristem would increase in size. In contrast, if cell division were restricted, then the meristem would decrease in size [5].

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Chapter 21
Shoot and Root Apical Meristems

We can lick gravity, but sometimes the paperwork is overwhelming.

Wernher von Braun

Abstract The structure and organization of the shoot and root apical meristems is presented. Their functional zones and mechanism of functioning are described. The shoot apical meristem is presented as a dynamic structure that changes during leaf and stem formation. The generation of primary roots from the primary (apical) root meristem and of secondary roots from the secondary root meristems is described and schematically presented. Finally, similarities of plant stem cells in different types of meristems, at the molecular level, are presented.

21.1 Shoot Apical Meristems

The vegetative shoot apical meristem generates the aerial organs of the plant—the stem and the lateral organs attached to the stem (leaves and lateral buds). The shoot apical meristem typically contains a few hundred to a thousand cells, although the *Arabidopsis* shoot apical meristem has only about 60 cells. The shoot apical meristem is located at the extreme tip of the shoot, but it is surrounded and covered by immature leaves (Fig. 21.1).

These are the youngest leaves produced by the activity of the meristem. One should distinguish the shoot apex from the meristem proper. The shoot apex consists of the apical meristem plus the most recently formed leaf primordia. The shoot apical meristem is the undifferentiated cell population only and does not include any of the derivative organs. The shoot apical meristem is a flat or slightly curved region, 100–300 μm in diameter, composed mostly of small, thin-walled cells, with a dense cytoplasm, and lacking large central vacuoles. The shoot apical meristem is a dynamic structure that changes during leaf and stem formation. Besides, in many plants it shows seasonal activity, like the entire shoot. Shoot apical meristems may grow rapidly in the spring, enter a period of slower growth during the summer, and become dormant in the fall, with dormancy lasting through the winter. The size and

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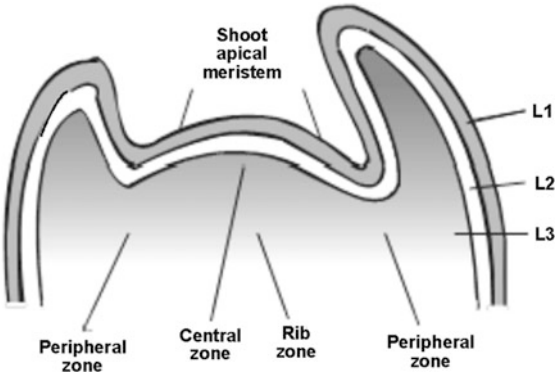

Fig. 21.1 Scheme of the enlarged longitudinal section through the center of the shoot apical meristem. The outer (L1) layer generates the shoot epidermis; the L2 and L3 layers generate internal tissues. The central zone (CZ) contains the stem cells, which divide slowly and produce the tissues that make up the plant body. The peripheral zone (PZ), in which cells divide rapidly, surrounds the central zone and produces the leaf primordia. A rib zone is located below the central zone

structure of the shoot apical meristem also changes with seasonal activity. Shoots develop and grow at their tips, like the roots, but the developing regions are not as stratified and precisely ordered as they are in the root. Growth occurs over a much broader region of the shoot than in the roots [1, 2].

21.1.1 Functional Zones and Layers of Shoot Apical Meristem

The shoot apical meristem consists of different functional regions. They can be distinguished by cell size, activity, and by the orientation of the cell division planes. The angiosperm vegetative shoot apical meristem usually has a highly stratified appearance, typically with three distinct layers of cells. These layers are designated L1, L2, and L3, where L1 is the outermost layer (Fig. 21.1). Cell divisions are anticlinal in the L1 and L2 layers; that is, the new cell wall separating the daughter cells is oriented at right angles to the meristem surface. Cell divisions tend to be less regularly oriented in the L3 layer. Each layer has its own stem cells, and all three layers contribute to the formation of the stem and lateral organs. Active apical meristems have an organizational pattern called cytohistological zonation. Each zone is composed of cells that may be distinguished not only on the basis of their division planes, but also by differences in size and by degrees of vacuolation (Fig. 21.1). These zones exhibit different patterns of gene expression, reflecting the different functions of each zone [3, 4]. The center of an active meristem contains a cluster of relatively large, highly vacuolate cells called the central zone. The central zone is comparable to the quiescent center of root meristems. Another region of smaller cells, called the peripheral zone, abuts the central zone. A rib zone is

beneath the central cell zone and reaches the internal tissues of the stem. These distinct zones represent different developmental domains. The peripheral zone is the region in which the first cell divisions will occur, leading to the formation of leaf primordia. The rib zone contains cells that become the stem. The central zone contains the group of stem cells, some portion of which stays uncommited, while others fill the rib and peripheral zone populations [5].

21.2 Root Meristems

Roots grow and develop from their distal ends. Four developmental zones can be distinguished in a root tip: the root cap, the meristematic zone, the elongation zone, and the maturation zone (Fig. 21.2).

In the Arabidopsis root these zones occupy about a millimeter of the tip. The developing region is larger in other species, but growth is still confined to the tip. With the exception of the root cap, the boundaries of these zones overlap. The root cap protects the apical meristem from mechanical injury during root growth through the soil. Root cap cells are formed by specialized root cap stem cells. As the root cap stem cells produce new cells, older cells are progressively displaced toward the tip, where they are eventually sloughed off. During process of

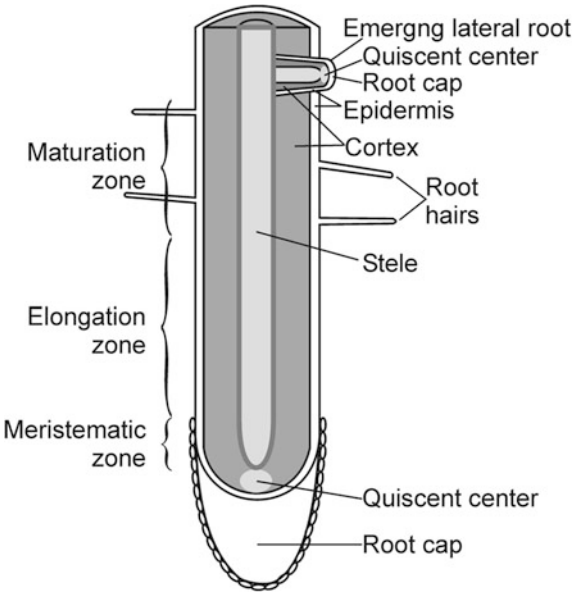

Fig. 21.2 Scheme showing longitudinal section through the center of the root with the root cap, the meristematic zone, the elongation zone, and the maturation zone. Cells in the meristematic zone have small vacuoles and expand and divide rapidly, generating many files of cells

differentiation, the root cap cells acquire the ability to perceive gravitational stimuli and secrete mucopolysaccharides that facilitate root penetration through the soil.

The meristematic zone is located just under the root cap. In Arabidopsis it is about a quarter of a millimeter long. The root meristem generates only primary root. It produces no lateral roots. The elongation zone is the location of rapid cell elongation. Although some cells may continue to divide during elongation within this zone, the rate of division decreases progressively to zero with increasing distance from the meristem. The maturation zone is the region in which cells acquire their differentiated characteristics. Cells enter the maturation zone after cessation of division and elongation. Although differentiation may begin much earlier, cells do not attain the mature state until they enter this zone. The radial pattern of differentiated tissues becomes obvious in the maturation zone.

Lateral roots grow from the pericycle in mature regions of the root. Cell divisions in the pericycle produce secondary meristems that grow out through the cortex and epidermis (Fig. 21.2) establishing a new growth axis [6]. In both primary and secondary root meristems divisions of the cells in the meristem produce progenitors of all the cells of the root.

Not all cells in the meristematic region divide at the same rate. Typically, divisions of the central cells are considerably slower than of the surrounding cells. These centrally located cells are called the quiescent center of the root meristem (Fig. 21.2). They are resistant to radiation and chemical damage. In the root tip, when viewed in longitudinal section, stem cells generate longitudinal files of cells. Most cell divisions in the root tip are transverse, or anticlinal, increasing root length.

Root apical meristems of seed plants contain several types of stem cells [2, 7, 8], while in the primitive vascular plants there is one single stem cell.

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Chapter 22
Lateral Meristems

The distance between insanity and genius is measured only by success.

Bruce Feirstein

Abstract In the plants exhibiting secondary growth (most trees, shrubs and some herbs), besides primary meristems there are secondary (lateral) meristems, which are described in this chapter. Accent is given on the lateral meristems in the stems of woody plants.

In many plant species primary growth is the only characteristic. Vascular plants also exhibit secondary growth. Most trees, shrubs, and some herbs have active lateral meristems, made of meristematic tissue within the stems and roots (Fig. 22.1). Before secondary growth begins, primary tissues continue to elongate as the apical meristems perform primary growth. As secondary growth begins, the lateral meristems produce secondary tissues, and the stem's girth increases. The effects of secondary growth are most remarkable in woody plants which have two lateral meristems. Within the bark of a woody stem there is the cork cambium, a lateral meristem that produces the cork cells of the outer bark. Cork tissues, whose cells become impregnated with *suberin* shortly after they are formed and then die, constitute the outer bark. Beneath the bark there is the vascular cambium, a lateral meristem that produces secondary vascular tissue. The vascular cambium forms between the xylem and phloem in vascular bundles, adding secondary vascular tissue on opposite sides of the vascular cambium. Secondary xylem is the main component of wood. Secondary phloem is very close to the outer surface of a woody stem. Tissues formed from lateral meristems, comprising most of the trunk, branches, and older roots of trees and shrubs, are known as secondary tissues and are collectively called the secondary plant body [1, 2].

Herbaceous stems do not produce a cork cambium. The stems are usually green and photosynthetic, with at least the outer cells of the cortex containing chloroplasts. Herbaceous stems commonly have stomata, and may have various types of trichomes (hairs) [1].

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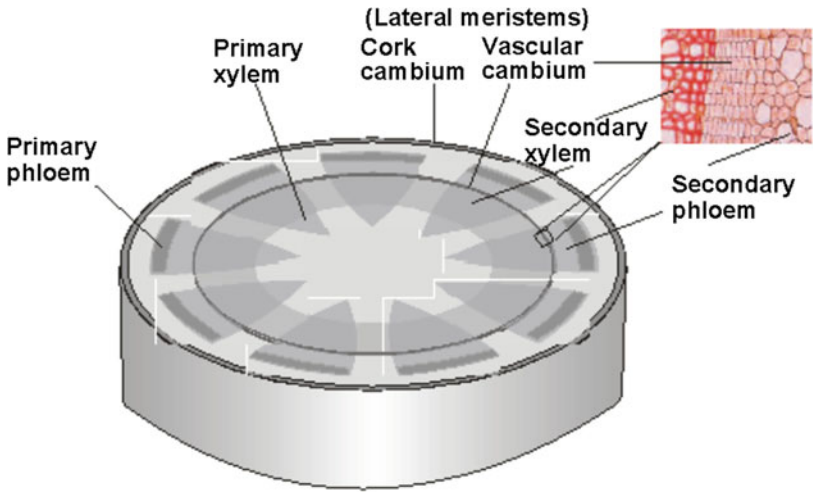

Fig. 22.1 Scheme showing vascular cambium and cork cambium (lateral meristems) in a stem, and formation of the secondary xylem, secondary phloem, and cork

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Chapter 23
External Control of the Plant Stem Cells

The next major explosion is going to be when genetics and computers come together. I'm talking about an organic compute—about biological substances that can function like a semiconductor.

Alvin Toffler

Abstract This chapter describes the effect of the external factors such as elevated CO_2 or mechanical sensations on the activity of meristems, and adaptive meaning of these relations. The role of modeling in such kind of studies is mentioned.

In the 1960s, Jack Van't Hof demonstrated in the experiments that meristematic cells were dependent on carbon supply. When pea root tips were cultured in medium depleted of sucrose, cells arrested in G1 or G2 and the roots stopped growing [1]. They also arrested if supplied with uncouplers of oxidative phosphorylation even if those roots were supplied with a carbon source [2]. Similarly, plant cells in culture will arrest if deprived of phosphate. In regard to the nutrient sensing by meristems, it was found that the unique plant cell cycle gene, cylin-dependent kinase B1;1 may be particularly responsive to sugar signaling pathways. Also, the homeobox gene, STIMPY, emerges strongly as a link between sugar sensing, plant cell proliferation and development. Both meristem identity and organ identity genes could be differentially sensitive to sucrose and glucose signals. Meristems also respond to elevated CO_2 [3].

It was shown that light has an influence on the shoot apical meristem (SAM) by affecting auxin distribution [4]. Light promotes cytokinin signaling in the central meristem zone, which relieves CLV-mediated inhibition of meristem propagation, thus supplying a source of cells for organogenesis. This cytokinin-dependent meristem growth promotes organ initiation harmonized with the auxin signaling pathway. It is proposed that cytokinin is required for meristem propagation, and that auxin redirects cytokinin-inducible meristem growth toward organ formation [5].

In plants mechanosensitive control over growth and morphogenesis is an adaptive trait. The mechanosensitive control of SAM morphogenesis was studied, mostly by using specific biomechanical and/or mechanobiological models, to find

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how mechanosensitive cells signal meristematic cells. The models consider cell geometry, growth, cell wall mechanical properties, and microtubule orientation, in the SAM cells. It is proposed that mechanosensing reactions include regulation by auxin and Ca^{2+} influx (reviewed in Moulia et al. [6]).

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Chapter 24
Signaling and Genetic Regulation of the Plant Stem Cells

Eat food. Not too much. Mostly plants.

Michael Pollan

Abstract This chapter presents the up-to-date knowledge on signaling mechanisms and genetic regulation in plant meristems. First, regulation in shoot and root apical meristems is given. Transcriptional and posttranscriptional control is described, that enable maintaining the boundaries between pluripotent stem cells and differentiating descendants. The involvement of signaling molecules, such as hormones auxin and cytokinines, is presented with corresponding schematic view. The regulation in lateral meristems is also described, where several key regulators of stem cell maintenance revealed surprising similarities to the apical meristems. Regulation of grass meristems is further described, since many agricultural plants belong to this group. The shoot architecture in such plants is critical to reproductive success, and thus to agronomic yield. Phyllotaxy, or pattern of leaf initiation, and floral induction is described, since they are important for the members of the grass family. Corresponding genetic and hormonal regulation is briefly presented. Similarities at the molecular level between different plant stem cells, in terms of regulation and maintenance, are depicted.

Stem cells are maintained in specific environments, the stem cell niches, which provide signals to block differentiation. In plants, stem cell niches are situated in the shoot, root, and vascular meristems—selfperpetuating units of organ formation [1].

24.1 Signaling and Regulation in Shoot Apical Meristem

The organization of the shoot apical meristem (SAM) is extremely stable. The structure of SAMs is such that cell division patterns need to be coordinated among layers both to maintain the identity of SAM layers and zones and to position organs. Cell division rates also need to be coordinated in organ primordia. This coordination needs to involve signals.

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The signaling pathways in SAM communication remained unclear until the era of molecular genetic studies in Arabidopsis. Mutants in three genes with a variety of phenotypes, including extra leaves, extra floral organs with club-shaped carpels, altered phyllotaxy, and flattened stems, were identified and named *clavata1* (*clv1*), *clv2*, and *clv3* [2–4]. Molecular analysis revealed that *CLV3* encodes a secreted protein [5], CLV1, a leucine-rich repeat (LRR) receptor-like protein kinase (RLK) [6], and *CLV2* a receptor-like protein resembling CLV1 but without a kinase domain [7]. Analysis of cell type–specific expression of *CLV1* and *CLV3* suggests that the *CLV3* signal initiates from the L1 and L2 in the CZ, while *CLV1* is expressed in L3 cells of the CZ (Fig. 24.1). As a result of *CLV1* activation by *CLV3*, the expression of *WUSCHEL* (*WUS*), which encodes a transcription factor required for stem cell maintenance, is restricted to four to eight cells in the L3 [8]. There is evidence that *WUS* or a downstream target of WUS then acts in a feedback loop to upregulate *CLV3* [9, 10]. This signaling pathway could be analogous to the signaling from L3 to L1 and L2 observed in floral meristems in tomatoes, but these signals and receptors have not yet been identified in any plant [11].

24.1.1 Regulation by WUS *Transcription Factor*

A central role in shoot meristem maintenance is played by the transcription factor WUSCHEL (WUS) as in corresponding mutants a SAM is initiated but arrests after having produced a few organs [12]. Its precise targets and regulators are largely unknown, but it has been well established that it interferes with hormone signaling cascades, in particular cytokinins [13, 14]. WUS is involved in a negative-feedback loop with the CLAVATA receptor kinase signaling cascade (CLV), whose activity limits the size of the pool of stem cells (see Fig. 24.1 for expression patterns of the genes).

Seedlings of the *wuschel* (*wus*) mutant lack a shoot meristem and display partially differentiated cells at the position of the stem cells, suggesting that *WUS* is required to prevent differentiation of stem cells [8, 12]. In contrast to *stm* mutants,

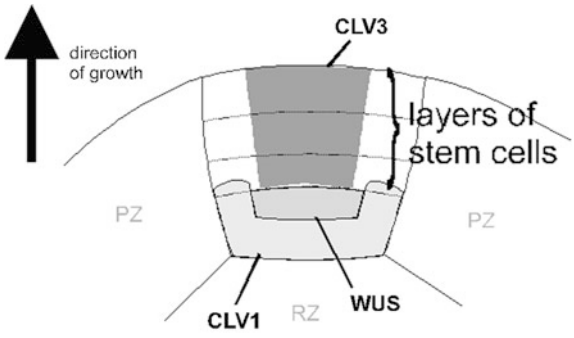

Fig. 24.1 Scheme showing WUS-CLAVATA signaling cascade

wus mutants can initiate adventitious shoot meristems postembryonically, although these terminate after the generation of a few organs. Conversely, overexpression of *WUS* leads to enlarged meristems, suggesting that *WUS* is also sufficient to promote stem cell identity [10, 15, 16]. This ability appears to be limited to immature tissues, implying that other factors are also required. *WUS* encodes a plant-specific homeodomain protein and is the founding member of the *WUSCHEL*-related homeobox (*WOX*) gene family, which regulates diverse aspects of development [17]. *WUS* expression in the shoot meristem defines the organizing center (OC), which in seedlings is located in the fourth- and fifth-outermost cell layers, underneath the three stem cell layers (Fig. 24.1). In addition to maintaining the undifferentiated nature of the stem cells, *WUS* is required for expression of the *CLAVATA3* (*CLV3*) gene in the stem cell region of shoot and floral meristems [10, 15]. Because all three layers of stem cells are affected by *WUS* expression in the OC, it was proposed that a stem cell-promoting signal arises from the OC [8]. Yadav and colleagues [18] demonstrated that the WUS protein moves from the OC into the central zone (CZ), where it binds directly to the *CLV3* promoter, which is an example of moving plant transcription factors in cell–cell communication [19, 20]. This intercellular movement appears to be critical for stem cell maintenance, since decreasing WUS mobility results in loss of the shoot meristem.

It has been shown that WUS transcription factor produced in cells of the niche, migrates into adjoining cells where it specifies stem cells. Yadav et al. [21], by means of high-resolution genomic analysis, have provided direct evidence that WUS protein represses a large number of genes that are expressed in differentiating cells including a group of differentiation-promoting transcription factors involved in leaf development. They have also shown that WUS directly binds to the regulatory regions of differentiation promoting transcription factors: KANADI1, KANADI2, ASYMMETRICLEAVES2, and YABBY3, thus repressing their expression. A computational model, combined with live imaging, revealed that WUS-mediated repression prevents premature differentiation of stem cell progenitors. This is part of a minimal regulatory network for meristem maintenance. The authors claim that direct transcriptional repression of differentiation-promoting transcriptional factors is an evolutionarily conserved logic for stem cell regulation.

In summary, control of *WUS* gene expression and multiple levels of lateral inhibition contribute to robustly maintaining the boundaries between pluripotent stem cells and differentiating descendants.

In addition to transcriptional control, posttranscriptional regulation by microRNAs (mi-RNAs) plays an important role in meristem function. These include, for example, the miR164 and miR156/157 families, which respectively target the CUC and SPL genes [22, 23].

Schoof et al. [10] proposed that in addition to the repressive *CLV3* signal, stem cells also result from a graded signal that promotes *WUS* expression and thus anchors the stem cell niche to the tip of the plant. Several recent observations are consistent with a model in which cytokinin might be involved in this process.

24.1.2 *Regulation by* KNOX *Genes*

There is another pathway where stem cell pluripotency is balanced against differentiation. This is achieved by the *KNOX* families of homeodomain transcription factors (Fig. 24.2). *KNOX* stands for *KNOTTED1*-like homeobox. These proteins act as strong inhibitors of differentiation. It was the first isolated plant stem cell regulator gene, from the maize leaf mutant *knotted* (*kn*) [24]. *KN* encodes a homeodomain protein that in the dominant active *kn-1* mutant isectopically expressed in leaf veins, resulting in proliferating "knots." *KN* is the founding member of the *KNOTTED1-like homeobox* (*KNOX*) genes. Its normal expression is in undifferentiated cells of the shoot meristem dome, but it is absent from the cells in leaf anlagen, which is consistent with a model in which *KN* promotes the undifferentiated cell state in the shoot meristem [19]. These genes are responsible for regulation of the Cytokinin/Gibberellic Acid Balance, which is involved in regulation of organ boundaries. A pool of pluripotent stem cells is maintained by a *WUS/CLV3* negative-feedback loop, while the cytokinin biosynthesis is activated [1].

24.1.3 *Signaling Molecules: Auxin*

It has been known for many years that auxin plays a central role in meristem function and organ formation. Auxin is not homogeneously distributed at the shoot apical meristem (SAM) and it is thought that this distribution is interpreted in terms of differential gene expression and patterned growth. Auxin plays two general roles in the meristem. First, auxin has long been thought to be the signal or one of the signals that regulates the initiation of primordia in a defined pattern, or phyllotaxy, in the peripheral zone of the SAM. A second role for auxin is in the differentiation of organ primordia once they are initiated [11, 25].

Auxin influx and efflux carriers control auxin distribution at the SAM. A number of synthetic auxins and auxin transport inhibitors affect phyllotaxy (reviewed in

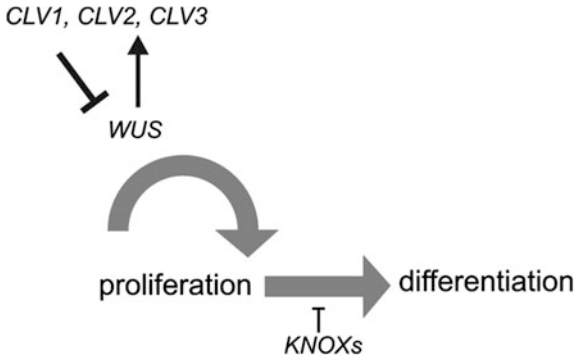

Fig. 24.2 Interplay among the *WUS-CLAVATA* and *KNOX* regulation pathways

Lyndon [26]). Okada et al. [27] identified an Arabidopsis mutant called pin-formed 1 (pin1) in reference to its needle-like inflorescence stem, unable to initiate flowers. Identification of the gene showed that it encoded a transmembrane protein [28] and there is now overwhelming evidence that the PIN1 protein is the founding member of a family of auxin carriers that transport auxin across membranes [29]. Second sets of transporters associated with auxin distribution at the SAM are the AUX/LAX influx carriers. AUX1, the founding member of the gene family [30], is expressed in the L1 surface layer of the shoot apical meristem [31]. The protein seems to be evenly localized over all membranes, indicating that the protein is not involved in the creation of hormone fluxes. Instead, it might rather concentrate auxin at the meristem surface. Altogether, the available data indicate that the formation of local auxin maxima mainly depends on the action of PIN exporters at the meristem surface. AUX and LAX proteins would facilitate organ positioning, probably by guaranteeing a sufficient supply in the L1 layer.

Auxin is not only important for establishing the placement of primordia in the peripheral zone, but also plays a role in initiation of the growth of primordia [32]. Information from molecular and genetic studies has led to the development of tools and assays that are used to probe the specific role of auxin in a variety of processes.

The results of studies that examined DR5 and/or PIN1 expression in fixed tissue in SAMs suggested that auxin is first transported from more basal epidermal cells apically toward a new primordium (Fig. 24.3) [31, 33]. The new primordium then functions as a sink, pulling auxin from nearby cells. Localization experiments using

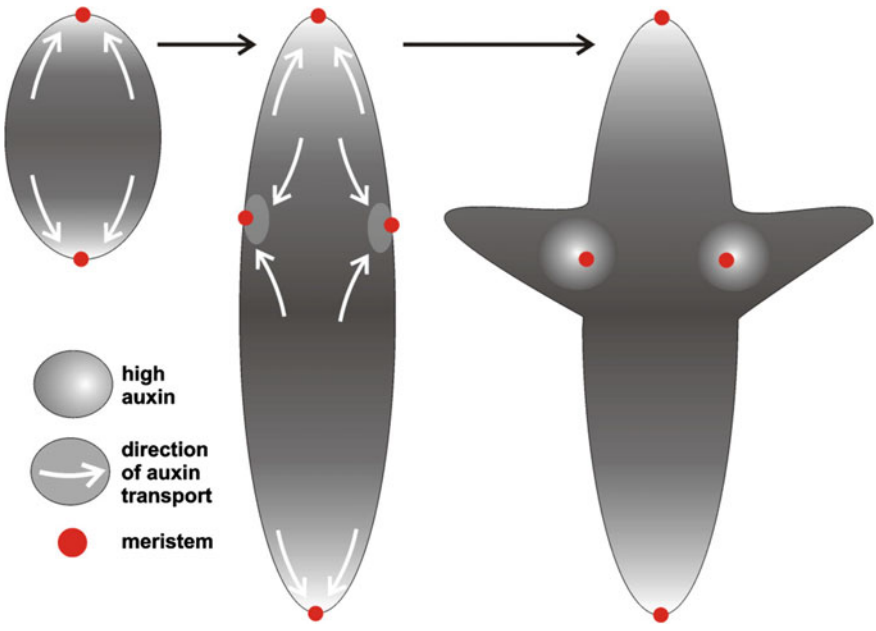

Fig. 24.3 Model for auxin dinamics in shoot apical meristem

a specific antibody to PIN1 suggest that the auxin in the primordium then moves into the vasculature and is transported basally out of the L1 of the SAM [31]. Since the primordium has locally depleted auxin from surrounding cells, a new primordium probably initiates growth at a region of high auxin concentration as far away from the old primordium as possible. High levels of auxin accumulation would therefore initiate primordia growth, and differences in auxin concentration could account for the patterns of phyllotaxis (patterned arrangement of aerial organs). The prediction of these results is that accumulation and depletion of auxin are important regulators of primordia initiation and phyllotaxis in inflorescence meristems (IM) epidermal cells and that auxin is not simply moved out of the way to the vasculature [31]. Therefore, auxin could be acting as a signaling molecule in that differences in concentration lead to different developmental outcomes. Heisler et al. [34] looked at several markers to correlate auxin movement with expression of the transcription factors mentioned above that regulate meristem maintenance and primordia differentiation.

In the past 20 years, mutants and their phenotypes have been the main tools used to study signaling pathways. While this approach has generated a number of well-established signaling pathways in plants, a major problem arises in that it becomes difficult to discern between the direct effects of the loss of a single gene and the indirect effects of the loss of that gene on other genes in the genome [35]. Indirect effects can be caused by the feedback regulation that is often found in signaling pathways. Reddy and Meyerowitz [36] addressed this problem by combining genetics and live imaging. The goal of these authors was to determine the function of *CLV3* in IM organization and growth. Using this approach, the authors were able to determine the role of *CLV3* in the regulation of IM size and in maintenance of the CZ. Reddy and Meyerowitz [36] described three mechanisms that may explain how *clv3* mutants produce larger SAMs. First, mutations in *clv3* simply result in an increase in cell division, resulting in an increased number of cells. Second, cells in the CZ are deterred from relocating to the PZ and differentiating. Third, it is suggested that cells at the CZ/PZ boundary dedifferentiate and revert to a CZ fate. Live imaging of single cells and their progeny showed that zone-specific signaling in the IM is involved in expansion as well is critical for maintaining the balance among the different zones. These results have shown that *CLV3* is involved in maintaining meristem organization both through regulation of cell fate determination as well as regulation of cell division. The implications from these results are that the boundaries between the different zones of the meristem are dynamic and rely on signals from across zone boundaries.

Modeling has been used to explore polar auxin transport regulation. The distribution of PIN at the meristem surface is complex and it is not obvious from simple visual inspection what the predicted auxin fluxes would be. Therefore, a set of careful localization studies were performed and the properties of the cellular transport networks analyzed using computer simulations [37]. This confirmed that PIN directs auxin to the sites where young primordia are being formed. The same computer simulations also identified additional properties of the transport network, and in particular a still undefined role for the meristem summit in auxin

redistribution was proposed [37]. Other models are able to explain the distribution of auxin transporters. Another hypothesis was based on the pioneering work of Sachs, who proposed the existence of a positive feedback between flux and transport [38]. It was subsequently shown that this mechanism is able to amplify small fluxes and can potentially create canals of auxin between hormone sources and sinks. A range of experiments supports the canalization hypothesis, at least in the inner tissues of the plant where it can account for the formation of venation patterns [39–41]. The existence of canalization would imply the coexistence of two radically different mechanisms for PIN allocation to the membrane, the one based on flux sensing (in the inner tissues) and the other on local concentration sensing (at the meristem surface). Stoma et al. [42] tested whether canalization could potentially also account for the behavior of auxin transporters at the shoot apical meristem surface. They proved this by using a computer simulation tool, thus providing a unifying concept for the control of auxin distribution in the plant [42]. Concepts like canalization are still relatively abstract and could represent a combination of processes. Although the exact mechanisms that control the dynamics of PIN1 polar localization are still unknown, accumulating evidence indicates that it depends on multiple processes, such as membrane traffic and cytoskeleton organization (for review: Kleine-Vehn and Friml [43]). A prominent set of data also points at the importance of phosphorylation and the PINOID (PID) serine-threonine protein kinase [44, 45].

Although the role of polar auxin transport during developmental processes, notably in the shoot meristem, has been extensively studied, little attention has been paid to the role of auxin metabolism until recent years. However, local concentrations of auxin in the shoot meristem are expected to be under the control of the combined action of polar auxin transport and auxin biosynthesis, but also of auxin catabolism and conjugation. The recent identification of the YUCCA (YUC) family of auxin biosynthetic genes encoding flavin monooxygenases has been instrumental in demonstrating that auxin biosynthesis is not homogenous in a given tissue and that a fine control of auxin biosynthesis in the shoot apex plays a key role in the regulation of the activity of the shoot and floral meristems. At least two nonredundant auxin biosynthesis pathways are thus implicated in organ initiation at the shoot or floral meristem. The phenotype obtained on inactivation of these pathways indicates that they are necessary to control locally auxin homeostasis at the shoot meristem and that auxin transport is probably not the only limiting mechanism for generating the spatial variations in auxin concentration implicated in lateral organ initiation [25].

The action of auxin does not only depend on the regulation of its synthesis or transport, but the competence to react to auxin seems also to be controlled in time and space. Both biochemical and genetic approaches have identified the members of the Aux/IAA and ARF family of transcriptional regulators as major effectors of auxin signal transduction [46]. It has been shown that the competence for organ initiation at the periphery of the meristem thus depends, at least in part, on a spatial modulation of auxin signal transduction [47]. The analysis of cell identity in the meristem of the pin1 mutant gave the first clues concerning the role of auxin in the

control of cell identity at the meristem [48]. Although meristem structure and maintenance were not severely affected in the mutant, major alterations occurred at the periphery of the pin1 meristem, where organ initiation should occur. The results also implied that auxin levels control the identities of the cells at the periphery of the meristem, thus impacting organ separation, positioning and outgrowth.

Auxin is not only involved in organ initiation but is also associated with the establishment of symmetry. In vivo imaging suggests that auxin maxima precede and might control the establishment of adaxial/abaxial symmetry [34]. Such a role was further supported by genetic studies, identifying genes that regulate organ asymmetry [49]. Another link between auxin and organ symmetry involves members of the KANADI (KAN) gene family, which regulate abaxial identity and laminar growth of lateral organs [50].

Studies on plant tissue regeneration have shown that a high cytokinin (CK) to auxin ratio can trigger the initiation of shoot meristems from undifferentiated callus but the molecular basis for such interactions in the shoot are just starting to emerge [14]. It has been shown that high CK signaling is essential for maintenance of the meristem through a direct effect on stem cell activity, as well as that CKs contribute to organ initiation together with auxin [51].

Auxin is also associated with another key class of hormones in the shoot meristem, the gibberellins (GA). The concentration of active GA is mainly controlled by GA 20-oxydases and GA 3-oxydases, required for GA biosynthesis, and by GA 2-Oxydases, which control GA deactivation. These enzymes are encoded by gene families, some of which show very specific expression patterns in the shoot meristems. In particular, biosynthetic enzymes are excluded from the meristem center and restricted to young organs, whereas deactivation enzymes are expressed at the base of the SAM, below the rib zone [52, 53]. Auxin treatments on seedlings suggest that 8 out of 13 GA oxydases (both activating or deactivating) expressed in seedlings are transcriptionally regulated by auxin [54].

An evidence was provided of importance of mechanical properties of the shoot apex cell walls in auxin signaling. By using AFM, it was shown that local accumulation of auxin in the shoot apex leads to tissue softening and, thus, organ outgrowth. Auxin signaling in the shoot apex acts through a mechanical bottleneck, namely demethyl-esterification of homogalacturonan (HG). This implies that the complex suite of changes induced by auxin within the apex cannot proceed without HG-mediated changes in cell wall rigidity. Coordinated localization of the auxin transport protein, PIN1, is disrupted in a naked-apex produced by increasing cell wall rigidity. These data indicates that a feedback loop between the instructive chemical auxin and cell wall mechanics may play a crucial role in phyllotactic patterning [55].

24.1.4 Signaling Molecules: Cytokinines

It has been demonstrated [56] that the cytokinin biosynthetic enzyme LOG4 is expressed in the epidermal layer (L1) of the SAM and floral meristem. Based on this,

a cell-based computational model has been formulated involving growth and division in the apical–basal axis. The model shows that epidermally derived cytokinin, together with the CLV-WUS genetic network, regulates cell division and positions the WUS expression domain within the SAM during growth. Besides, using the model in connection with experiments, it was revealed a feedback principle whereby WUS negatively regulates epidermally produced cytokinin biosynthesis in the SAM. This leads to an updated picture of how mechanisms of feedback control, which occur over space and time, pattern and maintain the SAM stem cell niche.

24.2 Signaling and Regulation in the Root Apical Meristem

Longitudinal root growth originates at the tips of the roots where the root stem cells reside. In the center of the root tip is the quiescent center (QC), which is mitotically relatively inactive in Arabidopsis. The stem cells directly surround the QC and give rise to the different cell files of the root, the stele, the ground tissue (consisting of endodermis and cortex), the epidermis, and the lateral root cap, as well as the columella (Fig. 24.4). Each stem cell division is asymmetric, generating one daughter cell that stays in contact with the QC and persists as a stem cell, and another that is located one cell away from the QC, can undergo several rounds of

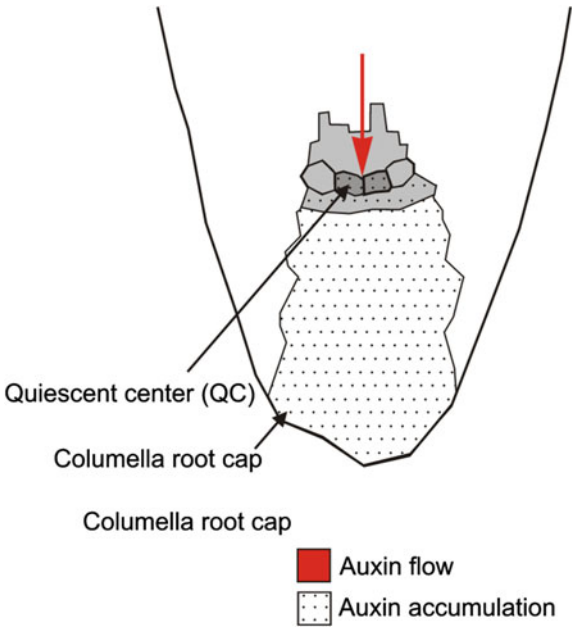

Fig. 24.4 Model for auxin dinamics in root apical meristem

cell divisions, and eventually differentiates. At the distal side of the QC are the columella stem cells (CSCs), whose daughter cells differentiate without additional rounds of cell divisions into starch-containing, gravity-sensing columella cells. Thus, the Arabidopsis root stem cells operate in a lineage-based mechanism similar to most animal stem cell niches and unlike the shoot meristem [57]. Notably, each stem cell in the root meristem gives rise to only one tissue, raising the question of whether the stem cell potential is limited. The ablation studies have shown, however, that the daughter cells differentiate according to signals from older differentiated cells and have the ability to switch fates if displaced to a new position [58].

The quiescent center is organizer of the root stem cell niche. Direct evidence that the QC plays a role in controlling stem cells came from laser ablation of individual Arabidopsis QC cells [58]. CSCs abutting the ablated QC cells ceased proliferation and differentiated into starch-containing columella cells, whereas abutting cortex and endodermis initials (CEIs) differentiated into CEI daughter cells. The fact that only cells in direct contact with the QC are maintained as stem cells might suggest short-range or contact-based signaling. That this is not the case was shown in an elegant experiment by Wildwater et al. [59] in which differentiation of stem cell daughters was blocked by RNAi-mediated downregulation of RETINO BLASTOMA-RELATED (RBR) activity, resulting in several layers of undifferentiated cells next to the QC. These extra cells lost their undifferentiated state when the QC was ablated, indicating that the stem cell-promoting signals from the QC can work over several cell diameters but normally are counteracted in cells without direct contact to the QC. Although the QC-borne signal molecules are still undiscovered, genetic and molecular studies have identified pathways that are essential for stem cell maintenance in the Arabidopsis root.

The *WUS* homolog *WOX5* is specifically expressed in the QC, and loss of *WOX5* function leads to differentiation of the CSCs, similar to what was observed upon QC ablation [60, 61]. Furthermore, overexpression of *WOX5* in the columella blocks differentiation and generates stem cell-like cells. In contrast to the excessive undifferentiated cells caused by *RBR* downregulation, QC ablation does not suppress the effects of *WOX5* overexpression, consistent with the hypothesis that no QC signal other than the one(s) generated by *WOX5* is required for stem cell maintenance. In addition to its effect on CSCs, *WOX5* is also required for maintaining proximal stem cells [61].

24.2.1 Control of the Root Stem Cells via Auxin and PLT *Genes*

The root stem cell niche is marked by an auxin maximum at the location of the QC [62, 63]. Computer modeling and the discovery of polar localization of PIN auxin transport facilitators to one side of a cell suggest that auxin accumulates in the QC region by a rootward-directed auxin transport in the vasculature and a shootward-directed transport in the lateral root cap and epidermis [64, 65]. After excision of

the root tip or ablation of the QC, a new auxin maximum is established a few cell layers apically from the new tip and a new stem cell niche is formed [62, 66], suggesting that the auxin maximum and the stem cell niche are functionally linked. Auxin function in the stem cell niche is mediated by PLT transcription factors [67]. The activity of the different PLT proteins is additive, and manipulating the expression levels suggests a dose-dependent readout, reminiscent of animal morphogens. The highest PLT levels are in the QC, and seem to be required for specifying and maintaining the stem cell niche; intermediate PLT levels in the proximal meristem are required for mitotic activity; and low levels correlate with differentiation (Fig. 24.4). Because the response of *PLT* expression to auxin occurs later than for other known auxin response genes, it has been postulated to be rather indirect [67]. Tyrosylprotein sulfotransferase (TPST) and the ROOT GROWTH FACTOR (RGF) tyrosine-sulfated peptides might link auxin to PLT protein levels [68, 69].

In summary, the hormone auxin not only promotes the root stem cell niche, but is also involved in restricting it.

The low mitotic activity of QC cells is caused by a prolonged G1 phase. For example, maize QC cells divide 10 times more frequently than the proximal meristem cells [70, 71]. It was shown how the quiescence of QC cells is controlled. First, QC-specific knockout of *RBR* function indicates that *RBR* suppresses cell divisions in the QC [72]. Second, the oxidized redox status of the QC has been proposed to cause arrest at the G1/S transition [73]. As this oxidized status is postulated to be due to auxin degradation, an interesting question is how auxin, redox regulation, and cell cycle control are linked. The biological significance of a low cell division rate in the QC is unknown. Under certain conditions, cell divisions of QC cells do occur and QC derivatives can replace stem cells. In Arabidopsis the QC is mitotically more active in older roots, and mitosis is induced by stress conditions, altered hormone levels, or a reduced redox status [74–77]. In other species with larger stem cell niches, such as maize, there is no clear boundary between mitotically almost inactive QC cells and the dividing stem cells of the proximal meristem [70, 78]. Therefore, the QC can be seen as a flexible and responsive organizer that is competent to replenish stem cells when necessary.

24.2.2 Control of the Root Stem Cells by Transcription Factors

Specific transcription factors (TFs) play important roles in maintaining stem cell homeostasis in the root. TFs regulate the expression of other genes, but data on direct targets of TFs involved in root apical meristem regulation are scarce, being known for only few cases. One of the most important TF regulating stem cell fate in the root is the homeodomain containing WUSCHEL-RELATED HOMEOBOX5 (WOX5). WOX5 is expressed in the QC in embryos and mature roots and maintains

the surrounding stem cells in a largely unknown non-cell autonomous way. Other TFs have been described to play important roles in root stem cell maintenance, e.g., the R2R3-MYB transcription factor BRASSINOSTEROIDS AT VASCULAR AND ORGANIZING CENTER (BRAVO). Transcriptional regulation is controlled by phytohormones and several TFs have been shown to be regulated by them. Phytohormones act mostly as long-range signals, other more short-range signals mediating TF regulations include small peptides, microRNAs, and movement of TFs. Due to their rigid cell walls, plant cells are not able to move and need to communicate with each other non-cell autonomously in order to integrate external and internal cues with development and growth [79]. About 17–29% of TFs are predicted to move either targeted or non-targeted from cell to cell [80, 81]. This TF movement is proposed to occur by transit through plasmodesmata, membrane-lined channels that interconnect plant cells symplastically, and thereby propagate signaling outputs.

24.3 Regulation in Vascular Meristems

The plant vasculature is the main route for long-distance transport of water and minerals. It is comprised of two main elements: xylem and phloem. The xylem stream transports upward water and dissolved mineral nutrients taken up by the roots, while the phloem is the primary downward transport route for photoassimilates, signaling molecules and some mineral ions throughout the plant [82, 83]. It also gives mechanical support for the growing stem. Although the cell types are the same in all vascular plants, the architecture of the vasculature varies, even between organs. In vascular plants principal secondary meristem is the vascular cambium (VC)—mitotic regions toward the exterior of roots, stems, and branches that produce the cells for continued growth in girth via the production of secondary vascular elements (Fig. 21.1). Most of the recent insights into VC function have come from work conducted in two plant models: Arabidopsis and Poplar (*Populus* spp.) [84]. Despite its herbaceous nature, Arabidopsis has proven to be an excellent model system for vascular development [85]. In the Arabidopsis root, the xylem is located in a central row of cells, with the protoxylem located on the marginal positions and the metaxylem in a central position (Fig. 24.5). On the perpendicular axis two poles of phloem are present and the intervening procambium consists of pluripotent stem cells. These tissues are surrounded by the pericycle and together form the vasculature (or stele) (Fig. 24.5). In the stem, a ring of vascular bundles is present with phloem on the outside, the procambium (or fascicular cambium) in the middle and the xylem on the inside (Fig. 24.5). During secondary growth, the fascicular cambium and the interfascicular cambium (IC) form a closed cambium ring. The current opinion, supported by transcriptional profiling in Poplar [86], is that the cambium contains stem cells with phloem mother cells on one side and xylem mother cells on the other [87].

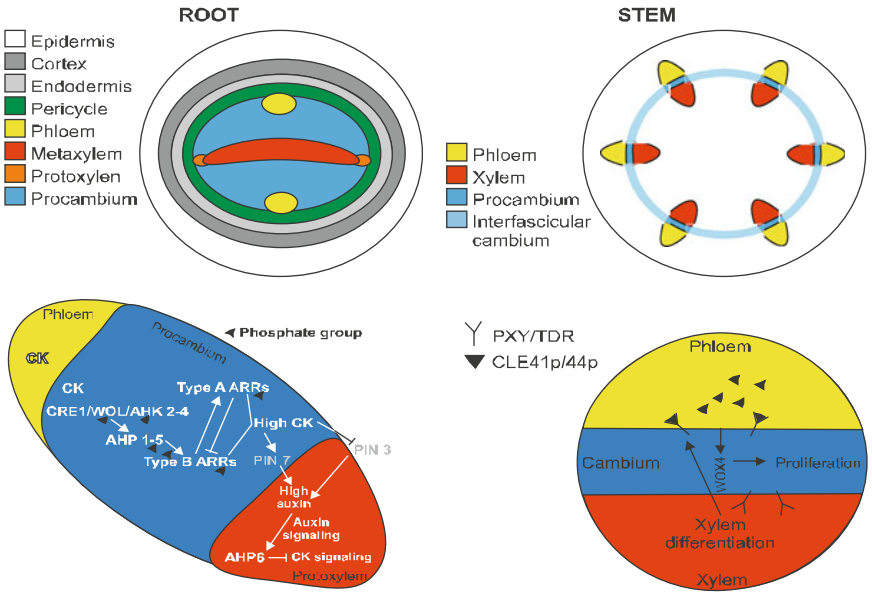

Fig. 24.5 Organization of the vascular cambium in the Arabidopsis postembryonic root and stem (*upper panels*), and regulatory pathways—and the CK-auxin pathway in the root and the CLE–PXY/TDR–WOX4 pathway in the stem

Several key regulators of stem cell maintenance in the procambium have been identified and revealed surprising similarities to the apical meristems. Ito et al. [88] set the stage by purifying the CLE peptide TDIF (tracheary element differentiation inhibitory factor), a repressor of xylem differentiation and promoter of cell proliferation in *Zinnia* cell culture. The differentiating phloem daughter cells provide stem cell-promoting signals and act like niche cells, similar to the QC [89, 90]. Ubiquitous overexpression of the ligand and the receptor represses xylem differentiation and causes more cells to accumulate in the vascular bundle and in the interfascicular region, exerting secondary growth initiation. The CLE41p–PXY/TDR module defines the boundary between vascular cell types and regulates the size of the vascular stem cell population (Fig. 24.5). Furthermore, the position of the CLE peptide-producing cells relative to the stem cells has been reported to correlate with the orientation of stem cell divisions, because ubiquitous and xylem-specific expression of *CLE41* induces disoriented procambial cell divisions, whereas overexpression of *CLE41* in the phloem (where it is normally expressed) induces only correctly oriented cell divisions [91].

Transcript profiling of Arabidopsis and poplar cambiums suggests that the known shoot meristem regulators CLV1 (expressed in the phloem and cambium) and STM (expressed in the cambium) might also play a role in vascular stem cell maintenance [86, 92].

A mutually inhibitory interaction between cytokinin and auxin determines boundary formation between procambium stem cells and the protoxylem [93]. Auxin polarization is also vital for the establishment of the primary vascular structures [48]. The vascularisation of developing leaves is a good example of the role played by auxin in vascular development. The establishment of procambial strands from isodiametric preprocambial cells in the ground tissue of the leaf primordium [40, 94] has been proposed to be driven by a self-reinforcing canalization of auxin flow. The canalization theory proposes that auxin exerts a positive feedback on the rate and polarity of its own transport [29, 95, 96]. Polarization of PIN1 amplifies and stabilizes small cellular fluxes of the hormone, reinforcing its directional movement and ultimately the establishment of laterally restricted channels of auxin flow [40, 97]. The expression domains of PIN1 and an auxin response element, MONOPTEROS (MP), are essential components in the process, indicating that primary vascular development is regulated by changes in both concentration and responsiveness to hormone [98]. During primary vascular development, PIN1 and MP expression domains overlap and become increasing restricted long with the zone of elevated auxin transport to specific cell files [98, 99]. Genetic analyses have shown that sterols are one of the additional factors required for vascular patterning. Genes responsible for encoding enzymes in the sterol biosynthetic pathway, are required for normal auxin distribution in the root, and also for formation of normally connected vascular systems (reviewed in Reinhardt [100]).

In the root, cytokinin (CK) and auxin determine boundary formation between the procambium and protoxylem [93]. CK is transported from the phloem and binds to hybrid histidine kinase receptors (CRE1/WOL/AHK4, AHK2, AHK3), inducing autophosphorylation. The phosphate group is subsequently transferred to Arabidopsis histidine phosphotransfer proteins (AHP1–5), which move into the nucleus and phosphorylate Arabidopsis response regulators (ARRs). Type B ARRs act as activators and directly induce type A ARRs, which act as repressors [101, 102]. The CK signal influences localization of PIN3 and PIN7, creating an auxin maximum in the xylem. There, auxin induces pseudophosphotransfer protein *ARABIDOPSIS HISTIDINE PHOSPHOTRANSFER PROTEIN 6* (*AHP6*), which acts as an inhibitor of cytokinin signaling and promotes protoxylem formation (Fig. 24.5).

24.4 Regulation in Grass Meristems

Shoot architectures displayed by members of the grass family are critical to reproductive success, and thus agronomic yield. Variation in shoot architecture is explained by the maintenance, activity and determinacy of meristems. In this regard vegetative phase meristems and the floral transition are in focus of current research.

Major areas of interest include: the control of meristem homeostasis by the CLAVATA–WUSCHEL pathway and by hormones such as cytokinin; the initiation of axillary meristems and the control of axillary meristem dormancy; and the environmental and endogenous factors that regulate flowering time [103]. The studies in both maize and rice suggest that the CLV pathway that negatively regulates stem cell maintenance is conserved in grasses [104, 105]. These observations demonstrate that stem cell maintenance is likely to be regulated by at least three related negative pathways in rice, and each pathway seems to contribute differently to this regulation depending on the type of meristem. In contrast to negative pathways in meristem maintenance, current understanding of factors that promote stem cell identity is still lacking. It is probable that *WUS* orthologs, or *WUSCHEL-RELATED HOMEOBOX* (*WOX*) genes, may also have such function in grasses. Although a few studies concerning the expression patterns of *WOX* genes have been published, no genetic or functional analysis has been reported in grasses. However, the presence of two *WUS* paralogs, *ZmWUS1* and *ZmWUS2*, with different expression patterns, suggests that some degree of subfunctionalization has occurred [106]. Functional identification of stem cell promoting factors, such as WUS, would be helpful to elucidate the genetic mechanism that regulates stem cell maintenance in grasses. A recent study reports that *WOX4*, a distinct member of the rice *WOX* gene family, acts as a positive factor in shoot meristem maintenance and is negatively regulated by FCP1 in rice [107].

Cytokinines. A genome-wide binding profile for KN1 was recently identified by chromatin immunoprecipitation-sequencing (ChIPseq), and targeted genes were compared with a list of genes differentially expressed in the kn1 loss-of-function mutant [108]. This analysis revealed that KN1 targets genes involved in four major hormone pathways (auxin, cytokinin, gibberellic acid and brassinosteroids), orchestrating a careful balance that promotes meristem maintenance. Direct targets also included many other transcription factors, placing KN1 at the summit of a regulatory cascade controlling shoot meristem function [108].

The *FLATTENED SHOOT MERISTEM (FSM)* gene is another factor required for meristem maintenance in rice, as mutants have a flatter and smaller SAM than wild-type plants [109]. *FSM* encodes a Chromatin Assembly Factor-1 (CAF1) subunit, and is the ortholog of the Arabidopsis gene *FASCIATA1 (FAS1)*. *FAS1* displays an enlarged meristem, suggesting that this layer of meristem maintenance may function quite differently in the monocots and dicots [109].

24.4.1 Phyllotaxy and Plastochron Regulation

Most members of the grass family display an alternate phyllotaxy, or pattern of leaf initiation, with one organ initiated at the flank of the meristem at a time, resulting in one leaf per node [51]. The pattern of leaf arrangement is important for plant traits such as stalk strength and optimal light capture. A complex interplay between auxin and cytokinin signaling regulates phyllotaxy and leaf initiation [110].

Another property of organ initiation from the meristem is plastochron, the elapsed time between the initiation of two leaves. Three rice mutants, plastochron1, 2, and 3, display greatly reduced plastochron length, with a large increase in the number of leaves originating from the SAM [111–113]. The plastochron phenotype is associated with larger meristems, with much higher rates of cell division than wild-type plants [111]. An analysis using various mutants with defects in the rate of leaf initiation found a correlation between meristem shape parameters (i.e., height/width ratios) and phyllotaxy and plastochron parameters; however, no such relationship existed with meristem size per se [114].

24.4.2 The Floral Transition

Grasses have evolved a spectrum of different pathways that coordinate the floral transition in response to environmental and endogenous factors. Some features of grass flowering pathways are conserved between all flowering plants, while others represent innovations specific to various grass lineages. For example, different species of grasses have different sensitivities and thresholds for day length-dependent flowering. Rice is considered a photoperiod–sensitive species, with a facultative short-day requirement. On the other hand, floral induction in maize reflects its domestication from a tropical grass, but subsequent breeding and improvement over a wide range of temperate environments. Most temperate maize inbred lines are essentially day-neutral, whereas tropical lines respond to short-day inductive cues [115]. Other temperate grasses, such as wheat and barley, have a long-day requirement with a vernalization switch [116]. Much of what we know about the floral transition comes from studies in Arabidopsis. The *CONSTANS (CO)* gene integrates the main outputs of the circadian clock, and synchronizes flowering time with long-day photoperiods [117]. Under long-day conditions, CO, a zinc finger transcription factor, is stable and activates the expression of *FLOWERING LOCUS T (FT)* in leaves. Subsequently, the FT protein product is translocated through the phloem to the SAM, where it interacts with the bZIP transcription factor FLOWERING LOCUS D (FD) and targets floral regulators. FT is regarded to fulfill the criteria for the universal leaf-derived flowering signal, 'florigen' [117]. This extensively characterized photoperiod-responsive flowering module is conserved in grasses. An endogenous pathway regulating the floral transition operates in parallel with the photoperiod pathway in grasses, and takes on an increased importance in day-neutral temperate maize [118].

24.4.3 Inflorescence Meristem Identity

Following the vegetative to reproductive transition, the inflorescence meristem (IM) functions like the vegetative SAM, initiating lateral leaf (bract) primordia in a

regular phyllotaxy, which are accompanied by apical meristems (AMs). Grasses have a program of bract suppression to limit leaf outgrowth, and thus the dominant features of the inflorescence are all derived from the AMs (e.g., spikelet and floral meristems) [119]. Not much is known about genes that regulate the identity and determinacy of the IM. A recent study revealed that *PANICLE PHYTOMER2* (*PAP2*) and three other *AP1-like MADS*-box genes are required to specify the identity of the rice IM downstream of the florigen signal [120]. Properties such as the determinacy, or persistence, of the IM have the ability to influence panicle size and ear length, and thus grain yield, greatly.

24.5 Similarities of Plant Stem Cells at the Molecular Level

The striking similarities between the shoot and root niches in both regulation and development have been interpreted as an indication of an evolutionary relationship [61], in line with paleobotanical views that the root evolved from a shoot [121]. The CLE/WOX modules have been identified for all three stem cell niches, but each acts distinctly at the molecular level. First, CLE peptide signaling negatively regulates *WUS* and *WOX5* expression in the shoot and root, whereas CLE41p positively affects *WOX4* expression in the vasculature [8, 10, 61, 122]. Second, the sources and sinks of the CLE peptides differ: in the shoot meristem, CLV3p signals from the stem cells to the organizing center, whereas in the root and in the vasculature, differentiated stem cell daughters signal back to the niche [10, 123]. Thus, without further studies, it is not possible to determine whether the repeated use of CLE/WOX modules is coincidental or reflects adaptations of an ancient stem cell-regulating mechanism. There are more striking differences in the stem cell niches. Cytokinin signaling is required in the shoot meristem and vascular stem cells, whereas auxin is important for root stem cell maintenance.

The data of Schrader et al. [86], particularly the specific cambial expression profiles of homologs of known apical meristem regulators like PttCLV1, PttANT, and PttKNOX, suggest that similar regulatory mechanisms are active in the cambium and apical meristems. Besides similarities on the level of stem cell maintenance, it seems to be a certain degree of conservation in the radial patterning system between the shoot apex and the cambial meristem. The expression profiles of genes like PttKAN1 and PttHB9 in the stem correspond well to the expression patterns reported for their Arabidopsis orthologs in leaves. Because the vasculature in leaves is continuous with that in the stem, it was assumed that such similarity might be the result of a common patterning mechanism.

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Chapter 25
Meristems and Primary and Secondary Growth in a Plant

Science is a way of life. Science is a perspective. Science is the process that takes us from confusion to understanding in a manner that's precise, predictive and reliable—a transformation, for those lucky enough to experience it, that is empowering and emotional.

Brian Greene

Abstract This chapter describes the role of primary and secondary growth and of related meristems, in establishing the basic body plan of the plant. The evolution of secondary growth and role of corresponding meristems in adaptations to terrestrial life is explained.

Coordination of primary and secondary meristematic growth produces the body of the adult sporophyte plant. Plant bodies do not have a fixed size. Parts, such as leaves, roots, branches, and flowers all vary in size and number from plant to plant —even within a species. The development of the form and structure of plant parts may be relatively rigidly controlled, but some aspects of leaf, stem, and root development are quite flexible. As a plant grows, the number, location, size, and even structure of leaves and roots are often influenced by the environment. A vascular plant consists of a root system and a shoot system (Fig. 19.1). The shoot system consists of the stems and their leaves. Flowers, fruits, and seeds are also formed on the shoot. The repeating unit of the vegetative shoot consists of the internode, node, leaf, and auxillary buds. Auxillary buds are apical meristems derived from the primary apical meristems, which allow the plant to branch or replace the main shoot if it is destroyed. When the plant has passed to the reproductive phase of development, these axillaries may produce flowers or floral shoots [1]. Primary and secondary growth play important roles in establishing the basic body plan of the organism. In the earliest vascular plants, the vascular tissues produced by primary meristems played the same conducting roles as they do in contemporary vascular plants. There was no differentiation of the plant body into stems, leaves, and roots. These three kinds of organs is a property of most present plants, due to the need for increasing specialization as a response to the demands of terrestrial existence. The evolution of secondary growth enabled to vascular plants

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to develop thick trunks (Fig. 21.1). This evolutionary advance contributed to development of forests and expansion of plants on the land. On the basis of found fossils, secondary growth evolved independently in several groups of vascular plants by the middle of the Devonian period 380 million years ago. The two types of conducting systems appeared in the earliest vascular plants. Sieve-tube elements conduct carbohydrates away from areas where they are produced or stored. Vessel elements and tracheids are thick-walled cells that transport water and dissolved minerals up from the roots. Both kinds of cells are elongated and arranged in strands making tubes. Sieve-tube elements are characteristic of phloem tissue, while vessel elements and tracheids are characteristic of xylem tissue. In primary tissues, formed in primary growth, these two types of tissue are typically associated with each other in the same vascular strands. In secondary growth, the phloem forms on the periphery, while a very thick xylem core develops more centrally [1, 2].

With increasing height, plants must ensure that their stems can carry their weight. Plant body weight can induce secondary growth in Arabidopsis stems, because the addition of artificial weights (placing a 2.5 g tube on the top of an immature plant) can induce interfascicular cambium (IC) imitation, probably through auxin signaling [3]. However, a recent study suggests that there is no linear correlation between plant height/weight and IC initiation. The authors instead found that manipulation of JA signaling affects secondary growth. Because the touch-inducible JA signaling gene *JAZ*10 is expressed at the base of the stem in the xylem and IC, it was hypothesized that intra-tissue tension might play a role. This tension may arise from divisions in the fascicular cambium or from xylem formation, which pushes the cambium outward, inducing JA signaling and thereby causing IC initiation [4]. Thus, body weight and tension might provide input into the cambium stem cell niche.

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Chapter 26
Propagation of Plant Stem Cells in Culture

The clearest way into the Universe is through a forest wilderness.

John Muir

Abstract This chapter describes advantages of cultivation of plant stem cells as a source of biologically active compounds for potential applications, over wild plants collection, plant cultivation, or non-meristematic plant cell culture propagation.

Plants are able to synthesize an enormous variety of compounds that have biological activity. There is a wide range of industrial sectors utilizing plant products, including pharmaceuticals, foods and drink, cosmetics, agrochemicals. There is also a large use of plant products in medicine in production of drugs [1]. Traditionally, these bioactive compounds have been directly extracted from raw plant material or obtained by chemical synthesis. A biotechnological alternative for its production is the use of plant cell cultures. It is evident an increasing interest in plant cell cultures, since a number of firms and academic institutions investigated possibility of massive production of very promising bioactive compounds. As an alternative to wild collection or plant cultivation, the production of useful and valuable plant bioactive compounds in large bioreactors is very attractive and efficient. It should contribute significantly to preservation of global biodiversity and alleviates associated ecological problems. The advantages of such processes include the controlled production according to demand and a reduced man work requirement [2]. Moreover different factors affect the culture growth and bioactive compound production in bioreactors: the gaseous atmosphere, oxygen supply and CO_2 exchange, pH, minerals, carbohydrates, growth regulators, the liquid medium rheology and cell density, agitation systems, and sterilization conditions. Therefore, efficient cultivation of plant cells requires comprehensive knowledge of biological as well as biochemical fundamentals (e.g., characteristics of cell growth and metabolism, cell line establishment, culture medium optimization) and related engineering principles, e.g., bioreactor design, process scale-up, and optimization [3].

Use of plant stem (meristematic) cell cultures has advantages over somatic cell cultures. Plant stem cells never undergo aging process, and are totipotent cells

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equipped with regenerative powers that facilitate plant growth and development. These cells have the ability to self-renew (by cell division) such that their number is maintained for indefinite periods in culture. Plant stem cells contain minerals, vitamins, essential fatty acids, and protective bioactive compounds. For example, biosynthesis of paclitaxel (Taxol), a compound with proven strong anticancer activity, in *Taxus cuspidata* is most expressed within the region containing cambial meristematic cells [4]. Since the meristematic cells are constantly dividing, they input energy in perpetual multiplication, not providing capacity for production of secondary metabolites. Thus, the extracts of their suspensions lack definitive secondary metabolites and therefore have the ability to provide clear product and brand differentiation. The strategy to increase the large-scale production process of these bioactive compounds by using plant stem cells is the elicitation, which consists in the induction of metabolites by elicitors that are introduced in small concentrations in a living cell system, improving the biosynthesis of specific compounds. Thus, using elicitors on plant stem cultures, high levels of bioactive metabolites are accumulated, and easily recovered directly from the culture media without cell biomass destruction. These quantities are significantly higher than by production using dedifferentiated plant cell cultures, i.e., those reminiscent of stem cells (undifferentiated) and derived from non-meristematic plant tissues [5, 6]. Besides, the meristematic cells retain fresh after indefinite cycles in culture, while the dedifferentiated cells in culture show signs of decay after certain time [5].

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Chapter 27
Cultured Plant Stem Cells as a Source of Plant Natural Products

Never memorize something that you can look up.
Albert Einstein

Abstract A detailed explanation of advantages of using meristematic cell cultures in comparison with dedifferentiated plant cell cultures is given. The examples are given of the known natural products derived from certain types of meristematic cells cultured in bioreactors. Additionally, meristematic cell cultures from reference species may also provide an important biological tool to explore plant stem cell function. The chapter further describes few plant stem cell extracts known up-to-date and used commercially as sources for regenerative therapy of human cells, in the field of cosmetics. The most common plant-derived stem cell used in skincare today is the Swiss Uttwiler Spätlauber apple; the regenerative effects of its extract on the skin properties are presented. Another known stem cell extract, used for skin cell protection from heavy metal damages, is derived from tomato (*Lycopersicon esculentum*).

Plant stem cells, originating from meristems, can theoretically divide unlimited number of times and thus can be considered immortal. Since the beginnings of plant tissue culture, cell suspension cultures have been routinely generated through what was believed to be a dedifferentiation process [1, 2]. Recent results evidence that this mechanism might not include a simple reverse reprogramming [3]. Regardless of the mechanism involved, this process results in mitotic reactivation of specialized cell types within a given organ, generating a multicellular mixture of proliferating cells.

It has been shown to be a complex process including chromatin reorganization associated with reprogramming of gene expression. It also includes selective destruction of proteins involved in maintaining the old function of a cell and a concomitant activation of proteins that are essential for the establishment of dedifferentiation and/or cell proliferation. Histone methylation activity is required for the establishment/maintenance of the dedifferentiated state and/or for inducing cells to reenter the cell cycle, at least partly, through activation of genes. The complexity of cellular dedifferentiation, particularly the occurrence of DNA recombination can

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lead to genome instability [4]. Suspension cultures derived from such cellular assortments often exhibit poor growth properties with low and inconsistent yields of natural products, due to harmful genetic and epigenetic changes that occur during this process [4, 5].

The solution to avoid this so-called dedifferentiation procedure is development of an innately undifferentiated cell line derived from meristematic cells, which function as stem cells. The further step is development of the cell suspension culture starting from this cell line. An example is undifferentiated cell line derived from cambium cells, or vascular stem cells. This mass of proliferating cells was distinct from dedifferentiated cells (DDCs) derived from a needle or embryo. The properties of these cells were studied on an example of culture of cambial meristematic cells (CMCs) derived from *Taxus cuspidata* [6], the source of the key anticancer drug, paclitaxel (Taxol). Their properties were compared with properties of the corresponding DDCs. A combination of deep sequencing technologies was used to compare the molecular signatures of these cells and those of typical DDCs. There are differences in gene expression in these two cell types. Digital gene expression tag profiling data are consistent with a CMC identity for these cultured cells. Also, genes encoding key enzymes integral to the biosynthesis of paclitaxel were induced more strongly in CMCs than in DDCs. Besides, growth properties were significantly better in culture of CMCs than in DDCs, even in the bioreactor conditions, such as a 20 L air-lift bioreactor, routinely used as a pilot for subsequent large-scale production. DDCs did not grow in this size bioreactor under the conditions tested and rapidly became necrotic. Conversely, CMCs always grew rapidly, increasing their d.c.w. from 3.65 g/l to 12.85 g/l within 14 d. Their relative tolerance of shear stress can likely be attributed to their small and abundant vacuoles (Fig. 26.1), reduced aggregation and thin cell walls. A key trait for the exploitation of plant cells on an industrial scale is the stability of their growth in suspension culture [4]. Opposite to the DDCs derived from the needles or embryo, the CMCs were successfully cultured over longer periods and with high performance, establishing their utility for growth on an industrial scale [6]. The magnitude of paclitaxel produced in the suspension was also compared for these two cell types. The amount of paclitaxel produced was strikingly greater in batch cultures of CMCs than that generated by either needle or embryo-derived DDCs, in both 3 and 20 L air-lift bioreactors. The CMCs are also significantly more responsive to elicitation compared to typical *T. cuspidata* suspension cells. These *T. cuspidata* suspension cultures were also monotored for the production of the abietane tricyclic diterpenoid derivatives, taxamairin A and taxamairin C, which have also been shown to possess antitumor activities. Elicitation of these cells within a 3 L air-lift bioreactor induced increases in both taxamairin C and especially taxamairin A in CMC cultures. The values were far greater than those produced in DDC cultures.

To establish whether CMCs derived from other plant species also exhibit superior properties regarding the biosynthesis of commercially relevant natural products, the above technology was used to produce such cells from some other plant species, including Japanese yew (*T. cuspidata*), ginseng (*Panax ginseng*), ginkgo (*Ginkgo biloba*), and tomato (*Solanum lycopersicon*). For example,

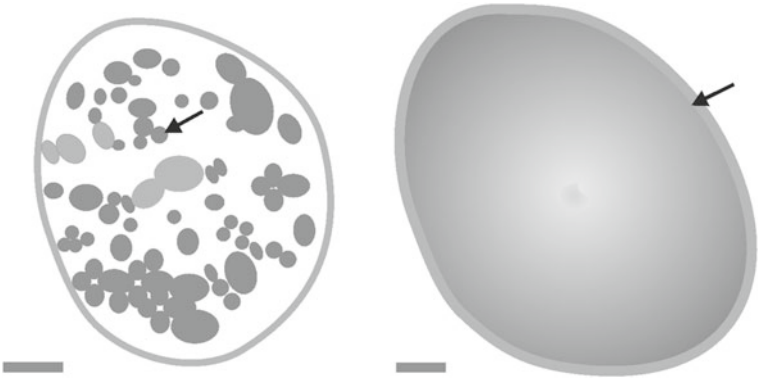

Fig. 27.1 Schematic presentation of the small vacuoles in a meristematic cell (*left*) as compared with a DDCs (*right*). The vacuoles are marked with arrows. Scale bar, 10 μM (based on the references Lee et al. [6] and Zirkle [7])

following elicitation of tap root-derived *P. ginseng* suspension cells, cultured using a 3 L air-lift bioreactor, ginsenoside F2, and gypenoside XVII accumulated to strikingly greater levels in *P. ginseng* CMCs relative to DDCs [6]. Ginsenosides are a class of triterpenoid saponins derived exclusively from the plant genus *Panax*. Ginsenosides have been reported to show multiple bioactivities including neuroprotection, antioxidative effects, and the modulation of angiogenesis [8].

Numerous medical and industrial products are derived from plant natural products [9, 10]. The above studies have shown that cultured CMCs may provide a cost-effective, environmentally friendly and sustainable source of important natural products. Furthermore, CMCs from reference species may also provide an important biological tool to explore plant stem cell function.

27.1 Plant Stem Cell Extracts as a Source for Regenerative Therapy of Human Cells

Most prominent results in application of plant stem cell extracts have been achieved in the field of cosmetics [11].

Two basic types of stem cells are present in the human body: embryonic stem cells found in blastocysts (structures found in the human pre-embryonic stage) that can grow and differentiate into one of the more than 220 different cell types which make up the human body, and adult stem cells located in some adult tissues that can only differentiate into their own or related cell types. These cells act as a repair system for the body but also maintain the normal turnover of regenerative organs, such as blood, skin, or intestinal tissues.

Currently in medicine, adult stem cells are already used particularly in transplant medicine to treat leukemia and severe burns. In the cosmetic field, scientists are focusing their research on adult stem cells located in the skin. They are studying the potential of this type of cells, their functioning and aging. This research is helping us understand how to protect skin stem cells. In the human skin, two types of adult stem cells have been identified: epithelial skin stem cells which are located in the basal layer of the epidermis, and hair bulge stem cells located in the hair follicle. Epidermal or skin stem cells replenish and maintain the balance of cells within the skin tissue and regenerate tissue damages during injury. However, with age, the number of skin stem cells decreases and their ability to repair the skin becomes less efficient [12].

Unlike humans, adult plants contain totipotent stem cells with the potential to regenerate a whole plant. The plant tissue culture technique is based on propagation of plant stem cells either to produce a whole plant, only tissue or just single cells in culture to harvest plant metabolites. This practice allows the production of plant material under sterile and standardized conditions independent of season and other environmental restraints.

The most common plant-derived stem cell used in skincare today is the Swiss Uttwiler Spätlauber apple. First cultivated in the eighteenth century for its hardy, long-lasting fruit, this tannin–rich fruit offers a rich source of anti-aging activity. They are selected for intensive cultivation and for a pleasant sweet flavor. In former times, good storage properties were an important factor for cultivar selection. Some of these old cultivars survived as isolated trees in areas with less intensive agriculture. The Uttwiler Spätlauber is an apple tree that was cultivated especially because of its good storage properties. A success full liquid culture in bioreactors of Uttwiler Spätlauber stem cells could be established. An extract of these cells was tested in a series of studies for anti-aging efficacy in skin and hair [13]. Reduced viability and premature senescence or apoptosis of stem cells is a principal cause for tissue aging. The extract of Uttwiler Spätlauber stem cells positively influences viability and resistance against senescence and apoptosis of human stem cells. In this way, the plant stem cell extract promotes regeneration of skin and hair and delays the appearance of skin aging signs.

Anti-Wrinkle Effect On Crows Feet The anti-wrinkle effect of Malus Domestica was evaluated in a study with 20 volunteers aged from 37 to 64. An emulsion containing 2% of PhytoCellTec™ Malus Domestica was applied twice daily for 28 days to the crow's feet. Wrinkle depth was determined by means of PRIMOS (phase-shifting rapid in vivo measurement of skin). Results showed a significant and visible decrease in wrinkle depth for 100% of the subjects.

Age-Delaying Effect on Hair Follicles of Apple Stem Cells Hair follicles are mini organs that represent a natural combination of epidermal and melanocyte (cells in the bottom layer of the skin) stem cells. The follicles can be maintained in a growth medium where they elongate until about day 14. Then the follicle cells gradually become senescent or undergo apoptosis—effectively they deteriorate and start to die—which is caused by the lack of blood circulation. Isolated hair follicles

represent a good test model to analyze actives ingredients, such as Malus Domestica, that can delay the cell deterioration and death process. Isolated human hair follicles were incubated with Malus Domestica extract. Addition of 0.2% of this extract was found to slightly but clearly postpone deterioration and necrosis: follicles kept in presence of the Malus Domestica stem cell extract continued to elongate until day 18, whereas the control follicles started to shrink after day 14.

Maintenance of Stem Cell Growth An in vitro test was conducted on blood stem cells with Malus Domestica stem cell extract. The influence of Malus Domestica stem cell extract on blood stem cell growth was evaluated by counting the cell number after incubation. Results showed that Malus Domestica stem cell extract has a positive effect on stem cell growth thus maintaining the growth and the proliferative activity of stem cells.

Protection Against UV Radiation Another test was conducted on blood stem cells. The protective effect against UV damage of Malus Domestica stem cell extract was evaluated by scientific analysis. Cells were incubated with different concentrations of Malus Domestica stem cell extract for 24 h and were then exposed to UV radiation. The analysis, which measures the number of cells still living and therefore the damage from UV, was performed 48 h after UV radiation. Results showed the capacity of Malus Domestica to protect cells from UV damage even at low concentrations.

Further Swiss studies showed that incubating fibroblast cells—the building blocks of collagen and other skin structural tissue proteins—in a 2% Uttwiler Spätlauber apple extract neutralized factors that lead to aging and, in some cases, actually reversed the process.

Another promising regenerative product for skin cell protection from heavy metal damages is tomato (*L. esculentum*) stem cell extract [14]. Plants have evolved sophisticated mechanisms to protect their cells from heavy metal toxicity, including the synthesis of metal chelating proteins and peptides, such as metallothioneins and phytochelatins (PC), which capture the metals and prevent the damages on the cellular structures. It was developed a cosmetic active ingredient from tomato cultured stem cells, to protect human skin cells from heavy metal toxicity. This product, besides its high content of antioxidant compounds, contains PC, effective in the protection of skin cells toward heavy metal toxicity. It was demonstrated protection of DNA from heavy metal damages by applying this product, by neutralyzing the effect of heavy metals on collagen degradation.

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Chapter 28
Mitochondria, a Vital Organelle in Stem Cell Maintenance

Science without religion is lame, religion without science is blind.

Albert Einstein

Abstract The energy metabolism in animal normal and cancer stem cells is described. Mitochondria in plant stem cells are characterized, with focus on the specific architecture of mitochondria in shoot apical and leaf primordial meristems, and on the role of dysfunctional mitochondria in meristem regulation. A description of molecular differences in mitochondria between plants and animals is given.

Mitochondria are the main source of ATP energy in eukaryotic cells, being also energetic hub of the cell. This is reason why they are of key importance for stem cells, which have high energy demands. They are among the most plastic organelles regarding form and distribution [1–4]. Furthermore, changes in their architecture and their ability to translocate rapidly throughout the cytoplasm are of critical importance for their cellular functions. It is known that mitochondria assemble around cellular areas with high energy requirements [2, 5]. Also, in both mammalian and plant cells, they constantly undergo fission, fusion, and branching changes while moving to different cellular locations [2, 5–7].

28.1 Mitochondria: Energy Metabolism in Animal Normal and Cancer Stem Cells

The essential role of mitochondria in animal cells is to synthesize energy reach compound ATP, through two vital cellular cycles: respiration, and glycolysis. These processes lead to ATP synthesis, which is then a macromolecule, reach in phosphor-ester bonds (3 total), capable of splitting and releasing energy needed for endergonic reactions of the cell, such as DNA synthesis. It is obvious that all kind of normal cells, normal stem cells, and cancer stem cells will need it.

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As already mentioned, in Chap. 11, metabolic pathway-the breakdown of glucose by enzymes, releasing energy and pyruvic acid, occurs in cytosol and brings up to 5% of total ATP synthesis (on ATP-ases of the cell plasma membrane). This process is also known as anaerobic glycolysis, since it occurs without presence of oxygen and yields to 2 mol of ATP per mole of glucose. The other part is aerobic glycolysis—within mitochondria and contributes to much higher production of ATP (4 mol ATP/mol of glucose).

The rest of ATP (95%) is synthesized on the ATP-synthase of the inner mitochondrial membrane. In the process of cellular respiration, within intramitochondrial matrix and inner mitochondrial membrane, three essential processes that enable this reaction to happen are: separation of the electricity/charge (positive and negative) on the inner mitochondrial membrane, (H^+ and e^-), synthesis of ATP on ATP-asis of the inner mitochondrial membrane and synthesis of endogenous water (from H^+ and O^{2-}) within matrix of mitochondria. The process is known as oxidative phosphorylation and contributes to 36 mol ATP/mol glucose. Thus, animal cells have mitochondria for cellular respiration and ATP synthesis releasing CO_2 in the air which is used by plants in photosynthesis within the chloroplast in order to synthesise starch and ATP within plant mitochondria.

Additional source of energy reach compound is the **Krebs cycle**, the sequence of reactions by which most living cells generate energy during the process of aerobic respiration. It takes place in the matrix of mitochondria, consuming oxygen, and producing carbon dioxide and water as waste products, as well as converting ADP to energy-rich ATP. Glycolysis is linked to respiration (aerobic glycolysis) via pyruvate which gives oxaloacetate as transportable form for mitochondria and starts, as well as ends up, the Krebs cycle.

Mitochondria is dividing and it has its own, circular DNA, the genes of which (about 150) code for enzymes of respiratory chain, and some proteins of human body. It is important from the point of inheritance since paternal mitochondria are lost during zygote formation and if wrong, the particular mother's genes can transfer disease to offspring, such as hemophilia, etc., due to the lack of "healthy"/male counterpart. There is a set of diseases known as mitochondrial diseases for which the mother is carrier.

Beside energy, mitochondria are known to be a storage of calcium which is released on specific ionophores when needed in the cell [8].

Deviation in metabolic aspects of mitochondria is described in Chap. 11 and their possible role in cancer stem cells explained. The uncoupling effect due to production of uncoupling proteins as a part of metabolic reprogramming, prevents cancer cell to couple respiration to oxidative phosphorylation which in return, causes the lower ATP synthesis due to block in the transfer of reducing equivalents from cytosol to mitochondria.

There are also suggestions that morphology of cancer stem cell (CSC) mitochondrial as well as their molecular composition are different [9]. The idea that mitochondrial function correlates with tumor malignancy recently arose together with indication that respiration and processing of different nutrients, as well as the generation of reactive oxygen species (ROS), play a mandatory role during key

steps of tumor progression [10]. While some cancers have mutations in nuclear-encoded mitochondrial tricarboxylic acid (TCA) cycle enzymes that produce oncogenic metabolites, there is negative selection for pathogenic mitochondrial genome mutations [9]. Eliminating mtDNA limits tumorigenesis, and rare human tumors with mutant mitochondrial genomes are relatively benign [9]. Thus, mitochondria play a central and multifunctional role in malignant tumor progression, and targeting mitochondria provides therapeutic opportunities [9].

The impact of mitochondria upon development of anticancer strategies is also described (Chaps. 11 and 19). The latest report describes among cancer cell subsets a phenotype with: addiction to mitochondrial function, activation of anabolic pathways, achievement of stem-like traits, resistance to stress and therapy, ability to undergo epithelial/mesenchymal transition [9]. Then microenvironmental factor, such as fibroblast and macrophages, cytokines, and oxygen/glucose shortage are of deep influence upon this complex phenotype. It is obvious that extensive investigation is necessary to identify common molecular pathways for these very aggressive CSC phenotype in order to define a targeted and effective therapeutic strategy against such a strongly malignant cell subset.

28.2 Mitochondria in Plant Stem Cells

28.2.1 Specific Architecture of Mitochondria in Shoot Apical and Leaf Primordial Meristems

The cell cycle-dependent changes in mitochondrial architecture have been studied in different *Arabidopsis thaliana* cell types. While mitochondria of cells from most plant organs are always small and dispersed, shoot apical and leaf primordial meristematic cells contain small, discrete mitochondria in the cell periphery and one large mitochondrial mass in the perinuclear region. Serial thin-section reconstructions of high-pressure-frozen shoot apical meristem (SAM) cells demonstrate that during G1 through S phase, the large, central mitochondrion has tentaculate morphology and wraps around one nuclear pole. In G2, both types of mitochondria double their volume, and the large mitochondrion extends around the nucleus to establish a second sheet-like domain at the opposite nuclear pole. During mitosis, approximately 60% of the smaller mitochondria fuse with the large mitochondrion, whose volume increases to 80% of the total mitochondrial volume, and reorganizes into a cage-like structure encompassing first the mitotic spindle and then the entire cytokinetic apparatus. During cytokinesis, the cage-like mitochondrion divides into two independent tentacular mitochondria from which new, small mitochondria arise by fission [11]. Reticular mitochondria have been reported as a characteristic feature of unicellular organisms, such as trypanosomes, yeast, fungi, Chlamydomonas, Chlorella, and Euglena [12, 13]. In contrast, the mitochondria of animal cells are typically discrete round or sausage-like organelles (for review, see Bereiter-Hahn [2];

Bereiter-Hahn and Voth [14]). Similarly, higher plant mitochondria are also round to sausage-like organelles [7, 15]. Most of the documented examples of reticular mitochondria come from mitochondrial mutants [16, 17] or from cells subjected to experimental perturbations [7, 18]. The presence of a large tentaculate/cage-like mitochondrion in SAM and leaf primordial (LP) meristematic cells makes these cell types unique (Fig. 28.1). Why these cells contain mitochondria with this type of morphology? It has been postulated that the proliferative function of SAM cells and the fact that they are the precursors of, among others, the germ cell lines can explain the presence of a reticulate mitochondrion in these cells. The cage-like mitochondrial architecture provides a means for efficient delivery of ATP to cell proliferation-related activities, and also provide a structural framework for efficient mixing and recombination of mtDNA.

It has been hypothesized that the cell cycle-dependent changes in the cage-like mitochondria are expressed in those cell lines destined to originate germ lines, avoiding the accumulation of undesirable mtDNA mutations. This capability would be characteristic of vegetative development, being expressed in SAM cells as well as in cells directly derived from them, including the LP cells. However, once the proliferative phase of the LP cells stops and differentiation to a mature leaf occurs, such a mechanism is no longer required. This explains why these complex mitochondrial morphologies have not been observed in cells of differentiated organs, including mature leaves. This also applies to any other SAM-derived differentiated cell, tissue, or organ, since after meiosis and gamete formation there is no need for this feature until a new seedling started a new cycle of vegetative development. Also, this hypothesis provides a rationale for why reticulate mitochondria have not been observed in other meristematic tissues such as root meristems, since they do not give rise to future germ cell lines [11].

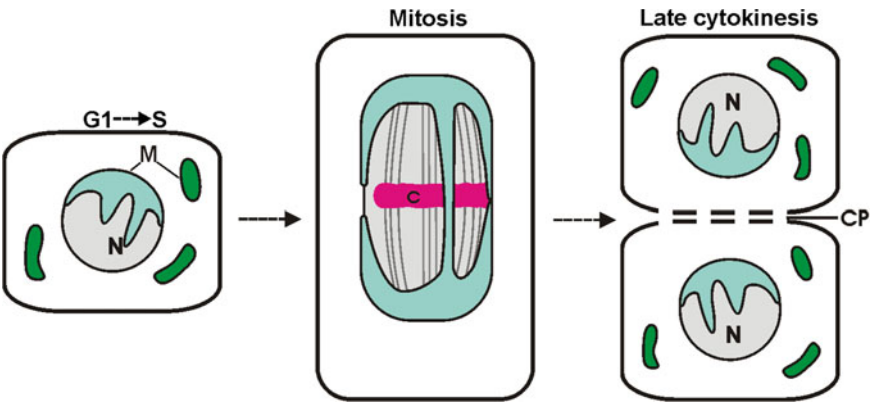

Fig. 28.1 Scheme of the changes in mitochondrial architecture in Arabidopsis SAM and LP meristematic cells at different stages of the cell cycle. There is mitochondria fusion during mitosis forming a cage-like mitochondrial structure, followed by fission during cytokinesis. *N* nucleus, *M* mitochondria, *C* chromosomes, *CP* cell plate (based on the reference [11])

28.2.2 Role of Dysfunctional Mitochondria in Meristem Regulation

Perturbation of mitochondrial structure and function affects plant growth and development. Most mitochondrial mutants have reduced growth rates and have a short root phenotype. The rate of cell production in a root meristem is proportional to the number of dividing cells times cell division rate. It is suggested that cell division rate in the root meristem rarely changes [19]. By contrast, changes in meristem length by setting the boundaries for meristem and elongation zone play a key regulatory role in root growth [20]. On the basis of experimental results it has been proposed that dysfunctional mitochondria may play an important role in the regulation of the boundaries for meristem and elongation zone in the root, and control of the cell division arrest front in the leaf [21], which regulates cell proliferation. Auxin-associated redox regulation is involved in the establishment and maintenance of QC [22]. It has been shown that mitochondria play an important role in the regulation of redox homeostasis in the QC [23]. Recent studies have implicated that mitochondrial perturbation negatively affects auxin signaling. Besides, one of the mitochondrial retrograde regulated genes is directly involved in auxin homeostasis [24]. Thus, retarded root growth observed in mitochondrial mutants may be explained by the interactions between dysfunctional mitochondria and auxin signaling.

28.3 Molecular Difference in Mitochondria Between Plants and Animals

Although both plant and animal cells contain mitochondria, there is a prominent difference at molecular level between mitochondria of these two kingdoms. Since mitochondria are of crucial importance for cell energy production and maintaining, this difference may be caused by the specific life conditions of plants, as sessile organisms, and may respond to specific demands imposed to plant meristems, to enable constant growth.

A feature of the plant mitochondrial electron transport chain (ETC) is the presence of two terminal oxidases. In addition to cyt oxidase, which exists also in the animal cells, a non-energy conserving alternative oxidase (AOX) is present in plant cells, which directly couples the oxidation of ubiquinol with the reduction of O_2 to H_2O. AOX is present in many animal phyla, but absent from vertebrates and arthropods. While respiratory carbon oxidation pathways, electron transport, and ATP turnover are tightly coupled processes, AOX provides a means to relax this coupling, thus providing a degree of metabolic homeostasis to carbon and energy metabolism. Beside their role in primary metabolism, plant mitochondria also act as "signaling organelles," able to influence processes such as nuclear gene expression. AOX activity can control the level of potential mitochondrial signaling molecules,

such as superoxide, nitric oxide, and important redox couples. In this way, AOX also provides a degree of signaling homeostasis to the organelle. It has been evidenced that AOX function is particularly important during stress. As sessile organisms, land plants are subjected to many stressors in their environment, such as high or low temperature, drought, nutrient deficiency, salt, and metal toxicity, hypoxia, and pathogen attack. Since the net carbon gain of a plant is equal to CO_2 uptake by photosynthesis minus CO_2 release by respiration, changes in either of these processes during stress will impact overall plant growth and productivity. Diverse mitochondrial dysfunctions, often associated with oxidative stress, result in the induction of AOX at the transcript and protein level. As a result, AOX is now often used as a general marker of mitochondrial dysfunction and/or cellular oxidative stress. Further, numerous abiotic and biotic stress conditions are known to elevate AOX amount, supporting the idea that such stresses impact mitochondrial function and that AOX might represent an important acclimation response [25].

The rate of ROS generation by mitochondria depends on the reduction state of ETC components. In animals, this reduction state is generally dependent on the rate of electron transport and the membrane potential, which in turn are primarily dependent on the rate of dissipation of membrane potential, particularly by oxidative phosphorylation. Thus, when ADP is readily available and being actively phosphorylated to ATP, dissipation of the proton gradient lowers membrane potential and O_2 generation is lower than when ADP is limiting. In plants, however, the relationship between electron transport, oxidative phosphorylation and ROS generation is more complex because electron flow from ubiquinol to AOX does not contribute to membrane potential. Hence, AOX could provide a means to maintain significant electron flow, even when ADP is limiting, while still preventing the overreduction of the ETC [26, 27]. Studies have now provided direct *in planta* evidence that AOX acts to prevent the overreduction of ETC components that leads to single electron leak [28]. Stress induces changes in the mitochondrial metabolism, when changes in AOX activity are needed for rapid response to these changing demands. AOX activity can also directly impact the level of potential important signaling molecules, thus providing an important link between mitochondrial function, signal transduction, and acclimation to stress. Similarly, in plant meristems there are specific demands in regard constant maintaining plant growth. In such conditions AOX may provide a similar support to that in stress response.

The respiratory parameters at each age are not identical in the different regions of a plant organ. It has been shown that AOX protein is localized in the apical meristem and in developing xylem. The temporal evolution of AOX in different plant organs [29, 30] indicates that this protein is responding to some developmental cues. The specific expression of AOX in xylematic tissues and its coordinated decline during root and hypocotyls development, suggest a role for this enzyme connected with xylem differentiation. Since heat is a major product of the alternative pathway [31], it has been speculated that localized warming at precise times might be important in the promotion and/or coordination of specific processes during morphogenesis. A small or moderate temperature difference between determined cells with high

AOX activity and the surrounding tissues or medium, rather than a high global temperature elevation, could mediate some event in developing tissues [29].

AOX is encoded in small multigene families in plants. Functional analysis of the *A. thaliana* alternative oxidase 1c (AtAOX1c) promoter, an AOX gene not induced by oxidative stress, provided insight into the regulation of AOX expression under non-stress conditions and during growth and development. The results indicated that regulation of expression was complex, with the upstream promoter region containing positive and negative response regions. Comparison with the promoter region of soybean (*Glycine max*) alternative oxidase 2b (GmAOX2b), another AOX gene not induced by oxidative stress, revealed that they contained seven sequence elements in common. All elements were active in the promoter region of AtAOX1c in suspension cells and in leaf tissue from wildtype and mutant plants, where a mitochondrial protein import receptor was inactivated. Analysis indicated that AtAOX1c was coregulated with components involved with cell division and growth, being regulated by growth and developmental signals [32].

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Chapter 29
Molecular Similarities Between Plant and Animal Stem Cells

If we knew what it was we were doing, it would not be called research, would it?

Albert Einstein

Abstract This chapter presents the known similarities between animal and plant stem cells' maintenance and regulations. The RETINOBLASTOMA-RELATED (RBR) protein, the plant homolog of the RB tumor suppressor protein, which has a crucial role in both root and shoot niche regulation, is the rare known protein involved in stem cell function that is conserved between the animal and plant kingdoms. Another similarity between plant and animal stem cells refers to the fact that damaged stem cells in the root can be replaced by symmetric division of an adjacent stem cell that subsequently adopts the tissue fate according to positional signals, which is also observed in several animal stem cell niches. The role of miRNA in maintaining the position of the functional stem cell niche within a dynamic structure is an important mode of regulation in both plants and animals. Breast cancer susceptibility gene 1 and matching protein (BRCA1), as well as another BRCA2 protein, are required for the efficient repair of double strand breaks by homologous recombination, in the cells of plant *Arabidopsis thaliana*, like in animal cells. Pluripotent stem cells in both animals and plants contain open chromatin compared with differentiated cells.

Main differences between plant and animal cells are presence of cell wall compartment around plant plasma membrane, as well as vacuoles and chloroplasts inside the plant cells. The function and significance of vacuoles varies significantly according to the type of cell in which they are present, and have much greater importance in the cells of plants, fungi, and certain protists than those of animals and bacteria. Plant vacuoles are much larger than those in animal cells. They can store food or various nutrients that a cell might need to survive. They can also store waste products to protect the cell from contamination. Vacuoles maintain internal hydrostatic pressure or turgor within the plant cell, and maintain an acidic internal pH. They allow plants to support structures such as leaves and flowers due to the pressure of the central vacuole [1].

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It has been shown that animal stem cells contain a large number of small vacuoles in comparison to the plant cells, although the number is not set up and varies, dependent on the type and phase of the life cycle. [2]. In plant stem cells a great number of vacuoles has also been found, yet, considerably smaller than those in adult cells, with respect that they have also One Large Central Vacuole but there is no analogous structure in animal cells [3, 4].

29.1 Stemness Factors

It still remains unrevealed whether stem cells have specific stemness factors that make them pluripotent, or they are simply any kind of cell that divides and is blocked from the next step of differentiation. Mutant analysis evidenced that chromatin factors and genome organization factors are crucial for stem cell maintenance, and for most of them, it has been shown that they regulate key stem cell regulators (like WUS, WOX5, or PLT) [5–9]. Thus, shoot and root plant stem cells might contain a chromatin state different from that of differentiated cells, which is similar to animal stem cells (see below). Transcriptional profiling experiments are also important in understanding the molecular nature of plant stem cells in depth. The cambial stem cells in poplar are characterized by signal transduction and transcriptional regulation factors [10]. The transcription profile of the root cortex/endodermis stem cells shows high expression of the G2/M-phase-specific genes [11]. Shoot stem cells have an overrepresentation of transcripts encoding factors involved in DNA metabolism, DNA replication and repair, chromosome organization, and biogenesis [12]. A systematic comparison of transcriptional profiles will be needed to elucidate if there is a signature common to all plant stem cells.

Plant stem cell niches are specified during embryogenesis, and, post-embryonically, they are located within the meristems, which are organized groups of dividing cells that are responsible for most post-embryonic development.

Dividing stem cell progeny in the meristem are equivalent to the animal transit-amplifying cells. The activity of meristems enables plants to continuously generate new organs and structures throughout their lifetime, thus determining plant architecture. This contrasts with animal development, as the animal body plan is mostly defined during embryonic development; adults generally lack pluripotent stem cells and multipotent stem cells mainly function to maintain tissue homeostasis and in tissue repair. Comparison between the two kingdoms has been reviewed by Heidstra and Sabatini [13].

In short, two opposite processes occur in meristems: stem cell self-renewal is stimulated to maintain the population of stem cells, and cells are recruited from the meristem into developing organs. The number of stem cells and their dividing daughter cells in the meristem remains constant, despite continuous displacement of differentiating cells into new organs. This indicates that the formation of new cells and cell differentiation are dynamically and almost perfectly balanced. On the other

side, the continuous organ production that is characteristic of plant growth requires a robust regulatory network to keep the balance between pluripotent stem cells and differentiating progeny. Maintaining stem cell homeostasis in the shoot and root stem cell niches is essential to ensure that an equal number of new cells are generated to replace those that are displaced from the niche, to differentiate and to enable the growth and formation of new tissues and organs. Interestingly, the RETINOBLASTOMA-RELATED (RBR) protein, the plant homologue of the RB tumor suppressor protein, has a crucial role in both niches [14, 15]. As in animals, RBR inhibits cell cycle progression by interaction with an E2F transcription factor homologue [16]. Reduced levels of RBR result in an increase in stem cell numbers, and increased RBR levels lead to stem cell differentiation, which indicates a prominent role for RBR in stem cell maintenance [17, 18]. At present, RBR is the only known protein involved in stem cell function that is conserved between the animal and plant kingdoms.

Another important protein in maintenance of a stable shoot stem cell pool is the WUS protein. It is regulated through expression of the *WUS* gene. The WUS protein acts as a nonautonomous signal to maintain stem cells and is sufficient to promote stem cell identity in meristems [19, 20]. The results suggest that WUS triggers local co-activators and co-repressors to target promoters, and that it may regulate gene expression in a concentration-dependent manner. The communication between stem cells and the organizing center is coordinated with environmental fluctuations to maintain a stable stem cell pool. Similar to the shoot niche, *WUSCHEL-RELATED HOMEOBOX 5* (*WOX5*), a homolog of *WUS*, maintains stable root stem cell pool, indicating that WOX5 is a conserved factor of a larger conserved module that regulates plant stem cell maintenance in general [21]. The results indicate existence of a ligand—receptor complex, which physically restricts the exchange of 'stemness information' beyond adjacent quiescent center and stem cells [22, 23]. The important role has the CLAVATA3/ESR-RELATED 40 (CLE40) peptide from differentiated columella cells, which signals through its receptors to counteract the WOX5 activity and to promote cell differentiation. CLE40 signaling by differentiated columella cells to control the self-renewal capacity of their stem cell progenitors resembles the regulation observed in several animal stem cell niches. Recent studies suggest that committed stem cell progeny provide flexible feedback signals to their stem cell parents, thus being a vital component of the niche [24].

Another similarity between plant and animal stem cells refers to the fact that damaged stem cells in the root can be replaced by symmetric division of an adjacent stem cell that subsequently adopts the tissue fate according to positional signals, which is also observed in several animal stem cell niches, for example, in the *D. melanogaster* germarium [25]. Dedifferentiation of stem cell progeny provides a second mechanism for stem cell replacement [26]. Dedifferentiation of transit-amplifying cells in animal systems is similarly governed by signals from the niche [27]. The ability of the quiescent center to replace (damaged) stem cells indicates a 'reserve' function that is able to restore the stem cell niche, which is similar to the plasticity observed during regeneration of the crypt base columnar

stem cell compartment in the mammalian intestine or similar to the reactivation of quiescent stem cells in the bone marrow after injury [28]. However, whereas in animal systems lost stem cells can be replaced by the reversion of a differentiating cell to a stem cell or by symmetric self-renewal of a surviving stem cell, in plants it seems that the (quiescent) stem cell function is a property of organizing cells within the niche.

The role of miRNA in maintaining the position of the functional stem cell niche within a dynamic structure is an important mode of regulation in both plants and animals. Repression of differentiation-promoting transcripts in stem cells by local miRNAs to promote self-renewal is also observed in animal systems [29], which provides another mechanistic analogy between plant and animal stem cell regulation. Maintaining the position of stem cells at the shoot apical meristem involves several inputs from surrounding tissues besides the organizing center (WUS), including the epidermis (cytokinin and miR394), the peripheral zone (auxin) and the provasculature. In the root, the plant hormone auxin is the main positional cue to maintain stem cell niche position [30].

29.2 Stem Cells in Plants and the Method of Clonal Analysis

Like in animals, stem cells in plants function as a source of new cells to grow or replace specialized tissues. To perform this function, these cells must divide to renew themselves, while some of their descendants eventually differentiate to build up new tissues. By this definition, stem cells are particularly important for plant growth, because virtually all tissues of the plant descend from small groups of stem cells located in their growing apices, within structures called the apical meristems [14, 31]. We know that just a few stem cells are the ultimate source of all new plant cells, by creating genetic differences between cells of the same plant, and track how the descendants of a single marked cell make up organs and tissues as the plant grows. The method is called clonal analysis because it shows how clones (genetically identical groups of cells derived from the same progenitor) build up the plant. This type of experiment showed that most marked cells divide just a few times and make a small contribution to growth of the plant, while a few cells within the meristems divide many times and their descendants make up all new tissues and organs.

Clear evidence that the whole shoot descends from a small number of constantly dividing cells within the apical meristem came from clonal analyses done in the 1970s [32]. By looking into variegated plants, (whose leaves and stems are not uniformly green, but have patches of white tissue)—a feature often selected in ornamental plants, they found that these white tissues descend from mutant cells that are unable to produce chloroplasts. Most of the time, the plants produce small colorless patches, but these scientists also found plants with colorless sectors composing nearly a third to half of the whole shoot, and which were continuously

produced by the meristem over long periods of time. These large, stable sectors of mutant cells could only be formed if all cells that make up the shoot descended from a small population of relatively stable, long-term progenitors—the shoot stem cells. It was also confirmed by comparable experiments that showed that root tissues also descend from a small set of stem cells [33–35]. In a new set of experiments the scientists used a reporter gene (an artificially introduced gene that encodes an easily detectable product) to mark cell lineages in the roots of the model plant Arabidopsis. First, the reporter gene was blocked by a transposon (a piece of DNA that can move around in the genome). Then the cells were genetically marked when the transposon moved, unblocking expression of the reporter in only a few cells of the root meristem, which allowed these cells and their descendants to express the reporter gene. Thus, a few large and stable sectors allowed the scientists to trace the progenitors of root tissues to just a few stem cells in the center of the root meristem.

In summary, by genetically marking stem cells, it was possible to show that small groups of stem cells function as a long-term source of new cells to build up the shoot and the root. However, the experiments described above also revealed that the large sectors originating from the stem cells were not maintained throughout the life of the plant. Eventually, although the meristems continued to function, they stopped producing the genetically marked cells. The marked cells could not revert to what they were before, so progenitor cells within the meristem that gave rise to marked sectors must have died or simply stopped functioning as stem cells. This observation is explained by another key feature of stem cells that is shared between plants and animals, as explained below. The ability of stem cells to constantly generate new tissues has to be coordinated with the needs of the whole organism. If stem cells do not divide frequently enough, tissues cannot grow or be replaced (as happens during normal aging); if stem cells proliferate out of control, they disrupt normal development (resulting for example in cancer) [36, 37]. So it is not surprising that the behavior of stem cells is strictly dependent on signals provided by other cells. Stem cells are normally maintained only in specific locations where these extracellular signals are available. As the cells proliferate, only the descendants that remain within reach of the maintenance signals continue to behave as stem cells, whereas those displaced away from the signals begin to differentiate. The locations where extracellular signals allow stem cells to exist are called stem cell niches. The maintenance of stem cells only within the niche explains the observation above that a genetically marked stem cell eventually stopped functioning as a stem cell. This can occur by chance if the marked cell is pushed away from the niche by the divisions of neighboring stem cells.

How do we know that signals from other cells are needed to maintain the plant stem cells? In the shoot, this was revealed by work on mutant Arabidopsis plants that are unable to maintain the apical meristem. Laux et al. [38] isolated Arabidopsis plants with mutations in the WUSCHEL (WUS) gene. In these plants, the shoot apical meristem starts to form, but then the meristem cells differentiate and the stem cells are lost. WUS turned out to encode a transcription factor (a protein that controls the activity of other genes) that is expressed in only a small

number of cells just beneath the stem cells in the shoot meristem [19]. Because the WUS mutation affects the adjacent stem cells, where the gene is not expressed WUS must be required to produce a signal that functions between cells to maintain the shoot stem cells.

In the root, evidence that stem cells are maintained by intercellular signals came from experiments in which the cells making the signal were selectively killed. van den Berg and colleagues [39] used a laser beam to kill single cells within the root meristem. When they killed specific cells that flank the stem cells (called the quiescent center cells, or QC for short) the adjacent stem cells differentiated. This was not simply a result of the injury caused by the laser, because killing neighboring cells other than the QC did not induce differentiation. Therefore, the QC must be the source of a signal that prevents differentiation of the root stem cells.

Thus plant stem cells depend on the function of other, neighboring, cells to continue behaving as stem cells, and therefore some sort of signal must be passed between cells to maintain stem cell behavior. In animals, the signaling molecules used within stem cell niches are often the same used to organize growth and tissue patterning during embryogenesis, such as homologs of the Notch, Wingless, and Hedgehog proteins from Drosophila [36, 37]. Plant genomes do not contain genes that encode proteins similar to these, so plants must use different signals, but a major gap in our knowledge is that we still do not know exactly what signaling molecules maintain plant stem cells.

29.3 Homologs of Breast Cancer Genes in Plants

We have seen above that there are intriguing similarities in the way stem cells function in both plants and animals to sustain growth and replace tissues. To perform their functions, stem cells are different from other cells in two ways. First, they are able to produce copies of themselves over long periods of time, at least until they become damaged or are separated from their niche. In contrast, other cells are programmed to divide at most during a relatively short period before all their progeny differentiate and stop dividing. The second difference is that stem cells are generally pluripotent, that is, they have the potential to produce different types of differentiated cells. Do similar mechanisms operate in plant and animal stem cells to maintain long-term division and pluripotency?

Breast cancer proteins were shown to work as tumor suppressors through their involvement in DNA-damage repair. Interestingly, homologs of these genes can be found in plant genomes, as well. As for the biological roles of these proteins in plants, in addition to the conservation of their function in DNA repair, new plant-specific characteristics have been revealed. Breast cancer susceptibility gene 1 and matching protein (BRCA1), as well as another BRCA2 protein, are required for the efficient repair of double strand breaks (DSB) by homologous recombination in

somatic cells of the model plant *Arabidopsis thaliana*. BRCA1 exhibits most of its functions in mammals in a heterodimer with the so-called BARD1 (BRCA1 associated RING domain protein 1) protein. Bioinformatic analysis indicates that, while most homologs of key components of the different mammalian BRCA1 complexes are present in plant genomes, homologs of most factors involved in the recruitment of BRCA1 to the DSB cannot be identified. Thus, it is not clear at the moment whether differences exist between plants and animals at this important step. The most conserved region of BRCA1 and BARD1 homologs in plants is a PHD domain which is absent in mammals and which might be involved in the transcriptional regulation of plant development. On the basis of the presence of a plant-specific domain, the current model for the evolution of BRCA1 homologs has been proposed and a new hypothesis suggested, in which is postulated that plant BRCA1 and BARD1 have one common predecessor that gained a PHD domain before duplication. Also, work in Arabidopsis demonstrates that—as in animals—BRCA2 homologs are important for meiotic DNA recombination. A BRCA2 homolog was shown to be involved in pathogen defense in plants. It reveals the need to learn more about the biological role of these genes in plants. The acquisition of additional, more accurate plant genome sequences is needed, in order to validate the hypothesis of BRCA1 and BARD1 evolution in plants and animals [40].

29.4 Chromatin States in Animal and Plant Stem Cells

The property of pluripotency is believed to depend at least in part on the way the chromatin is organized, that is, how the DNA is packed in the nucleus and how this affects the access of regulatory proteins to genes required for cell differentiation. Polycomb proteins play an important role in regulating the chromatin to repress differentiation genes and therefore maintain the pluripotency of animal stem cells [37]. In plants, Polycomb proteins also regulate the transition between pluripotent and differentiated states, but unlike in animals, they are required in the differentiating cells to repress genes that are normally expressed in the meristem. This is shown by Arabidopsis plants with mutated polycomb genes: in these plants, shoot meristem genes continue to be expressed in cells that are due to form leaves and consequently leaf development is abnormal [41, 42].

In animals, the Oct4/POU5F1, Sox2, and Nanog transcription factors are found in stem cells and are sufficient to reprogram differentiated cells into stem cells. This transcriptional program is implemented in the context of a different chromatin state in stem cells [43–45]. Pluripotent stem cells contain open chromatin compared with differentiated cells, meaning less heterochromatin, more loosely bound (or hyperdynamic) architectural chromatin proteins, less H3K9 methylation, and global transcriptional hyperactivity. Upon differentiation, the transcriptional program needs to be rapidly switched, which is possibly mediated by the presence of both activating and repressive chromatin marks (so-called bivalent domains) on lineage-specific developmental regulators. This is achieved in a process where these

regulators are silenced and at the same time calm for activation. In addition, embryonic stem cells are sensitive to reduced levels of key structural components of chromatin (cohesin and condensing complexes).

In conclusion, at least some of the genes that control the stem cell state in animals are also relevant for plant stem cells, but there may be variations in the way these genes are deployed. The Rb protein seems to function similarly in plants and animals to stop cell division and start differentiation in cells that leave the stem cell niche. Polycomb proteins are used to maintain a repressive chromatin state in both kingdoms but appear to function differently in the stem cells: they repress differentiation genes in animal stem cells, whereas in plants they are used to inhibit meristem genes in differentiated cells. It must be said, however, that we are far from understanding the molecular basis of pluripotency in any organism, so we cannot yet be sure whether pluripotency is controlled differently in plants and animals.

29.5 Just Coincidence?

As described above, plant and animal stem cells have some surprising similarities in their developmental roles, in the ways they are organized within tissues, and to some extent in the molecular mechanisms controlling their behavior. This is surprising not simply because plants are so different from animals, but because plants and animals very likely evolved from unicellular to multicellular organisms separately [46]. Therefore, stem cells probably evolved independently in both kingdoms as an advantageous solution to the problem of balancing the need to grow with the need to produce specialized cells, which often cannot divide. In both plants and animals, stem cell niches likely evolved as devices to match the location and proliferation rate of stem cells to the needs of the whole organism. Molecular similarities, such as the role of Rb proteins, probably result from adopting mechanisms in the stem cells to control cell division and differentiation that already existed in unicellular organisms [14, 31]. In conclusion, comparisons across large evolutionary distances, such as that between plants and animals, allow us to highlight the most fundamental principles of stem cell biology.

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