Stem Cells and Cancer Stem Cells 11
Therapeutic Applications in Disease and injury

M.A. Hayat
Editor

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Volume 11
Therapeutic Applications in Disease and injury

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Therapeutic Applications in Disease and injury

Edited by

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"Although touched by technology, surgical pathology always has been, and remains, an art. Surgical pathologists, like all artists, depict in their artwork (surgical pathology reports) their interactions with nature: emotions, observations, and knowledge are all integrated. The resulting artwork is a poor record of complex phenomena."

Richard J. Reed, MD

Preface

Stem Cells are nature's indispensable gift to multicellular organisms, including humans.

This is the eleventh volume of the fourteen-volume series, *Stem Cells and Cancer Stem Cells: Therapeutic Applications in Disease and Tissue Injury*. A stem cell is defined as a cell that can self-renew and differentiate into one or more specialized cell types. A stem cell may be pluripotent, which is able to give rise to the endodermal, ectodermal, and mesodermal lineages; an example is embryonic stem cells. A stem cell may be multipotent, which is able to give rise to all cells in a particular lineage; examples are hematopoietic stem cells and neural stem cells. A stem cell may be unipotent, which is able to give rise to only one cell type; an example is keratinocytes.

A cancer stem cell is a cell type within a tumor that possesses the capacity of self-renewal and can give rise to the heterogeneous lineages of cancer cells that comprise the tumor. In other words, a cancer stem cell is a tumor-initiating cell. A unique feature of cancer stem cell is that although conventional chemotherapy will kill most cells in a tumor, cancer stem cells remain intact, resulting in the development of resistance of therapy. All of these types of stem cells are discussed in this series.

Molecular mechanisms underlying the derivation and expansion of human embryonic stem cells are explained. Also, the role of specific proteins in the maintenance and inhibition of extraembryonic differentiation of these cells is explained. The role of signaling responsible for the self-renewal of mouse embryonic stem cells is detailed.

Potential clinical importance of cancer stem cells is discussed in nine chapters. The principle involved in the stemness regulation of somatic cancer cells is also explained. Different sources of cancer stem cells are discussed. The role of cancer stem cells in diseases is explained in detail; the diseases discussed include glioma, leukemia, ovarian cancer, pediatric sarcomas, and head and neck squamous cell carcinoma. The role of cancer stem cells is also discussed in epithelial-to-mesenchymal transition in spreading head and neck squamous cell carcinoma is elucidated.

Therapeutic application of stem cells of hematopoietic and non-hematopoietic origin for regeneration of bones in the preclinical and clinical setting, including in osteoporotic bone disease, is well established. The application of hematopoietic stem cells in bone regeneration is detailed. Because blood vessels are a key element to the growth of tumors and hematopoietic cells support angiogenesis, the use of these cells as biomarkers

facilitates the determination of a treatment. The use of these cells as biomarkers for pediatric solid tumors is elaborated. The advantages and limitations of hematopoietic stem cell transplantation, for example, in neuroblastoma, are pointed out. The details of neural stem cell engraftment in injured spinal cord are included. The regenerative potential of neural stem/progenitor cells of the newborn is explained. Cancer gene therapy potential using neural stem cells is also included.

By bringing together a large number of experts (oncologists, neurosurgeons, physicians, research scientists, and pathologists) in various aspects of this medical field, it is my hope that substantial progress will be made against terrible human disease and injury. This volume was written by 58 contributors representing 10 countries. It is difficult for a single author to discuss effectively the complexity of diagnosis and therapy, including tissue regeneration. Another advantage of involving more than one author is to present different points of view on a specific controversial aspect of cancer cure and tissue regeneration. I hope these goals will be fulfilled in this and other volumes of the series. I am grateful to all the authors for their promptness in accepting my suggestions. Their practical experience highlights their writings, which should build and further the endeavors of the readers in these important areas of disease and injury. I respect and appreciate the hard work and exceptional insight into the nature of cancer and other diseases provided by these contributors. The contents of the volume are divided into four subheadings: Embryonic Stem Cells, Cancer Stem Cells, Neural Stem Cells, and Hematopoietic Stem Cells for the convenience of the readers.

It is my hope that subsequent volumes of the series will join this volume in assisting in the more complete understanding of the causes, diagnosis, and cell-based treatment of major human diseases and debilitating tissue/organ injuries. There exists a tremendous, urgent demand by the public and the scientific community to address cancer diagnosis, treatment, cure, and hopefully prevention. In the light of existing cancer calamity, government funding must give priority to eradicating deadly malignancies over military superiority. I am thankful to Dr. Dawood Farahi and Philip Connelly for their encouragement to continue the endeavor to publish these volumes. I am also thankful to my students for their help in many ways in completing this project.

M.A. Hayat

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Part I

Embryonic Stem Cells

Maintenance of Human Embryonic Stem Cell Identity and Inhibition of Extraembryonic Differentiation: Role of CNOT1, CNOT2 and CNOT3

1

Raluca Dumitru and Guang Hu

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Abstract

Embryonic stem cells (ESCs) are defined by their capacity to self-renew and differentiate into all adult tissues. They hold the promise to generate models for human development and disease, establish new platforms for drug discovery, and develop therapies for regenerative medicine. To fully realize these potentials, it is crucial to understand the molecular mechanisms controlling ESC self-renewal and differentiation. In this chapter, we discuss the regulators of human ESC self-renewal, with the emphasis on a newly-identified protein complex, Ccr4-Not, in the maintenance of human ESCs and the inhibition of extraembryonic differentiation.

Introduction

The isolation of human ESCs opened a new chapter in stem cell biology by unraveling new and exciting avenues for the study of human development and regenerative medicine. Human ESCs are derived from the inner cell mass (ICM) of the developing blastocysts and have two important characteristics shared only by the pluripotent stem cells: unlimited self-renewal in vitro while maintaining a normal karyotype, and pluripotency or the ability to differentiate into cells comprising the three germ layers ectoderm, mesoderm, and endoderm. Due to their wide-range differentiation ability, human ESCs were proposed as a potential cell replacement

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source to treat conditions such as spinal cord injury, neurodegeneration, diabetes, heart diseases and more. They are also the gold standard to understand the molecular basis of self-renewal and pluripotency, as well as the processes controlling differentiation and lineage specification during embryonic development (Murry and Keller 2008).

In this chapter, we provide an overview of the molecular circuitry regulating ESC self-renewal and differentiation, with the emphasis on human ESCs and their differentiation toward the extraembryonic lineages. We will discuss the role of a newly identified self-renewal complex, the Ccr4-Not complex, in the maintenance of human ESCs and its function in preventing extraembryonic differentiation.

Human ESC Self-Renewal and Differentiation

ESC self-renewal depends on a plethora of signaling pathways, a complex transcriptional network, as well as a number of epigenetic regulators (Dejosez and Zwaka 2012). In the past few years it has become increasingly apparent that the factors needed to maintain the self-renewal of human ESCs are different from those necessary for mouse ESCs. These two types of ESCs are different in morphology, growth, differentiation behavior, and culture condition requirements. Although they share a limited number of the core regulators, they show different gene expression profiles and rely on different signaling pathways for their maintenance. Remarkably, human ESCs with characteristics of the mouse ESCs could be generated by continued overexpression of several pluripotency genes and culture in conditions that favor mouse ESC self-renewal, suggesting that the pluripotent state may be conserved across species to some extent (Blair et al. 2011).

Key Transcription Factors in Human ESC Self-Renewal

Human ESC self-renewal is maintained by a complex transcriptional regulatory network (Fig. 1.1). At the core of this network are three master regulators: *OCT4*, *SOX2* and *NANOG*. Shown to function as a protein complex, they co-occupy and activate the expression of self-renewal genes while repressing differentiation and lineage specific regulators, as well as bind to and regulate their own gene promoters. This intricate auto-regulatory network allows for a robust maintenance of the self-renewal state while permitting a rapid switch towards differentiation if conditions change (Dejosez and Zwaka 2012). During development, *OCT4*, *SOX2* and *NANOG* are required for ICM and epiblast development by counteracting differentiation toward the extraembryonic lineages including the trophectoderm and primitive endoderm (Niwa 2010). In ESCs, they are also required to prevent extraembryonic differentiation. Interestingly, in the appropriate molecular context, they seem to be able to regulate differentiation of ESCs into embryonic lineages as well (Dejosez and Zwaka 2012).

OCT4 is a member of the Pou-domain octamer family of transcription factors and is probably the best-understood pluripotency factor. In vivo, *OCT4* is required for the formation of the epiblast and its deletion results in pre-implantation lethality. The ICM of the *OCT4* null embryos only contain cells that have differentiated towards the trophectoderm lineage (Nichols et al. 1998). In mouse ESCs, modulation of *OCT4* expression can lead to different developmental outcomes. Increased OCT4 level directs the differentiation to PE and mesoderm, decreased OCT4 level gives rise to TE cells, whereas intermediate OCT4 level maintains ESC self-renewal. These data indicate that the levels of OCT4 have to be carefully modulated for the proper maintenance of ESCs (Niwa 2010). In addition to SOX2 and NANOG, OCT4 also interacts with many supplementary self-renewal factors, such as SALL4, HDAC2, SP1, DAX1, NAC1, TCFP2L1, and ESRRB. These interactions do not necessarily exist in a single protein complex, but rather allow the formation of multiple complexes with different compositions to control different aspects of self-renewal (Dejosez and Zwaka 2012).

SOX2 is a member of the SOX (Sex determining region Y-related HMG box) family of transcription

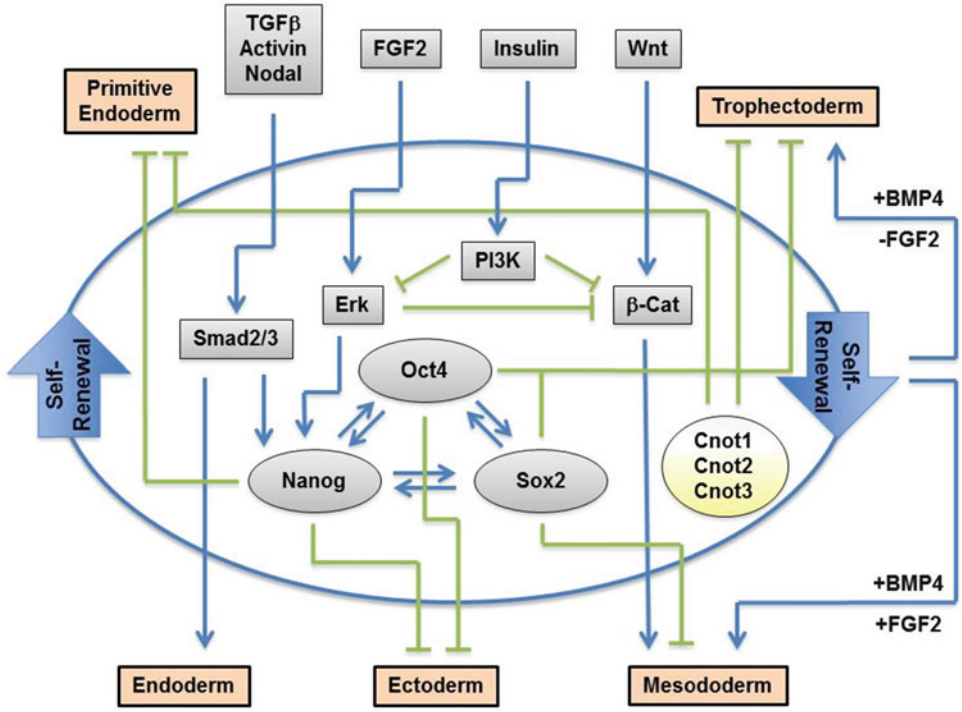

Fig. 1.1 Regulation of human ESC self-renewal and differentiation

factors that regulate tissue specific and cell fate determination. Gene targeting in mice revealed that the homozygous deletion of *SOX2* is embryonic lethal (Avilion et al. 2003). The mutant deciduae at around 6.0 dpc were found to lack the egg cylinder structure and the cells characteristic of the epiblast were replaced by trophectoderm cells. Like OCT4, its level in mouse ESCs is tightly controlled, as its deletion induces TE differentiation while its overexpression results in differentiation toward the neuroectoderm, mesoderm, and TE lineages. Intriguingly, a key feature of *SOX2* deletion appears to be an inability to sustain appropriate OCT4 levels, as forced *OCT4* expression can partially rescue the phenotype (Niwa 2010; Dejosez and Zwaka 2012).

NANOG was identified as the third principal component of the core transcriptional network important for ESC pluripotency and early mouse development (Chambers et al. 2003; Mitsui et al. 2003). It is a homeodomain-containing transcription factor. Although it is not required for ESC maintenance, it is required for the establishment of the pluripotent state both in vitro in the generation of the induced pluripotent stem cells and in vivo in the development of the epiblast cells. Its expression alone is sufficient to sustain ESC self-renewal and pluripotency under conditions that normally trigger differentiation. As is true of OCT4, NANOG interacts with a number of proteins that have been implicated in mouse ESC self-renewal (Dejosez and Zwaka 2012).

Although most of our knowledge about the three core self-renewal genes originated from studies in the mouse system, it is now apparent that their function in human ESCs is largely conserved. Like in mouse ESCs, silencing *OCT4*, *SOX2*, or *NANOG* in human ESCs by RNA interference (RNAi) leads to differentiation mostly toward the extraembryonic lineages (Avery et al. 2006). However, a recent study also indicated that *OCT4*, *SOX2*, and *NANOG* may be important for the specification of embryonic lineages in human ESCs (Wang et al. 2012). *OCT4* down-regulation was found to induce the differentiation of human ESCs to embryonic ectoderm in the absence of BMP4 and towards TE in the

presence of BMP4. *NANOG* down-regulation specifies embryonic ectoderm, while low *SOX2* induces meso-endoderm differentiation.

In summary, *OCT4*, *SOX2*, and *NANOG* are the key components in the regulatory network of self-renewal and pluripotency. They tightly control the ESC state through extensive interactions with other factors and transcriptional control of many developmental genes. Together, they play critical roles in the establishment and maintenance of pluripotency and in the suppression of extraembryonic differentiation.

Extrinsic Pathways that Regulate ESC Self-Renewal

Although human and mouse ESCs rely on the same three core factors for the maintenance of self-renewal, the signaling pathways engaged to prevent their differentiation are very distinct. While LIF/STAT3, BMP4 and Wnt/β-catenin pathways are important for mouse ESC self-renewal (Dejosez and Zwaka 2012), human ESCs do not respond to LIF, and BMP4 and Wnt/β-catenin induces their differentiation. In contrast, human ESCs require FGF2, Activin A or transforming growth factor β1 (TGFβ1), and activators of the phosphoinositide-3-kinase (PI3K) pathway such as insulin and insulin-like growth factor 1 (IGF1) (Fig. 1.1) (Levenstein et al. 2006; Vallier et al. 2009; Singh et al. 2012). Indeed, the signaling pathways that are absolutely required for the maintenance of self-renewal in human ESCs have recently become more apparent due to the development in the media formulation for these cells (Chen et al. 2011). It was shown that human ESCs can be maintained in a defined medium by FGF2, TGFβ, and insulin.

Fibroblast Growth Factor 2 (FGF2)

Expression of FGF receptors and their downstream targets was found to be high in the undifferentiated human ESCs and reduced upon differentiation. Indeed, FGF2 is the key ingredient for self-renewal in human ESCs and virtually all the media currently used for the maintenance of these cells contain high concentrations of FGF2 (Levenstein et al. 2006; Chen et al. 2011). FGF2 activates the mitogen activated kinase kinase – extracellular signal regulated kinase (MEK-ERK) pathway and plays several important roles for the maintenance of human ESCs: (1) It maintains the expression of pluripotency genes such as Nanog (Yu et al. 2011); (2) It induces the expression of TGF β1 and IGF-II signaling while inhibiting BMP4 signaling; (3) It inhibits caspase-mediated anoikis. Blocking FGF receptors in human ESCs led to the up-regulation of the TE as well as the primitive endoderm markers. Together, FGF2 is associated with many pleiotropic-positive effects on human ESCS, such as inhibiting differentiation, increasing proliferation, enhancing attachment and cloning efficiency, and reducing apoptosis, and thus maintain the cells in the optimal self-renewal state.

Transforming Growth Factor β (TGFβ)

The TGFβ/Activin/Nodal pathway is one major branch in the TGFβ superfamily signaling. It is evolutionarily conserved and is involved in key cellular decisions during embryogenesis. TGFβ/Activin/Nodal signals via the type I receptors ALK4, 5, 7 and activates SMAD2/3 to regulate downstream gene expression. It supports human ESC self-renewal together with FGF2, and its inhibition causes neuroectoderm differentiation. TGFβ/Activin/Nodal maintains self-renewal by directly and positively regulating pluripotency genes such as Nanog through SMAD2/3 and antagonizing BMP4 (Xu et al. 2008; Vallier et al. 2009). Importantly, it can act as a switch to control self-renewal and differentiation by integrating signals from multiple pathways involved in human ESC maintenance (see below, PI3K signaling). Several components of the TGFβ/Activin/Nodal pathway are expressed by human ESCs, including the ligands TGFβ1, GDF3, BMP2 as well as the secreted inhibitors LEFTY1, LEFTY2, Cerberus and Cripto. Together, this suggests that there may be feedback loops to fine tune the pathway.

Bone Morphogenic Protein 4 (BMP4)

BMP4 is also a ligand in the TGFβ superfamily. It signals via the type I receptors ALK1, 2, 3, 6 and activates SMAD1/5/8 to regulate downstream gene expression. In contrast to the situation in mouse ESCs where BMP4 maintains self-renewal (Dejosez and Zwaka 2012), in human ESCs BMP4 differentiates the cells to extraembryonic trophectoderm when FGF2 is not present, and to mesoderm when FGF2 is added (Yu et al. 2011). As a result, noggin and gremlin, two BMP inhibitors, can suppress SMAD1/5 signaling and prevent the differentiation of human ESCs (Avery et al. 2006).

Phosphoinositide-3-Kinase

PI3K signaling is important for the maintenance of human ESCs as it regulates the output from the TGFβ/Activin/Nodal signaling and controls the balance between self-renewal and differentiation (Singh et al. 2012). High PI3K signaling allows TGFβ/Activin/Nodal-Smad2/3 to perform a pro-self-renewal function by activating target genes like NANOG, while low PI3K and high Wnt signaling directs TGFβ/Activin/Nodal-Smad2/3 to promote differentiation.

Wnt

Wnt signaling plays important roles in many different types of stem cells, including hematopoietic stem cells, intestine stem cells, and ESCs (Nusse et al. 2008). In mouse ESCs, Wnt supports self-renewal, as its activation by Gsk3β inhibition stabilizes β-catenin and abrogates the negative regulation of self-renewal by Tcf3 (Wray et al. 2011). In human ESCs, Wnt activation by the expression of a stabilized β-catenin inhibits self-renewal and causes differentiation into the mesoderm progenitors. Such a differentiation depends on the cooperative action of β-catenin together with Activin/Nodal and BMP signaling pathways (Sumi et al. 2008). Consistently, it was recently shown that in the presence of low PI3K/Akt signaling, ERK can inactivate GSK3β and direct TGFβ/Activin/Nodal-Smad2/3 signaling to promote differentiation (Singh et al. 2012). Thus, Wnt signaling appears to have different roles between mouse and human ESCs.

Additional Regulators

Recently, additional regulators of human ESC self-renewal were discovered through genetic approaches. PRDM14 was discovered in a genome-wide RNAi screen as a determinant of human ESC identity (Chia et al. 2010). It was found to directly regulate the expression of Oct4 through its proximal enhancer. It co-localizes extensively with other key pluripotency transcription factors such as OCT4, NANOG, and SOX2, and may be an integral component of the core transcriptional regulatory network. In addition, components of the Ccr4-Not complex were identified in RNAi screens as regulators of self-renewal in mouse ESCs (Ding et al. 2009; Hu et al. 2009), and were later shown to be important for human ESCs as well (Zheng et al. 2012). Three of the subunits, CNOT1, CNOT2, and CNOT3 were shown to maintain self-renewal by repressing the expression of extraembryonic transcription factors and thereby inhibiting the differentiation to the extraembryonic lineages (Zheng et al. 2012).

ESC Differentiation Toward the Extraembryonic Lineages

Studies on mouse development showed that the early blastocyst is composed of two types of cells: the ICM and the trophectoderm (TE). TE is a single layer of cells that surrounds the ICM and will give rise to the majority of the fetal part of the placenta. By the time of implantation, the ICM further segregates into two lineages: the epiblast (EPI) that will give rise to the entire fetus as well as extraembryonic mesoderm, and the primitive endoderm (PE) that forms the extraembryonic endoderm layers of the visceral and parietal yolk sacs. The TE and PE extraembryonic lineages are required to support the growth of the

mammalian embryo and are sources of signals to the EPI to initiate axial patterning. The segregation between ICM and TE and EPI and PE are the earliest lineage specification events during development (Rossant 2007). Many genes important for these developmental choices have been identified and characterized, and not surprisingly they all play important roles in ESC self-renewal and differentiation (Niwa 2010).

ESCs are derived from the ICM of the blastocysts, which is established after the segregation of TE. Mouse ESCs can largely recapitulate the developmental potential of the ICM and differentiate into both the embryonic lineages and PE (Niwa 2010). Human ESCs, on the other hand, also have the capability to differentiate into TE (Xu et al. 2002).

Trophectoderm Differentiation

Mouse ESCs cannot contribute to the TE lineage after blastocyst injection, and they do not normally differentiate toward TE in vitro unless they are genetically manipulated. However, it is now known that mouse ESCs can be forced into TE differentiation by manipulating the expression of genes involved in TE specification, and they appear to be a useful system for identifying triggers of TE specification. Specially, reducing the level of *OCT4*, *SOX2*, or *CNOT1*, *CNOT2*, *CNOT3* (see below), as well as overexpression of *TEAD4*, *CDX2*, *EOMES*, or *GATA3* all leads to TE differentiation (Rossant 2007; Niwa 2010; Ralston et al. 2010; Home et al. 2012). In vivo, *OCT4* is required for the establishment of ICM and is thought to be a key player in the lineage choice between ICM and TE. *SOX*2 is likely to have a similar function, but additional experiments are needed to draw the conclusion due to its maternal expression. On the other hand, *TEAD4*, *CDX2*, and *EOMES* are all critical for TE development, and their deletions negatively impact TE development at various stages (Rossant 2007). CNOT1, CNOT2, CNOT3 are part of the Ccr4-Not complex and have been recently identified as important players in ESC maintenance both in mouse and human, and their silencing in mouse ESCs leads to TE differentiation (Zheng et al. 2012). However, their role in vivo during blastocyst development has not been carefully investigated.

Human ESCs are able to differentiate into the TE lineage much more easily than mouse ESCs. It has been shown that BMP4 treatment in the absence of FGF2 induces TE differentiation in human ESCs (Xu et al. 2002). In addition, like mouse ESCs, genetic perturbation of the key pluripotency genes, such as Oct4 and the Ccr4-Not genes, can also drive human ESCs into the TE lineage (Avery et al. 2006; Zheng et al. 2012).

Primitive Endoderm Differentiation

Both mouse and human ESCs are capable of differentiating into the PE. In mouse ESCs, PE can be generated by EB formation, as it closely mimics the post-implantation development of the ICM. It can also be generated by genetic perturbations such as reducing NANOG or elevating GATA4 or GATA6 levels (Niwa 2010).

In human ESCs, activation of the protein kinase C (PKC) pathway causes human ESCs to undergo an epithelial to mesenchymal transition and differentiates the cells to PE as exemplified by the up-regulation of SOX7, SOX17 and GATA6 (Feng et al. 2012).

The CCR4-Not Complex

The Ccr4–Not complex is conserved across the eukaryotic kingdom and acts at different levels to regulate gene expression (Collart 2003; Collart and Panasenko 2012; Doidge et al. 2012). It was originally discovered in budding yeast, with most components identified in genetic screens designed to find regulators of gene expression and some additionally identified in screens for other cellular functions. *Not1* and *Not2* were first identified as *Cdc39* and *Cdc36* in a screen for cell cycle arrest in G1, and *Not1*, *Not2*, and *Not4* were later identified as regulators of mating type and filamentous growth as well. *Ccr4* was discovered in a screen for genes that prevented the expression of alcohol dehydrogenase and was found to be

Table 1.1 Ccr4-Not complex components in different species

Saccharomyces cerevisiae	Musmus culus	Homo sapiens
Not1	CNOT1	CNOT1
Not2	CNOT2	CNOT2
Not3	CNOT3	CNOT3
Not4	CNOT4	CNOT4
Not5	N.A.	N.A.
Ccr4	CNOT6L	CNOT6/CCR4a
	CNOT6L	CNOT6L/CCR4b
Pop2/Caf1	CNOT7	CNOT7/CAF1a/hCAF1
	CNOT8	CNOT8/CAF1b/hPOP2/CALIF
Caf40	CNOT9/RQCD1/RCD1	CNOT9/hRCD1
Caf130	CNOT10	CNOT10

important for non-fermentative gene expression. The NOT genes acquired their names when the first four were identified in a screen for mutants that increased the expression of a compromised HIS3 gene. Mutations in these four genes increased mRNA generated from a TATA-less promoter of the HIS3 gene, suggesting that they were important for repressing the promoter. It was based on this phenotype that the four genes were named the "Negative on TATA-less" or NOT genes. Ccr4-Not was considered as a negative regulator of transcription since then, and additional genetic evidence supported such a role. Not too long after, it was shown that the NOT proteins and Ccr4 exist in one complex, and that the Ccr4-Not complex is the major cytoplasmic mRNA deadenylase in yeast.

The function of Ccr4-Not in gene regulation is conserved throughout evolution, and homologs of the core subunits have been identified in higher eukaryotes including mammals (Table 1.1). For the sake of relevance to human ESCs, we will primarily focus on the mammalian Ccr4-Not complex and refer to their yeast counterparts only when necessary and when information on the mammalian genes is not available.

Structure and Organization of the Complex

Yeast two-hybrid, co-immunoprecipitation, co-purification by gel-filtration, and structural studies have provided many details on how the subunits

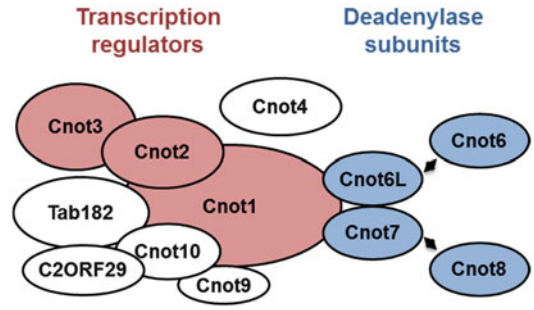

Fig. 1.2 The mammalian Ccr4-Not complex

interact with each other (Collart and Panasenko 2012). In mammals, the core Ccr4-Not complex is composed of 10 subunits (Fig. 1.2). CNOT1 is the scaffold of the complex and the largest subunit. Most other subunits of the complex interact with the middle and C-terminal region of CNOT1, but CNOT2 and CNOT9 also seem to interact with its N-terminus. Possibly because of its role as the scaffold, *CNOT1* is the only essential Ccr4-Not gene in yeast. CNOT2 is also important for the integrity of the complex, as it bridges the interaction between CNOT1 and CNOT3. CNOT3 only interacts with CNOT2, and loses its interaction with the complex in its absence. CNOT4 is not a stable subunit of the complex, and there is no ortholog of yeast *Not5* In mammalian cells. CNOT6 and CNOT6L are orthologs of the yeast deadenylases *Ccr4*, and have been found to be mutually exclusive in the complex. Similarly, CNOT7 and CNOT8 are orthologs of *Caf1* and appear in the complex in an alternating fashion. The interactions for the (rest of the complex)

components, such as CNOT9, CNOT10, TAB182, and C2ORF29, are less well defined.

In both yeast and mammals, the Ccr4-Not complex is known to exist in multiple distinguishable forms ranging from 0.5 to 2 MDa (Collart 2003; Collart and Panasenko 2012). Furthermore, different components of the complex seem to have very specific and only partially overlapping functions. For example, individual subunits have been associated with different cellular functions or enzymatic activities (see below). Deletion of silencing of individual subunit led to different patterns of gene expression changes and different phenotypes (Collart and Panasenko 2012). Thus, it is still unclear why these different subunits exist in one complex and how each subunit carries out its function. It is also unclear whether the Ccr4-Not complex is constituted of small subcomplexes that process unique functions outside of the whole complex. It was shown that the human Ccr4–Not subunits are nucleo-cytoplasmic shuttling proteins, although the majority of the subunits appear to be cytoplasmic, consistent with the role of the complex in cytoplasmic mRNA deadenylation (Doidge et al. 2012).

Molecular Function of the CCR4-Not Complex

Early studies in yeast suggested a strong role of the Ccr4–Not complex in transcription. Several subunits were initially described as transcription repressors, and later studies revealed that the complex can both positively and negatively regulate gene expression. The complex has both genetic and biochemical interactions with the general transcription factor TFIID, the TATA-binding protein TBP, and the SAGA histone acetyltransferase complex, supporting its role in transcription initiation. In addition, genetic studies and direct biochemical measurement also demonstrated that the complex regulate transcription elongation by RNA polymerase II (Collart and Panasenko 2012).

Consistent with its role in yeast, Ccr4–Not complex can also regulates transcription in mammalian cells (Collart and Panasenko 2012; Doidge et al. 2012). CNOT1 was found to interact with nuclear receptors and is recruited to gene promoters with other Ccr4-Not subunits to negatively regulate transcription. CNOT2 was found to repress reporter gene activity when targeted to DNA, possibly by a mechanism involving the N-coR/HDAC3 complex. CNOT9 was shown to interact with the MYB proto-oncogene and repress its target gene activation. CNOT6 and CNOT9 were also shown to function as nuclear hormone receptor co-activators and are required for target gene activation. CNOT7 interacts with the retinoid X receptor, and deletion of *CNOT7* in mouse embryo fibroblasts reduced binding of the receptor to a response element and retinoic acid-induced gene activation.

The other important function of the complex is deadenylation. Deadenylation is the first step in bulk mRNA decay, which leads to subsequent degradation of mRNA by the cytoplasmic exosome complex (Garneau et al. 2007). In mammalian cells, Ccr4-Not is one of the two major poly(A) nuclease complexes and is responsible for mRNA deadenylation (Bartlam and Yamamoto 2010; Doidge et al. 2012). The two deadenylase enzymes in the yeast Ccr4-Not complex are Ccr4 and Caf1, which belong to two different exoribonuclease superfamilies, the DEDD and EEP families, respectively. Mammals have CNOT6 and CNOT6L as orthologs of yeast Ccr4, and CNOT7 and CNOT8 for Caf1. While all four deadenylases can associate with the Ccr4-Not complex, only one Caf1-type and one Ccr4-type deadenylase subunits seem to be present in any given individual complex. CNOT6 and CNOT6L share high sequence similarity and partially overlapping function, and so do CNOT7 and CNOT8. However, despite the fact that both types of the deadenylases reside in the same protein complex, CNOT6/6L and CNOT7/8 appear to regulate distinct groups of mRNAs and have different effects on cellular phenotypes (Bartlam and Yamamoto 2010; Doidge et al. 2012).

In addition to the deadenylation enzymes, CNOT4 provides a second enzymatic activity in the Ccr4-Not complex. CNOT4 contains two functional domains in the N-terminus: a RING-finger domain that has the E3 ubiquitin ligase

activity and a RRM (RNA Recognition Motif) domain that has putative RNA-binding activity (Collart and Panasenko 2012). The structure of its RING-finger domain has been solved by NMR and its ubiquitin ligase activity has been demonstrated in vitro. In human cells, CNOT4 can polyubiquitinate JHD2, a histone H3 Lys 4 demethylase, and indirectly control gene expression. But in contrast to the situation in yeast, Cnot4 does not appear to be a stable subunit of the complex in mammalian cells, and it is therefore not clear how it contributes to the overall function of the Ccr4-Not complex (Collart and Panasenko 2012).

Ccr4-Not has been implicated in many cellular functions such as translational repression, miRNA-mediated mRNA decay, mRNA export and nuclear surveillance, and proteome assembly. As a result, defects in the complex have been associated with diverse phenotypes in DNA damage repair, cell proliferation, spindle positioning and microtubule length regulation, spermatogenesis, and adult heart function. It has been proposed that Ccr4-Not may act as a complex to connect to all aspects of the gene expression processes, and thereby provide cells with a "switchboard" for efficient adjustment of the gene expression program to respond to environmental cues (Collart and Panasenko 2012).

CCR4-Not in ESC Maintenance

The role of the Ccr4-Not complex in ES cell maintenance and self-renewal was first implicated in genetic screens carried out in mouse ESCs, in which CNOT1 and CNOT3 were identified as positive regulators of self-renewal by two independent studies (Ding et al. 2009; Hu et al. 2009). In a follow-up study, where all the core subunits of the Ccr4-Not complex were carefully re-examined, three subunits, CNOT1, CNOT2, and CNOT3, were confirmed to be required for mouse ES cell self-renewal, as silencing any of the three induced differentiation (Zheng et al. 2012). In contrast, silencing any of the deadenylases, either individually or in combination, had no obvious impact on self-renewal, suggesting that the deadenylase activity of the complex may not be essential. However, it is also possible that the functional redundancy between the deadenylases and/or the incomplete depletion of the enzymatic activity by RNAi may have masked the requirement for the deadenylases. The above findings were further supported by the reported phenotype of Ccr4-Not gene deletion in mice. *CNOT3* deletion in mice is embryonic lethal at or before implantation, and the ICM from the *CNOT3* null blastocysts has severe defect in outgrowth and failed to produce ES cell lines (Neely et al. 2010). On the contrary, *CNOT7* deletion does not negatively impact embryonic development and only has phenotypes in spermatogenesis at the adult stage (Berthet et al. 2004; Nakamura et al. 2004).

Biochemical and genetic epistasis analysis suggested that *CNOT1*, *CNOT2*, and *CNOT3* do not interact with other known self-renewal factors or pathways. In addition, chromatin immunoprecipitation followed by microarray indicated that the genomic occupancy of CNOT3 is different from that of the other pluripotency transcription factors such as NANOG, OCT4, and SOX2 (Hu et al. 2009). Thus, it was proposed that *CNOT1*, *CNOT2*, and *CNOT3* act in a parallel pathway to regulate self-renewal. Further experiments showed that they can repress the expression of TE transcription factors such as CDX2, and thereby maintain self-renewal by inhibiting TE differentiation (Zheng et al. 2012). Mechanistically, it is not clear whether the three Ccr4-Not genes repress the TE transcription factors transcriptionally or post-transcriptionally, as the Ccr4-Not complex can suppress gene expression through either transcriptional repression or mRNA deadenylation. It will be interesting if CNOT1, CNOT2, and CNOT3 indeed act through deadenylation, as most studies on self-renewal so far are focused on transcription regulation. Similar to what has been reported in other cell types, CNOT1, CNOT2, and CNOT3 form a complex in mouse ESCs, and they are mostly cytosolic with a small but significant fraction residing in the nucleus (unpublished result).

CNOT1, *CNOT2*, and *CNOT3* silencing in mouse ESCs resulted in differentiation predominantly into the TE lineage, as indicated by the

up-regulation of many TE markers and global gene expression analysis. As discussed above, mouse ESCs do not normally differentiate into the TE lineage, either in vitro or in vivo. Previously, only genetic perturbation of a small number of genes was known to induce TE differentiation. These include silencing or deletion of key self-renewal regulators such as *OCT4* or *SOX2* or overexpression of master TE transcription factors such as *CDX2*, *EOMES*, or *GATA3*, all of which play important roles in the separation and maintenance of the first two lineages (TE and ICM) during in vivo embryonic development (Niwa 2010). It will be instructive to determine whether *CNOT1*, *CNOT2*, and *CNOT3* are involved in the same lineage specification in early embryonic development. Thus far, the expression of *CNOT1*, *CNOT2*, and *CNOT3* appear to be enriched in the ICM of mouse blastocysts at E3.5 (Zheng et al. 2012), supporting the idea that they may be required for ICM development in vivo.

Similar to mouse ES cells, undifferentiated human ESCs express high levels of *CNOT1*, *CNOT2* and *CNOT3*, and down-regulate them upon differentiation. *CNOT1*, *CNOT2*, and *CNOT3* silencing by RNAi led to differentiation into both the TE and PE lineages based on marker expression (Zheng et al. 2012). Morphological changes induced by *CNOT1*, *CNOT2*, and *CNOT3* silencing appear different from those caused by Oct4 silencing, again suggesting that they may regulate self-renewal through separate mechanisms. Unlike mouse ESCs, human ESCs can differentiate into the TE lineages in the presence of BMP4 and absence of FGF2 (Xu et al. 2002), and adding FGF2 can divert BMP4-induced differentiation into the mesoderm lineage (Yu et al. 2011). Silencing the three Ccr4-Not genes induced high expression levels of TE markers and much lower levels of mesoderm markers even in the presence of FGF2, supporting the notion that at least part of their normal function in self-renewal is to inhibit TE differentiation. It remains to be determined if CNOT1, CNOT2, and CNOT3 inhibit extraembryonic differentiation in human ESCs by repressing master transcription factors like CDX2 and GATA6 as they do in mouse ESCs.

As mentioned in the previous section, human ESCs are significantly different from mouse ESCs in gene expression, morphology, growth kinetics, clonogenicity, differentiation behavior, requirement for culture conditions, and extrinsic signaling pathways. It has been proposed that mouse and human ESCs represent two different states of pluripotency: the naïve pluripotency and the primed pluripotency. The two states differ by the above mentioned characteristics, as well as a key difference that only the naïve pluripotent state is associated with the competency to contribute to blastocyst chimera and germline transmission. On the other hand, both states share the following common features: differentiation into three germ layers in vitro, terotoma formation, and expression of and requirement for *NANOG*, *OCT4*, and *SOX2* (Nichols and Smith 2009). The observation that *CNOT1*, *CNOT2*, and *CNOT3* are similarly required in both mouse and human ESCs suggests that they are likely a critical component in the self-renewal regulatory network shared by the naive and primed pluripotent states. To date, only a small number of core self-renewal transcription factors, such as *NANOG*, *OCT4*, and *SOX2*, have been shown to be required for the maintenance in both the naive and primed pluripotency. The identification of additional genes like *CNOT1*, *CNOT2*, and *CNOT3* provides a new opportunity to understand and dissect the similarities and differences between mouse and human ESCs and between different pluripotent states on the molecular level.

Concluding Remarks

Since the first derivation of human ESCs in 1998, extensive investigations have started to reveal the genes and pathways that regulate their pluripotency, self-renewal, and differentiation. Although much information is available, a comprehensive and unifying model describing the underlying molecular circuitry is still lacking. Recent genome-wide functional genetic screens provided a wealth of novel players in human ESC self-renewal. Defining the roles of these newly-identified regulators and their relationships with the known

pathways will offer new insights and greatly help the construction of such a model.

The Ccr4-Not complex is involved in almost all the stages of gene expression and in numerous biological processes. The discovery that components of this complex, CNOT1, CNOT2, and CNOT3, are required for human ESC maintenance raised the question whether and how self-renewal can be regulated at the post-transcriptional level. Whatever the answer may be, the apparently conserved role of the Ccr4-Not genes in mouse and human cells and in the prevention of extra-embryonic differentiation suggest that the complex, or a specific version of it, plays a critical role in the maintenance of the pluripotent state. Further studies of this complex in ESCs will be instructive to the understanding of both the complex itself and the molecular basis of pluripotency and self-renewal.

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Investigating the Role of the Embryonic Stem Cell Self-Renewal Gene NANOG in Neoplastic Processes

2

Collene R. Jeter

Contents

Abstract

Embryonic stem cell transcription factors are among the myriad of stem cell (SC)-related genes implicated in neoplastic disease processes. NANOG, a homeobox domain transcription factor essential to embryogenesis promotes proliferation and self-renewal integral to both normal and tumor development. NANOG mRNA species and protein have been detected in numerous types of tumor cells ranging from malignant pluripotent teratocarcinoma to somatic tumors of the brain, liver, breast and prostate, among others. Functionally, NANOG has been implicated as an important mediator of tumor cell clonogenic growth, cellular proliferation and tumor development. Further, NANOG has been shown to promote cancer progression, such as the manifestation of resistance to conventional chemotherapeutic drugs. However, it remains unclear how NANOG affects these biological responses at the cellular and molecular level. In this chapter, we shall review some of the evidence supporting the notion that NANOG is an important pro-tumorigenic molecule and consider the upstream regulatory mechanisms and downstream effectors integrating NANOG into cancer cell molecular circuitry.

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Introduction

NANOG is an essential transcription factor in development and maintains the primitive stem cell state in embryonic cells of the inner cell mass of the blastocyst (Chambers et al. 2003; Mitsui et al. 2003). NANOG is a unique homeodomain protein that acts in concert with other core embryonic transcription factors, particularly OCT4 (POU5F1) and SOX2 to maintain pluripotency (Chambers and Tomlinson 2009; Young 2011). NANOG performs this crucial biological function collaboratively with these and other transcriptional regulators in large multimeric protein complexes to both activate the expression of genes promoting self-renewal and repress genes promoting differentiation (Chambers and Tomlinson 2009; Young 2011). At the appropriate time during development, falling NANOG protein levels relieves suppression of differentiation genes, thereby permitting a differentiation prone state (Silva and Smith 2008). Embryonic stem cells (ESCs), cultured cells derived from the inner cell mass, are also sensitive to NANOG protein levels, displaying full 'ground state pluripotency' only in the presence of sufficient NANOG. This remarkable transcriptional control mechanism allows ESCs (both native and their ex vivo cultured counterparts) to self-renew, yet remain poised to differentiate, processes essential for normal development.

Tumorigenesis is also a developmental process, albeit aberrant. Somatic tumors have also been shown to possess cells exhibiting certain stem cell (SC) biological attributes and overlapping gene expression programs (Ben-Porath et al. 2008; Wong et al. 2008). Tumor 'recapitulation', functional regeneration of the heterogeneous primary tumor upon transplantation suggests that, like developing organs, tumors harbor stem-like cells with a high proliferative capacity, multi-lineage differentiation potential and self-renewal, the cardinal property of stem cells (SCs) (Tang et al. 2007; Visvader and Lindeman 2008). Renewing neoplastic cells, commonly referred to as cancer stem cells (CSCs), may arise in any cellular compartment by a combination of genetic mutations and epigenetic alterations, such that the cell-of-origin may be a somatic SC or, alternatively, a progenitor cell or terminally differentiated cell that acquires self-renewal as part of 'immortalization' requisite of transformation (Visvader 2011). Taken together with clonal evolution and more recent evidence of bidirectional interconversion between CSC and non-CSC, SC-related gene expression programs at any point in the disease process could serve as neoplastic engines driving malignant progression.

OCT4 overexpression in the skin and intestinal tract in transgenic mice has been shown to induce hyperplastic and dysplastic growths in a remarkably short time frame, unequivocally demonstrating that ESC self-renewal transcription factors possess powerful pro-tumorigenic properties (Hochedlinger et al. 2005). Ectopic expression of mouse and human NANOG in NIH3T3 cells, respectively, has been shown to promote entry into S-phase and promote foci formation in soft agar (Piestun et al. 2006; Zhang et al. 2005). Overexpression of NANOG in immortalized but benign HEK-293 cells has been shown to be sufficient for malignant transformation, increasing proliferation, anchorage-independent growth in soft agar and, importantly, tumor formation upon transplantation in athymic nude mice (Lin et al. 2011). Furthermore, *RB1*$^{-/-}$ mouse fibroblasts cultured under sphere-forming conditions upregulate *Nanog* mRNA concurrently with reprogramming to a CSC phenotype, including the acquisition of a cytotoxic-drug effluxing Side Population (SP), upregulated expression of SC- (and CSC) related genes (e.g., CD44 and CD133) and tumor-initiating cell capabilities upon transplantation (Liu et al. 2009). Mouse Nanog overexpression in hematopoietic SCs failed to enhance the self-renewal of these cells but unexpectedly induced the deve-lopment of a lymphocyte hyperproliferative disorder (Tanaka et al. 2007). Finally, studies of reprogrammed induced pluripotent stem (iPS) cells, have shown that transformed iPS cells (relative to non-transformed iPS cells) are uniquely dependent on NANOG for survival and self-renewal. Taken together, these findings serve as proof-of-principal that NANOG has oncogenic potential (Ji et al. 2009).

Here, we undertake a review of evidence that NANOG is expressed in human cancer cell lines,

xenografts and, most importantly, in primary human patient samples. Further, we will consider the possible origins of NANOG in tumor cells and how NANOG's expression may be regulated in a cell-type dependent manner. We shall subsequently examine NANOG's putative biological roles in neoplastic disease processes, focusing on somatic tumors. Finally, we will contemplate some mechanisms by which NANOG may modulate the molecular circuitry of cancer cells.

NANOG Origins and Expression in Cancer

Numerous groups have published reports of NANOG in multitudes of somatic cell tumors, often correlating with markers or phenotypes ascribed to CSCs (Bourguignon et al. 2012b; Chiou et al. 2008; Jeter et al. 2011; Noh et al. 2012a; Zbinden et al. 2010; Zhang et al. 2008). Many of these findings only report a detected RT-PCR product or a cryptic band on a Western blot. However, NANOG may arise from at least two sources with important implications in the regulation of *NANOG* gene expression, and possibly the biochemical activity of NANOG protein.

Embryonic or ESC *NANOG* mRNA arises from the *NANOG1* locus encoded on chromosome 12 and has four exons and three introns with a 915-bp open reading frame (ORF) (Booth and Holland 2004). Given that genes expressed abundantly during early embryogenesis are subject to transposition and retention in the germ line, one tandem duplication and nine retrogene-derived (intronless) genetic variations of NANOG exist scattered throughout the genome (Booth and Holland 2004). Only one, *NANOGP8* encoded on chromosome 15 is intact, without truncations, deletions or frame shifts and encoding a protein 99% identical to NANOG1 (Booth and Holland 2004). Among five conserved nucleotide differences between *NANOG1* and *NANOGP8* in the ORF, only one (nucleotide 759) induces an amino acid change (Q253H) (Booth and Holland 2004; Jeter et al. 2009).

Cancer cell NANOG has been reported to arise from both *NANOG1* and *NANOGP8* loci. In the case of pluripotent embryonal carcinoma and the seminoma class of testicular germ cell tumors, amplification of chromosome 12p including *NANOG1* is consistent with known expression from the *NANOG1* locus in normal primordial germ cells (Chambers et al. 2003; Ezeh et al. 2005; Korkola et al. 2006). Retinoic acid treatment of these cancer cells promotes neuronal differentiation coincident with NANOG downregulation (along with a cluster of other genes in close proximity to *NANOG1*) concurrent with attenuation of tumorigenicity (Giuliano et al. 2005). Thus, the most logical origin of NANOG in germ cell tumors and EC is primarily (although not necessarily exclusively) *NANOG1*.

In contrast, somatic tumor NANOG seems to arise primarily from the *NANOGP8* locus. Initially revealed by cloning and sequencing of NANOG RT-PCR products, Zhang and colleagues (Zhang et al. 2006) reported *NANOGP8* mRNA expression in a variety of cultured cancer cells, including OS732 (osteosarcoma), MCF-7 (breast adenocarcinoma) and HepG2 (hepatoma) as well as in primary breast and bladder tumor tissues; of note, *NANOG1* transcripts were also detected, albeit less frequently. Cloning and sequencing of full-length NANOG transcripts from prostate cancer cells, including the LNCaP cell line and several primary tumor specimens also provides indisputable evidence of *NANOGP8* mRNA expression (Jeter et al. 2009). Sequencing of NANOG RT-PCR products amplified from colorectal cancer cell lines and primary tumors, taken together with DNA fingerprinting by differential restriction enzyme cutting due to a unique AlwN1 cut site present in *NANOGP8* transcripts arising from the G→A transition at nucleotide 144, has provided further evidence that somatic cancer cell NANOG predominantly originates from the *NANOGP8* locus although *NANOG1* is also detected less frequently and generally in lower abundance (Ibrahim et al. 2012). Furthermore, in human *glioblastoma multiforme*, sequencing of NANOG transcripts as well as *NANOG1* versus *NANOGP8*-specific shRNA loss-of-function analysis has provided compelling evidence that *NANOGP8* is the primary source of NANOG in the U87 cell line

and primary glioma tumor specimens; however, on the basis of both transcript sequencing and *NANOG1* promoter-reporter assay, *NANOG1* mRNA was also expressed albeit at a lower level relative to *NANOGP8* (Niu et al. 2011). All together, NANOG origins in cancer cells is heterogeneous and may be derived from *NANOG1* or *NANOGP8* loci, although the predominant source in somatic cancers appears to be *NANOGP8*.

NANOG mRNA and protein are not abundantly expressed in somatic cancers relative to NANOG levels in ESCs or embryonal carcinoma. Nevertheless, NANOG protein has been detected via Western blot or in situ via immunohistochemistry (IHC) or immunofluorescence staining in various types of somatic tumors (Bourguignon et al. 2008, 2012b; Chiou et al. 2008; Gibbs et al. 2005; Jeter et al. 2009; Mathieu et al. 2011; Niu et al. 2011). Curiously, a wide range of molecular weights has been reported for NANOG protein, including many bands larger than that predicted (~35 Kd), some of which might represent post-translational modifications (e.g., ubiquitination, sumoylation and phosphorylation) (Jeter et al. 2009). In the case of NANOG1, splice variants derived from alternative exon usage are a distinct possibility as has been shown for mouse *Nanog* (Das et al. 2011). Although the identity of the various anti-NANOG immunoreactive bands has not been unequivocally proven in most cases, tryptic digest and nanoscale electrospray ionization mass spectrometry has been used to conclusively identify NANOG peptide sequences in breast patient tumor specimens, providing concrete evidence that NANOG protein is present in human patient neoplastic cells (Alldridge et al. 2008).

NANOG expression is heterogeneous in somatic tumors and has been reported to be associated with stem cell and CSC markers. In the prostate, we reported enrichment of NANOG mRNA in FACS sorted CD133 single positive and CD133/CD44 double positive primary human prostate tumor cells relative to their negative counterparts (Jeter et al. 2009). Furthermore, immunofluorescence staining of Du145 prostate cancer cells demonstrated NANOG protein is expressed as a gradient and positively correlates with CD44, a marker of tumor-initiating cells in the prostate (Jeter et al. 2009). In freshly derived (i.e., early passage) ovarian adenocarcinoma cell lines cultured under anchorage-deprived conditions in serum-free media supplemented with various growth factors (insulin, epidermal growth factor, and basic fibroblast growth factor), tumorigenic spheroids arose enriched for NANOG protein and co-expressing the SC markers CD44 and CD117 (Zhang et al. 2008). In oral cancer, IHC analysis of head and neck squamous cell carcinoma (HNSCC) patient tumors has shown NANOG to be associated with the CD44v3 isoform and high levels of the detoxifying enzyme aldehyde dehydrogenase-1 (ALDH1), another marker of CSCs and associated with chemotherapy resistance (Bourguignon et al. 2012b). In brain cancer, IHC analysis of a large cohort of glioma patient tumors has revealed that CD133 and NANOG are frequently co-expressed, particularly in high grade, poorly differentiated tumors (Niu et al. 2011); furthermore, *NANOGP8* knockdown in CD133+ cell subsets inhibits glioma tumor regeneration upon transplantation in immunocompromised mice in vivo (see below). Also, in a liver cancer mouse model (alcohol fed transgenic mice with hepatocyte-specific expression of the hepatitis C viral non-structural protein NS5A) $CD133^{+}$ hepatocellular carcinoma cells were found to be $NANOG^{+}$ and functionally dependent (Machida et al. 2009).

In sum, these reports, taken together with other literature beyond the reference limitations of this review, suggest that NANOG, originating predominantly from the NANOGP8 locus, is preferentially expressed in stem-like subsets of cancer cells. The presence of this powerful ESC transcription factor in neoplastic cells, particularly in subsets of tumor cells with properties ascribed to CSCs, naturally leads one to wonder what are the biological functions of NANOG in cancer cells?

NANOG Biological Functions in Neoplastic Processes

Renewing malignant neoplastic cells are tumor-maintaining cells functionally synonymous with 'tumor-initiating cells' in transplantation assays.

Regardless of the cell-of-origin, renewing tumor cells are intrinsically resistant to anti-cancer therapeutics, enriched upon experimental and clinical treatments and may mediate tumor recurrence and distant metastases. Therefore, renewing tumor cells may represent particularly salient clinical targets for the development of novel therapeutics, and the molecular underpinnings of these stem-like cancer cells may represent the Achilles' heel of tumor cells. With this in mind, we shall review the literature describing what is currently known about how NANOG functionally mediates the development and progress of neoplastic diseases, focusing on somatic cancers.

Self renewal and differentiation, the hallmark SC characteristics of tumor cells are mirrored by the high proliferative capacity and phenotypic plasticity of tumor cells and recent findings suggest that NANOG may promote these vital pro-tumorigenic properties in cancer cells. To evince that tumor-initiating cells possess long-term self-renewal, the cardinal property of SCs, serial transplantation assays are often performed and such methods have shown that NANOG is functionally associated with self-renewing tumor cells. For example, NANOG-expressing $CD44^{+}CD117^{+}$ ovarian cancer cells have been shown to be uniquely tumorigenic (relative to bulk $CD44^{-}CD117^{-}$ cells) in serial transplantation assays, giving rise to 2° and 3° tumors histologically reconstituting the parental tumor (Zhang et al. 2008). Loss-of-function of NANOG by shRNA has been shown to decrease prostate cancer serial tumor development, as well as serial sphere formation (Jeter et al. 2009), also supporting the premise that NANOG mediates renewing characteristics of cancer cells. Intriguingly, in hepatocellular carcinoma, the ESC cell surface marker CD24 marks relatively quiescent NANOG-expressing liver tumor cells with serial sphere- and tumor-forming capabilities, metastatic potential and the capacity to differentiate in vitro (Lee et al. 2011). That NANOG was epistatic to CD24 and critical for the tumorigenicity of these cells was demonstrated by the ability of NANOG overexpression to rescue tumor development in CD24 knockdown cells and to enhance serial sphere formation (Lee et al. 2011). In the central nervous system, normal neural stem cells in the brain as well as medullablastoma tumor cells abundantly expressed NANOG and RNA interference has evinced that this expression is essential for neural stem cell self-renewal (Po et al. 2010). Finally, in related studies of glioma cancer cells, NANOGP8-specific shRNA expressing GFP-labeled xenograft cells cotransplanted orthotopically with RFP-labeled control cells were lost in the first passage, providing compelling evidence that NANOGP8, in particular, is essential for glioma tumor growth and development in a cell-autonomous fashion (Zbinden et al. 2010).

The self-renewal of human ESCs is fostered by a short G1-phase (Becker et al. 2006), and NANOG is known to drive G1-S transition in ESCs by modulating the levels of cell cycle regulatory genes (Zhang et al. 2009). We have reported that NANOG knockdown decreased proliferation in prostate and breast cancer cells, both in vivo and in vitro (Jeter et al. 2009). Consistent with these findings are observations that NANOG knockdown in MCF7 and MDA-MB-231 breast cancer cells induced G0/G1 cell cycle arrest (Han et al. 2012) and that NANOG knockdown inhibited glioma sphere formation concordant with reduced proliferation, particularly in the $CD133^{+}$ cell subset (Zbinden et al. 2010). On the other hand, we have observed that NANOG overexpression short-term in prostate (Du145 and LNCaP) and breast cancer (MCF7) cells failed to promote proliferation under standard conditions (Jeter et al. 2011). However, upon androgen-deprivation via castration of host mice, normally androgen-dependent LNCaP prostate cancer cells adopted a castration-resistance, androgen-independent growth phenotype, including increased tumor development in accord with increased expression of the proliferation marker Ki67 (Jeter et al. 2011). Thus, a basal level of NANOG may be necessary for cancer cell proliferation, probably contributing at least in part to NANOG-mediated clonogenic and tumorigenic properties; however, NANOG overexpression may not be sufficient to promote tumor cell proliferation in all conditions.

NANOG has been broadly reported to promote cancer cell survival in response to a wide variety of biological stresses. In prostate cancer therapeutics,

androgen-deprivation therapy is commonly used to successfully treat invasive tumors; however, tumor recurrence in the form of castration-resistant prostate cancer invariably occurs heralding lethal disease. By overexpression analysis and *NANOGP8*-promoter reporter analysis, we have observed that NANOG correlates with androgen-independent prostate cancer growth (Jeter et al. 2011). Taken together with our observations that NANOG knockdown in undifferentiated Prostate Specific Antigen (PSA) negative cells inhibited prostate cancer xenograft tumor regeneration (Qin et al. 2012), these findings suggest that NANOG has the potential to be a key regulatory factor mediating castration resistance and may therefore represent a critical, clinically relevant target for treatment of lethal, late-stage prostate cancer. Of broader clinical relevance, NANOG siRNA has been shown to potentiate the effects of chemotherapeutic drugs whereas NANOG overexpression promoted resistance to chemodrugs in various cancer cells, such as in response to doxorubicin in breast and ovarian cancer (Bourguignon et al. 2008) and in response to cisplatin in HNSCC (Bourguignon et al. 2012b). In a cohort of primary ovarian patient tumors, increased nuclear NANOG has been shown to be significantly associated with reduced chemosensitivity and poor survival (Siu et al. 2012), suggesting that these findings are relevant to human disease.

The native tumor environment can be hostile to tumor cell survival and NANOG could, conceptually, promote adaptation to changing tumor microenvironments. Hypoxia-induced transcriptional reprogramming of cancer cells to an ESC-like gene expression profile, including upregulated *NANOG* mRNA (as well as other markers, such as *OCT4*, *SOX2*, *KLF4*, *cMYC* and *miR-302*) in various cancer cells lines, including those derived from prostate, brain, colon, liver and breast, among others (Mathieu et al. 2011). Furthermore, NANOG protein positively correlated with HIF1α in primary prostate tumors (Mathieu et al. 2011). These findings have been corroborated by independent observations of hypoxia-mediated upregulation of *NANOG* mRNA in Du145 and PC-3 prostate cancer cells, predominantly arising from *NANOGP8* (Ma et al. 2011). IHC and Western blot also demonstrated a strong upregulation of NANOG protein and biological characterization further revealed that pretreatment with 1% oxygen for 48 h (followed by culture in normoxic conditions) led to enhanced colony formation, an increase in the cytotoxic-dye-effluxing SP percentage, and expression of CSC markers such as CD44 and the multi-drug effluxing ATP-binding cassette transporter ABCG2 (Ma et al. 2011). Thus, NANOG upregulation in response to hypoxia may promote properties attributed to CSCs.

Hypoxia-induced tumor cell adaptive responses include acquisition of resistance to cytotoxic T lymphocyte (CTL)-mediated tumor cell lysis. NANOG, upregulated in hypoxic lung carcinoma cells, has been implicated to play a role in this biological process by loss-of-function analysis correlating with attenuation of resistance to hypoxia-induced resistance to CTL-dependent tumor cell killing (Hasmim et al. 2011). Independent reports have also shown NANOG to be upregulated in a human papillomavirus (HPV) type16-associated cervical cancer model passaged in vivo via resistance to HPV antigen E7-specific CTLs and associated with increased expression of CSC markers (e.g., CD133 and CD44), and increased clonogenic and tumorigenic potential (Noh et al. 2012b). The necessity for NANOG in mediating this tumor cell immune resistance was evinced by NANOG-siRNA, rendering tumor cells sensitive to CTL-mediated killing concomitant with reduction in tumor growth (Noh et al. 2012b). A similar study was also performed in a murine colon cancer model in which long-term animal survival was greatly enhanced by NANOG siRNA together with adoptive transfer of tumor-reactive CTLs (Noh et al. 2012a). Taken together, these studies suggest that NANOG in evolving cancer cell subsets may promote tumor cell immune system tolerance and evasion.

ESC-like gene signatures have been identified in poorly differentiated, aggressive human tumors, including NANOG transcriptional networks (Ben-Porath et al. 2008). Considering that NANOG is a critical cellular reprogramming factor in the derivation of iPS cells, it is reasonable to speculate that NANOG may drive the dedifferentiation

of cancer cells, particularly in late stage disease and/or permit reversible metaplastic phenotypes allowing for the acquisition of drug resistance, invasive or other malignant cancer cell properties. However, despite observations that NANOG overexpression can promote the expression of CSC markers in some cellular contexts, such as the upregulation of CD133 in MCF7 cells and reduce the expression of some terminal differentiation markers, such as Androgen Receptor (AR) in LNCaP prostate cancer cells, we have failed to detect upregulation of the CSC marker CD44 in prostate cancer cells cultured in androgen supplemented conditions (Jeter et al. 2011). These observations suggest that in a cell-context dependent manner, NANOG expression alone may not be sufficient for non-CSCs to undergo complete reprogramming to CSCs.

Revised CSC theory includes cellular plasticity and this paradigm shift has been conducive to the convergence of CSC concepts with those of Epithelial-Mesenchymal-Transition, a process often associated with cancer invasion and metastasis (Kong et al. 2011); (Singh and Settleman 2010). NANOG has been reported to induce EMT in human colorectal, lung and prostate cancer cells (Chiou et al. 2010; Jeter et al. 2011; Siu et al. 2012). In LNCaP prostate cancer cells, we have previously reported that NANOG overexpression endowed enhanced migration to these relatively benign, well-differentiated cells (Jeter et al. 2011). In ovarian cancer, NANOG knockdown in OVCAR-3 and SKOV-3 cells impeded migration and invasion whereas overexpression in OVCA420 cells enhanced these malignant characteristics (Siu et al. 2012). Overexpression of NANOG together with OCT4 in lung adenocarcinoma increased expression of CD133, activated expression of key EMT regulatory proteins, including SLUG (SNAI2) and enhanced tumor development and metastasis in vivo (Chiou et al. 2010). Furthermore, IHC and Kaplan-Meier analyses of a large cohort of lung adenocarcinoma patient samples have demonstrated a statistically significant correlation between NANOG, SLUG and OCT4 expression and survival, with lower expression of any of the three molecules associated with better survival rate in contrast with high expression of all three molecules being the worst prognostic (Chiou et al. 2010). Thus, NANOG appears to have the potential to promote migration, invasion and metastasis and this propensity appears to be relevant to human disease.

Taken all together, these studies provide convincing evidence that NANOG promotes neoplasia, often in association with the expression of CSC markers and phenotypic traits. An important outstanding question is by what molecular mechanisms does NANOG drive cells to acquire neoplastic phenotypes and biological properties?

NANOG Pro-Tumorigenic Molecular Mechanisms

NANOG does not appear to be a classical oncogene. Nevertheless, this expanding compendium of NANOG pro-tumorigenic biological properties is well evinced. In contrast to the well-studied molecular mechanisms by which NANOG regulates gene expression in ESCs, the NANOG-mediated signaling networks in cancer cells have only been scarcely elucidated. Here, we shall review our current understanding of the candidate molecular mechanisms by which NANOG expression may be regulated in cancer cells and how NANOG may confer malignant properties upon neoplastic cells.

As a retrogene derivative, *NANOGP8* possesses distinct promoter elements relative to *NANOG1*. For example, TRANSFAC analyses of the *NANOGP8* promoter *in silico* fail to identify OCT4/SOX2 elements present in *NANOG1* (Jeter, unpublished observations). As a consequence of these cis-element differences, transfactors regulating *NANOG* mRNA transcriptional activation or repression by promoter occupancy would most likely be distinct between *NANOGP8* and *NANOG1*. Therefore, the mechanisms by which *NANOG* is transcriptionally regulated in cancer cells will vary depending on the locus-of-origin for *NANOG* expression in particular cancer cells. Furthermore, since ESC NANOG promotes self-renewal by repressing differentiation genes and activating the expression of pluripotency genes via complex combinatorial regulatory mechanisms

involving promoter co-occupancy and direct protein-protein interactions in large multimeric complexes, contextual differences between ESCs and various cancer cells will engender distinct NANOG-mediated transcriptional programs.

NANOG has been reported both upstream and downstream of numerous molecules implicated in self-renewal. In human *glioblastoma multiforme*, NANOG has been reported to be epistatic to an important self-renewal regulatory signaling pathway Sonic Hedge Hog (SHH) and a critical GLI1 downstream effector promoting glioma tumor development (Po et al. 2010; Zbinden et al. 2010). Furthermore, as NANOG and GLI1 were coordinately expressed reciprocally with p53, Zbinden et al. (2010) proposed that NANOG functions in glioma as a molecular 'nexus' connecting a positive regulatory loop with GLI1 and a negative regulatory feedback via p53. It is currently unclear the relative contribution of *NANOG1* and *NANOGP8* in glioma as both origins have been evinced; however, GLI1-binding sites have been experimentally demonstrated in *NANOG1* via promoter reporter (Po et al. 2010) and p53 is known to regulate *NANOG1* mRNA expression in ESCs whereas no p53 binding site is evident in the *NANOGP8* promoter region (Jeter, unpublished observations). Regardless, although loss of p53 leads to an upregulation of SHH and a corresponding increase in NANOG, modulation of SHH can effectively regulate NANOG levels even in the absence of p53 (Po et al. 2010). Thus, that NANOG is an important mediator of SHH-GLI self-renewal in normal and glioma SCs is well documented by independent researchers; however, the degree to which *NANOG1* and *NANOGP8* loci contribute to this phenomenon and how exactly p53 inhibits this process is unclear.

STAT3 is another important self-renewal associated molecule also implicated in tumorigenesis and a known member of NANOG-signaling networks in ESCs (Chambers and Tomlinson 2009; Young 2011). In liver cancer cell lines and xenografts, STAT3 has been reported to regulate NANOG expression in $CD24^+$ tumor-initiating cells as demonstrated by phospho-STAT3 (Tyr705) occupancy of *NANOG1* in chromatin immunoprecipitation assay (ChIP) and downregulation of NANOG expression upon treatment with a STAT3 antagonist (Lee et al. 2011). Furthermore, *NANOG1*-promoter reporter GFP-tagged cells were enriched in $CD24^+$ cells and decreased in response to STAT3 pharmacological inhibition, although *NANOGP8* was not evaluated in these cells (Lee et al. 2011). Therefore, STAT3 may be upstream of NANOG in cancer cells, particularly in tumors expressing NANOG primarily from the *NANOG1* locus. It is particularly interesting that these self-renewing $CD24^+$ liver cancer cells were enriched by chemotherapy, implicating NANOG as a potential downstream mediator of chemoresistance (Lee et al. 2011).

NANOG has been purported to mediate chemotherapy resistance of MCF-7 breast cancer cells downstream of hyaluronan (HA)-induced CD44 activation via complex formation with STAT-3 (Bourguignon et al. 2008). Curiously, NANOG was described as initially cytoplasmic in these cells and associating with the intracellular domain of CD44 as demonstrated by co-immunoprecipitation assay (Bourguignon et al. 2008). HA stimulation enhanced the NANOG-CD44 interaction leading to NANOG translocation to the nucleus, whereupon NANOG interacted in turn with STAT3 (co-immunoprecipitation) to mediate transcriptional upregulation of the multidrug transporter MDR1 (p-glycoprotein) and effect resistance to chemotherapeutic drugs (Bourguignon et al. 2008). More recently, protein kinase Cε has been proposed to be an intermediate in NANOG-mediated drug resistance by direct phosphorylation of NANOG leading to increased miR-21 levels and upregulation of anti-apoptotic proteins (IAPs) and drug resistance mediators (MDR1) in both breast cancer cells (Bourguignon et al. 2009) and in HNSCC (Bourguignon et al. 2012a).

NANOG-mediated resistance to other forms of cytotoxic cellular stress may have overlapping NANOG-dependent molecular mechanisms. NANOG attenuation of hypoxia-promoted resistance to CTLs tumor cell lysis also appears to involve STAT3 phosphorylation and nuclear translocation as these processes are inhibited

by NANOG knockdown (Hasmim et al. 2011). Hypoxia and drug resistance mediated by NANOG may also share other downstream molecular mechanisms, such as the ESC regulatory microRNA family encoded by miR-302. Detected as part of the ESC 'signature' (including NANOG) in various cancer cell lines subject to hypoxia (Mathieu et al. 2011), miR-302 is also upregulated in HNSCC downstream of NANOG, OCT4 and SOX2 and pivotal to chemotherapy resistance in HA-stimulated CD44v3^{high}ALDH1^{high} HNSCC cells (Bourguignon et al. 2012b). Candidate upstream regulators of *NANOG* expression in response to hypoxia may include Hypoxia-Inducible Factor 1α (HIF1α) as HIF1α-siRNA ablates hypoxic induction of NANOG protein in lung cancer cells (Hasmim et al. 2011). IHC analysis has also demonstrated a correlation between NANOG and HIF1α in prostate and glioma (Mathieu et al. 2011), although other hypoxia-inducible factors (e.g., HIF2α) may also contribute. It remains to be determined to what degree hypoxia-induced cancer cell NANOG arises from *NANOG1* versus *NANOGP8* and whether, or not, this regulation is via direct transactivation or an indirect phenomenon.

Cancer cell adaptation to changing tumor microenvironments may also encompass EMT and NANOG-mediated EMT has been recently linked to CSCs and conversion of early neoplasms to invasive malignancies (Kong et al. 2011; Singh and Settleman 2010). EMT is associated with differential expression of EMT transactivators such as Slug (SNAI2), Snail and E-box binding proteins ZEB1 and ZEB2, as well as the miR-200 family of microRNAs. NANOG overexpression has been reported to promote the migration of colorectal cancer cells in association with the upregulation of Slug and Snail (Meng et al. 2010). Furthermore, TGFβ (Transforming Growth Factor β)-induced EMT in these cells was associated with a Snail-dependent upregulation of NANOG, suggesting that NANOG may be both an inducer and a recipient of EMT signals (Meng et al. 2010). In a separate study, PDGF (Platelet Derived Growth Factor) has been shown to stimulate EMT-like changes in prostate cancer cells associated with an upregulation of NANOG concordantly with a decrease in miR-200 family miRNAs, and upregulation of ZEB1 and the Let-7 regulatory protein Lin28 (Kong et al. 2010). The precise epistatic relationship in regards to NANOG in response to PDGF remains to be defined, although authors have surmised that *NANOG* mRNA levels are regulated post-transcriptionally by Let-7. Finally, in EMT in ovarian cancer cells, reciprocal expression of NANOG and Forkhead (FOX) family proteins has been observed, such that overexpressing NANOG is associated with decreased FOXO1, FOXO3a, FOXJ1 and FOXB1 together with increased migration, proliferation and invasion, whereas the opposite pattern occurred in response to NANOG knockdown (Siu et al. 2012). FOXJ1 siRNA was also observed to enhance EMT in ovarian cancer cells depleted of NANOG, leading authors to speculate that FOXJ1 is a downstream molecule presumably repressed during NANOG-mediated cell migration and invasion (Siu et al. 2012). How FOX proteins such as FOXJ1 impede EMT and how NANOG regulates FOX proteins in this process are unknown.

The progression of prostate cancer to androgen-independent, castration-resistant disease also requires cancer cell adaptation and survival mechanisms. In androgen-deprived LNCaP cells overexpressing NANOG1 and NANOGP8, we observed an increase in oncogenic c-Myc protein and, via chromatin-immunoprecipitation PCR assay, we observed NANOG1/P8 c-Myc promoter occupancy, suggesting c-Myc may be a direct target of NANOG in cancer cells (Jeter et al. 2011). Potential downstream targets upregulated at the RNA level include Insulin Growth Factor Binding Protein 5 (IGFBP5), a molecule implicated in castration-resistance and the chemokine receptor CXCR4, a molecule potentially important in mediating increased cancer cell migration (Jeter et al. 2011). In breast cancer cells, we observed that both NANOG1 and NANOGP8 overexpression promoted an increase in CD133 mRNA and protein, an increase in the drug-resistance gene ABCG2 transcript levels, and an increase in aldehyde dehydrogenase enzymatic activity [ALDH^{high} Aldefluor assay; (Jeter et al. 2011)]. Independent evidence that these molecules may be NANOG

target genes implicated on the basis of increased levels of RNA or protein in cancer cells expressing varying amounts of NANOG include: the drug-resistance gene ABCG2 (Jeter et al. 2011; Ma et al. 2011) stem cell markers such as CD133 (Jeter et al. 2011; Mathieu et al. 2011; Po et al. 2010; Zbinden et al. 2010), aldehyde dehydrogenase enzymatic activity (Jeter et al. 2011; Santini et al. 2012) and the potent oncogene c-Myc (Han et al. 2012; Jeter et al. 2011; Mathieu et al. 2011), among many others.

NANOG: Outstanding Questions and Future Directions

As a transcription factor, NANOG can affect changes in the levels of downstream target genes and proteins directly by interacting with chromatin to transactivate or repress transcription. Alternatively, NANOG may mediate effects on target genes indirectly via altered expression of an intermediate. For example, altered expression of a particular miRNA (e.g., miR-302) in response to NANOG could affect the mRNA levels of a myriad of target genes further downstream. A comprehensive understanding of the NANOG-regulated transcriptome in cancer cells awaits integrative genomic analyses via chromatin immunoprecipitation and sequencing (ChIP-Seq) and RNA-Seq. A caveat of such studies is that in consideration of the impact of varied and dynamic cellular contexts, these analyses will need to be performed with numerous cancer cell types and under various conditions. For example, in the case of prostate cancer, it would potentially be the most informative to perform ChIP-Seq and RNA-Seq both in the presence and absence of androgen to determine how the absence of androgen may redirect NANOG targeting to mediate castration-resistance.

In ESCs, NANOG interacts with chromatin, transcriptional regulators and chromatin remodelers, either as a dimer or in large multimeric complexes, driving transcription of self-renewal genes, repressing differentiation genes and directing chromatin remodeling (Chambers and Tomlinson 2009; Young 2011). NANOG-interacting proteins in cancer remain largely obscure, particularly chromatin remodelers. Conceptually, neoplastic cells could be considered to undergo cellular 'reprogramming' and the epigenomic alterations in cancer cells are known drivers of cancer progression. Future investigations will be required to address the intriguing possibility that NANOG-mediated chromatin remodeling occurs in cancer cells as in ESCs. Also uncertain is whether NANOG1 and NANOGP8 have unique biochemical properties resultant from the single amino acid change conserved between these two NANOG variants. Whether due to contextual or biochemical/structural differences between NANOG isoforms, NANOG (either NANOG1 or NANOGP8) may have non-canonical binding partners in neoplastic cells relative to those in ESCs. Future research will undoubtedly reveal novel NANOG-interacting proteins in cancer cells, potentially including chromatin remodelers as well as other transcriptional activators or repressors.

The transcriptional regulation of *NANOG* in cancer cells, particularly *NANOGP8,* remains poorly characterized. In colorectal cancer, ectopic expression of β-catenin/TCF4 promoted whereas and shRNA-mediated knockdown of C-Jun, β-catenin or TCF4 inhibited endogenous *NANOG* mRNA expression, providing evidence that these molecules could potentially regulate *NANOG* transcription in cancer cells (Ibrahim et al. 2012). However, despite observations that *NANOGP8* mRNA is considerably more abundant than *NANOG1* in these cells, *NANOG1-luciferase* promoter reporter constructs were used to provide evidence that *NANOG1* may be regulated by AP1 (c-JUN) as well as β-catenin/TCF4. Further experiments to address candidate upstream regulators of *NANOGP8* await further investigation.

Potentially obfuscating these and other NANOG-related mechanistic studies is that NANOG1 protein has been reported to regulate its' own expression in ESCs in *trans* via *NANOG1* promoter occupancy (Das et al. 2011). Thus, in cancer cells in which *NANOG1* chromatin is not strongly epigenetically silenced by DNA methylation and/or repressive histone modifications, conceptually, the highly similar NANOGP8 protein may be sufficient to transactivate the

expression of *NANOG1*. Finally, it is important to bear in mind that NANOG-regulatory miRNAs or proteins may be conserved between NANOG variants encoded by *NANOG1* and *NANOGP8*. Thus, heterogeneous NANOG expression in cancer cells may be subject to complex but interrelated regulatory mechanisms that remain to be fully understood.

In summary, the outstanding questions regarding NANOG in cancer cells include: What are the key NANOG downstream signaling networks and molecular targets mediating the pro-tumorigenic properties of NANOG? What are the critical upstream transcriptional regulators of NANOG's expression in cancer cells? How can NANOG's expression or biological properties be disrupted in cancer cells? In this review, we have summarized evidence that NANOG-expressing tumor cells are biologically plastic, renewing, tumor-maintaining cells intrinsically resistant to anti-cancer therapeutics and possibly mediating tumor recurrence and metastases. As such, tumor cells expressing NANOG may represent critical, clinically relevant targets in the treatment of cancer and it is our sincere hope that further understanding of the NANOG circuitry in cancer cells will inspire the development of novel anti-cancer therapeutics in the future.

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Derivation and Expansion of Human Embryonic Stem Cells Under Xeno-Free, Defined Conditions

3

Guoliang Meng and Derrick Rancourt

Contents

Abstract

Human embryonic stem cells (hESCs) hold great promise in regenerative medicine and cell therapy due to their unique properties: unlimited self-renewal and the pluripotency to differentiate into all cell lineages in the body. However, the overwhelming majority of currently available hESC lines have been directly or indirectly exposed to materials containing animal-derived components during their derivation, propagation, and cryopreservation. The use of animal-derived components would prevent the use of hESCs for clinical purposes, due to the possibility of xenogeneic bimolecule and pathogen contamination. Therefore, the establishment of clinical-grade hESC lines in xeno-free, chemically defined conditions is the first and key step. In this chapter, we review and summarize the history and current state of derivation, propagation and expansion of hESCs in static and suspension cultures in xeno-free, defined conditions. The main part of this review focuses on the recent advances in the generation and expansion of hESCs in xeno-free, chemically defined conditions. Based on previous studies, we also put forward the possible means for deriving and expanding hESC lines in xeno-free, defined conditions under current good manufacturing process (cGMP) standards that will enable the generation of clinical-grade hESC lines for the clinical purposes.

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Introduction

Human embryonic stem cells (hESCs) are derived from preimplantation human embryos (Thomson et al. 1998; Reubinoff et al. 2000). Their two important characteristics, unlimited self-renewal ability and the pluripotency to differentiate into all cell types in the body, give them broad application prospects for human cell and tissue replacement therapy. While it is true that hESCs hold great promise in regenerative medicine, the overwhelming majority of hESC lines have been directly or indirectly exposed to animal-derived components during their derivation, propagation, and cryopreservation, which would prevent their use in clinical trials. The presence of animal products in hESC culture media, as well as feeder cell populations, drives two main concerns (Martin et al. 2005). First, animal products may contain toxic proteins or immunogens that evoke an immune response and thus lead to rejection upon transplantation of hESCs. Second, the use of animal products increases the risk of hESC contamination by animal pathogens, such as viruses or prions. The presence of undefined components of animal or human origin in these systems, such as feeder cells and Matrigel, is also problematic. First, unknown factors in Matrigel or produced by feeder cells may interfere with the interpretation of the effect of exogenous agents on the growth and differentiation of stem cells. Second, components such as Matrigel, unknown growth factors, and feeder cells are prone to significant variability (Gokhale et al. 2010).

To address these issues, some progress in the design of xeno-free, chemically defined culturing practices and components have been made in recent years (Villa-Diaz et al. 2010; Melkoumian et al. 2010; Rajala et al. 2010; Meng et al. 2012). Xeno-free culture media and recombinant human protein matrices are commercially available from several companies. Under these culture conditions, some attempts have been made to derive and expand hESCs (Rajala et al. 2010; Ilic et al. 2012). Even though hESCs have been successfully cultured and expanded in completely xeno-free, defined conditions by several groups, no cell line has been established under these culture conditions. A major limitation of static (adherent) culture is low cell yield, which is not able to meet the requirements of the clinical trials. The need for large-scale expansion in clinical therapies could be overcome by cell propagation in suspension culture. Recently, researchers tried to expand hESCs in suspension for prolonged culture and have made some interesting progress on this (Amit et al. 2010; Krawetz et al. 2010; Steiner et al. 2010; Abbasalizadeh et al. 2012). Yet to date, there are no reports on expansion of hESCs in suspension culture under completely xeno-free, defined conditions. In this chapter, we review and summarize research findings from three aspects: isolation and derivation of hESCs, culture and expansion of hESCs in static conditions, large-scale expansion of hESCs under xeno-free, defined conditions (Table 3.1).

Derivation of Human Embryonic Stem Cell (hESC) Lines

Human embryonic stem cell (hESC) lines are derived from the inner cell mass (ICM) of preimplantation blastocysts, early stage embryos or blastomeres. The unique properties of hESCs give them the potential to provide an unlimited source of cells for transplantation therapy, and regenerative medicine. Recently, hESCs have been moved into clinical trials in more and more countries and we can only assume that more trials will begin in the coming years. As mentioned above, the major challenge of hESCs used in regenerative medicine is that the overwhelming majority of hESC lines have been directly or indirectly exposed to materials containing animal-derived components during their derivation, propagation, and/or cryopreservation.

For derivation of hESCs, an immunosurgical method has been routinely used to isolate human inner cell mass (ICM) by selectively killing surrounding trophoblasts within blastocysts (Thomson et al. 1998). This method, which requires animal-derived antibodies and complement, runs the risk of contaminating resulting hESC lines with animal pathogens and foreign molecules. So, xenogenic exposure to hESC lines

Table 3.1 Summary of recent advances toward the xeno-free, defined derivation and expansion of hESCs

Cell lines	Derivation and/or expansion	Isolation of ICM	Substrate	Media or main components	Passages in culture	Reference
H1	Expansion	N/A	Human laminin	Animal-free SFM	40	Li et al. (2005)
1 new line	Derivation and expansion	Acid Tyrode's solution	Xeno-free HFF feeders	HS medium	>20	Ellerström et al. (2006)
5 new lines	Derivation and expansion	Mechanical isolation method	Human foreskin fibroblasts	KSR medium	13–20	Ström et al. (2007)
45 new lines	Derivation and expansion	Laser-surgery	MEF feeder cells	KSR, FBS and plasmanate	Not specified	Chen et al. (2009)
I6	Expansion	N/A	Human fibronectin (CellStart)	Xeno-free, defined hKSR medium	7	Swistowski et al. (2009)
2 new lines	Derivation and expansion	Mechanical isolation method	Human feeder cells	Xeno-free, defined RegES medium	>80	Rajala et al. (2010)
H9, CA2	Expansion	N/A	Decellularized HFF ECM	HEScGRO medium	>20	Meng et al. (2010)
H9, BG01	Expansion	N/A	Synthetic polymer coatings	Human-cell-conditioned-medium and StemPro medium	25	Villa-Diaz et al. (2010)
H1, H7	Expansion	N/A	Synthetic peptide-acrylate surfaces	TeSR2	>10	Melkoumian et al. (2010)
H1, H9, CA1	Expansion	N/A	Vitronectin, Poly-D-Lysine, etc.	Xeno-free, defined TeSR2, HEScGRO medium, etc.	15, 34, etc.	Meng et al. (2012)
5 new lines	Derivation and expansion	Laser-assisted dissection method	Human foreskin fibroblasts and decellularized HFF ECM	Xeno-free, defined KO-SR and TeSR2	Not specified	Ilic et al. (2012)

established by using immunosurgery would hinder their clinical application.

To eliminate the need for animal-derived products during the process of ICM isolation, whole embryo culture method has been used successfully for derivation of hESCs. This involves the outgrowth of zona-free embryos onto mitotically inactivated feeder cells (Suss-Toby et al. 2004). Since trophoblasts can interfere with ICM-derived ES-like cells inside outgrowth, two alternative mechanical approaches have been developed to remove trophoblast cells. First, ICM can be isolated mechanically from expanded human blastocysts by microdissection with sharpened metal needles (Ström et al. 2007). This mechanical method proved to be an effective way to isolate ICM in xeno-free conditions. One drawback of this technique requires specialized metal needles and complicated operation. Meng et al. (2010) also described a simple method as an option for removing trophoblast cells prior to seeding ICM on feeder cells. Here, a 30G1/2 needle attached to a 1 mL syringe was substituted for a dissecting needle and holding needle and used to isolate human ICMs by removing the majority of trophoblast cells. This syringe needle approach is fast and convenient for subsequent non-enzymatic passaging of colonies in the early derivation stage by cutting hESC colonies into cell clumps. It is also efficient for removing differentiated regions within colonies. More recently groups have developed laser-based systems for isolating ICMs that can give rise to hESC lines. Chen et al. (2009) reported that the derivation efficiency of hESC lines can reach as high as 52% by using laser-assisted system when isolating ICM from day 6 blastocysts.

Derivation of hESC lines from blastocyst-stage embryos has raised political and ethical issues. In order to address these issues, the researchers have devoted much work to isolate hESC cells from earlier stage embryos without embryo destruction. Klimanskaya et al. (2006) found a method to generate hESC lines from blastomeres isolated from eight-cell embryos. The serious defect of this method is that it required co-culture of blastomeres with existing hESCs previously derived in xenogeneic conditions. The same group (Chung et al. 2008) reported the derivation of five hESC lines without embryo destruction, by using a modified approach using culture media supplemented with laminin. Also, by using optimal culture conditions, one blastomere-derived hESC cell line was established without co-culture using existing hESCs in feeder-free conditions. This strategy substantially improved the efficiency of the hESC derivation to rates comparable to whole embryo derivations. hESC lines have also be generated from naturally dead embryos (Zhang et al. 2006; Gavrilov et al. 2009). Based on these studies, probably researchers can generate new hESC lines using xeno-free, defined conditions, while avoiding ethical issues.

Simón et al. (2005) and Vemuri et al. (2007) reported that they derived hESC lines under animal-free or xeno-free conditions. However, the serum replacement (SR) medium they used to drive hESC lines contained large amounts of animal derived bovine serum albumen. For the first time, Ellerström et al. (2006) reported the first derivation of one hESC line under xeno-free conditions. In this study, the researchers derived and cultured hESCs on inactivated human foreskin fibroblasts (HFFs) in human serum containing medium. However, human serum is not optimal for supporting hESC derivation and expansion, as human serum can cause extensive differentiation of hESCs, presumably due to human serum batch variations and human serum containing unknown factors that promote the differentiation of human ES cells. Rajala et al. (2007) tested nine previously reported xeno-free culture media formats, including human serum containing medium and concluded that none could maintain the undifferentiated growth of human ES cells., Rajala et al. (2010) developed an optimized xeno-free, defined medium (RegES medium) and used it to generate two hESC lines on human feeder cultured in human serum containing medium. Although this culture system included human feeder cells cultured in human serum containing medium that was not chemically defined, this study made great progress toward to derivation of clinical-grade hESC lines.

Most recently, Ilic et al. (2012) successfully derived two normal and three specific mutation-carrying (SMC, Huntington's disease, and myotonic

dystrophy 1) genomically stable hES cell lines, under totally xeno-free conditions. In this study, inner cell mass (ICM) was isolated with laser-assisted dissection method. For derivation of hESC lines, ICM was initially placed on mitotically inactivated HFFs in Quinn's advantage protein plus blastocyst medium supplemented with human bFGF. Then medium was replaced with xeno-free KO-SR medium supplemented human bFGF. Under these conditions, five hESC lines were established.

Up to now, even if animal components can be completely removed from hESC media, we are still limited by the feeder cells for deriving hESC lines in an undifferentiated state. Although human feeder cells were shown to support hESCs for prolonged cultures while maintaining hESC properties, they still have some disadvantages. First, the need for feeder cells limits the large-scale culture of hESCs. Second, the culture system cannot be precisely defined because of unknown factors secreted by feeder cells or what is included in serum containing media used for feeder cell culture. Third, there exists large batch to batch variability amongst feeder cells. Fourth, the expansion of hESC lines on human feeder cells runs the risk of contamination with human pathogens, which can be transmitted to patients (Rodríguez et al. 2006). So the optimal expansion of hESCs should be in an animal-free, feeder-free, and chemically defined culture system.

Culture and Expansion of Human Embryonic Stem Cells

Since the first derivation of hESCs, two culture systems, feeder-dependent and feeder-free, have been widely used to maintain hESC growth in an undifferentiated state (Meng et al. 2012). Traditionally, the feeder-dependent culture system consists of knockout serum replacement (KSR) medium and feeder cells, either mouse embryonic fibroblasts (MEFs) or human fibroblasts. The feeder-free culture system consisting of mTeSR1 medium or feeder cell conditioned media and Matrigel coating matrix. hESCs cultured or expanded in both culture systems are not suitable for clinical trials, as KSR and mTeSR1 media contain a large amount of xenogeneic bovine serum albumin (BSA), and Matrigel, a reconstituted extracellular matrix (ECM) derived from Engelbreth-Holm-Swarm mouse tumor. As for feeder cells, mouse embryonic fibroblasts (MEFs) derived from day −12.5 to −13.5 post-coitum fetuses, or human fibroblasts are, in most cases, derived and cultured in FBS containing media.

As stated above, the use of animal products in hESC culture system can contain toxic proteins or immunogens, which may evoke an immune response that can lead to rejection upon transplantation, and the use of animal products, increase the risk of hESC contamination from animal pathogens, such as viruses or prions. The presence of undefined components of animal or human origin in these systems, such as feeder cells and Matrigel, is also problematic. First, unknown factors in Matrigel or produced by the feeder cells may interfere with the interpretation of the effect of exogenous agents on the growth and differentiation of the stem cells. Second, components such as Matrigel, unknown growth factors, and feeder cells are prone to significant variability (Gokhale et al. 2010).

For clinical purposes, a totally xeno-free and defined cell culture environment should be created to culture hESCs. To replace undefined, animal- derived feeder cells or matrix, several defined, xeno-free recombinant proteins and synthetic matrices have been developed and used to support hESC growth as alternative substrates (Villa-Diaz et al. 2010; Melkoumian et al. 2010; Rajala et al. 2010; Meng et al. 2012). Purified extracellular matrix, a combination of fibronectin, laminin, collagen IV, and vitronectin was first reported as part of a defined hESC culture system. However, the extracellular matrix (ECM) derived from human tissues still cannot rule out the possibility of human pathogen contamination and generally are not xeno free. Recombinant protein matrices, such as laminin and vitronectin, produced in animal-free conditions have been reported to support long-term growth of hESCs. Li et al. (2005) reported the development of a completely defined system that is devoid of

animal-sourced products for long-term culture of hESCs in undifferentiated state. In this study, hESCs cultured in defined medium, which contains only human sourced and recombinant proteins on human laminin coated surface retain the characteristics and replicative potential of hESCs. Swistowski et al. (2009) described another defined, xeno-free culture system. This system comprised a completely humanized base medium with hKSR supplemented with growth factors and a defined substrate – fibronectin (CellStart). Rajala et al. (2010) reported the development of a fully defined xeno-free medium (RegES) capable of supporting the derivation and long-term (>80 passages) expansion of hESCs on human feeder cells. Most recently, Ilic et al. (2012) tested the growth of five newly derived hESC lines on native decellularized HFF ECM in the presence of two commercially available xeno-free culture media, KO-SR XF (Invitrogen) and TeSR2 (Stemcell Technologies). The results demonstrated that native decellularized HFF ECM and TeSR2 was an optimal combination for maintaining all hESC lines tested in an undifferentiated state.

In 2010, two groups reported that synthetic polymer coatings (Villa-Diaz et al. 2010) and synthetic peptide-acrylate surfaces (Melkoumian et al. 2010) could sustain the long-term growth of hESCs. In the meantime, several of these research groups also reported that some kinds of xeno-free, defined media could effectively support hESC growth in conjunction with purified proteins from human tissues, recombinant proteins, and synthetic matrices. Most recently, our group (Meng et al. 2012) demonstrated that the synergy between medium, matrix, and exogenous factors is critical for hESC adhesion and proliferation in defined, xeno-free culture environment. We also found that two culture systems, Vitronectin matrix/TeSR2 medium and Poly-D-Lysine matrix/HEScGRO (Y27632) medium, were optimal for maintaining the long-term culture of three hESCs lines and two hiPSC lines in an undifferentiated state under defined, xeno-free culture conditions. Our results also suggested that not every pair of medium and matrix made for hESCs can support hESC adherence and proliferation effectively. The combination of medium and matrix must meet the requirement for signaling pathways to maintain hESC attachment and growth in an undifferentiated state. Despite the ability of these xeno-free conditions to support hESC maintenance, the utility of these systems in deriving hESC lines has not yet been tested.

Dissociation and Passaging of Human Embryonic Stem Cells

During hESC isolation and the early passaging of newly isolated hESC colonies, a mechanical method is usually used to dissociate ICM-derived colonies or putative hESC colonies into cell clumps using finely drawn glass pipettes or syringe needles (Ström et al. 2007; Meng et al. 2010). This mechanical passaging method can be used to remove differentiated colonies as well as differentiated areas appeared within colonies in the process of establishing hESC lines. As the number of cells increase, mechanical dissection is insufficient to meet the scale up expansion of hESCs. Therefore, enzymatic dissociation is used as the main method to dissociate hESC colonies in their expansion. For enzymatic dissociation, collagenase IV and dispase are most commonly used to detach and dissociate hESC colonies into cell clumps. A combination of mechanical and enzymatic methods is very useful and practical for hESC propagation. The differentiated colonies and differentiated areas that appear within colonies should be removed with mechanical dissection before enzymatic dissociation of hESC colonies, as propagation of hESCs only with enzymatic dissociation can easily accumulate differentiated cells in hESC populations. For hESC derivation and culture under xeno-free conditions, commercially available animal origin free collagenase IV and dispase can be used to dissociate hESC colonies in their expansion. In addition, another two commercially available xeno-free enzymes, Accutase (Innovative Cell Technologies) and TrypLE (Invitrogen) are used to dissociate hESCs to single cells and have been reported to provide higher cell survival after passage (Bajpai et al. 2008; Ellerström et al. 2010). These products

may prove to be useful in expansion of cell lines under xeno-free conditions.

hESCs grown in either xenogeneic or xeno-free culture system are passaged in cell clumps, which hinder the use of hESCs in many aspects, such as clonal selection, electroporation, flow cytometry. However, hESCs undergo massive cell death in culture after single cell dissociation and single hESCs are very sensitive to routine manipulations, such as passaging, cryopreservation and gene transfection. These shortcomings illustrate the need for new reagents and technical improvement for single cell expansion. A major breakthrough which helped to resolve this problem was the identification of Y-27632, a selective inhibitor of the p160-Rho-associated coiled kinase (ROCK). Previous studies confirmed that Y27632 is a highly efficiency and safe reagent, which can dramatically enhance the cell survival rate after single cell dissociation (Watanabe et al. 2007). However, the molecular mechanism of action of Y-27632 has not been fully understood. The results of some groups suggested that cell-cell contact, not anti-apoptosis, is predominantly responsible for the hESC growth in static culture system (Meng et al. 2012). As Y27632 can greatly promotes hESC attachment and viability but not proliferation, Y27632 containing medium should be replaced with fresh medium without Y27632 the second day after seeding or thawing dissociated cells.

Expansion of hESCs in Suspension Culture

Low cell yield of hESCs cultured in static conditions will not meet the actual demand of the clinic in the future. Recent years, researchers have reported different culture methods for scale-up of hESCs in suspension culture. Suspension culture of hESCs results in the formation of aggregates, a process which can be controlled by several parameters, such as inoculating cell concentration, rate of initial aggregate formation, passage time, stirring speed, pH, culture medium, and addition of growth factors. Some investigators focused on adaptation of static culture of hESCs to suspension culture by microcarriers. Microcarriers are support matrices allowing for the growth of adherent cells in bioreactors by providing an enlarged attachment surface. For the first time, Phillips et al. (2008) reported their developed method for supporting undifferentiated hESCs on trimethyl ammonium-coated polystyrene microcarriers for feeder-free, suspension culture. However, they only achieved initial hESC expansion, cell growth ceased over successive passages. This probably means that trimethyl ammonium may not be an ideal matrix for supporting the long-term growth of hESCs in this suspension culture system. Oh et al. (2009) described a simple and robust method for maintaining hESCs in suspension culture on Matrigel-coated microcarriers, which yielded two to fourfold more cells than those in static cultures. They also successfully expanded hESCs on microcarriers in two serum-free defined media (StemPro and mTeSR1) with high cell yield. In these studies, hESC attachment and proliferation depended on matrix coated microcarriers. However, Matrigel is a poorly defined matrix, and therefore can produce a source of variability in experimental results. So it is also not suitable for expansion of hESCs utilized for the clinical purposes. Also, the use of microcarriers might increase technical and operational difficulties with respect to hESC generation and expansion for clinical purposes.

To establish a culture system for hESC expansion in scalable, suspension cultures, independent of additional matrix, Krawetz et al. (2010) developed a novel 100 ml bioreactor protocol that yields a 25-fold expansion of hESCs over 6 days by using mTeSR1 supplemented with 10 μM rock inhibitor, Y-27632 and 0.1 nM Rapamycin. In this report, the authors demonstrated that hESCs cultured in bioreactor retained high levels of pluripotency and a normal karyotype. Singh et al. (2010) reported a method for expansion of hESCs in suspension culture via forming controlled aggregates from single hESCs in mTeSR1 medium by using Y-27632 in combination with heat shock treatment. In this study, Y27632 and a heat shock were used to treat single hESCs, and then the treated single cells formed aggregates in

2 ml medium and progressively up-scaled to 10 ml, and finally 50 ml stirred suspension cultures. The medium mTeSR1 supported long-term culture of two hESC lines and retained high levels of expression of pluripotency markers, but only in one cell line.

Steiner et al. (2010) developed a method for deriving and expanding hESCs in suspension by using Neurobasal medium supplemented with Knockout serum replacement, growth factors (bFGF, Activin A), beta D-xylopyranose, neurotrophic factors, and ECM components (laminin, fibronectin, and gelatin). Although hESCs could be maintained and expanded in this culture system, the cell yield was lower compared with static culture on feeder cells due to increased cell loss.

Amit et al. (2010) described a radically different approach for expansion of hESCs in suspension cultures. To start suspension cultures, hESCs were detached and dissociated into small cell clumps from culture dishes using type IV collagenase and cultured in suspension in Petri dishes. Before transferring to an Erlenmeyer, cell clumps were cultured in Petri dishes for at least one passage. In this study, the researchers found that hESCs were prone to differentiation after five passages, even at high concentration of bFGF (10–40 ng/ml). The combination of full-length IL6RIL6 chimera and bFGF could support hESC and hiPSC expansion in suspension for long-term culture. However, in this paper, the researchers didn't provide further information on whether this culture system could support single cell expansion.

Most recently, one group (Abbasalizadeh et al. 2012) developed a novel bioprocess platform for large-scale expansion of hESCs and hiPSCs in stirred suspension bioreactors. This bioprocess platform utilized the stepwise optimization of both static and dynamic suspension culture conditions. Also, in this study, the authors identified and addressed the most critical scale-up challenges through stepwise optimization of the key parameters for each bioprocess step. These key parameters included: (1) aggregate dissociation and single cell passaging, (2) cell inoculation density, (3) hydrodynamic culture conditions, (4) oxygen concentration, and (5) aggregation kinetics under dynamic conditions. However, although they claimed that the eight media that they used in this study were xeno-free, in fact none of them were really xeno-free, as these media contained a large amount of BSA. So far, although suspension culture of hESCs in fully defined, xeno-free conditions has not been reported yet, it enables the propagation and expansion of hESCs in the absence of feeder cells and matrices, which makes a big step forward in facilitating the clinical application of hESCs.

Strategies for Deriving and Expanding Clinical-Grade hESCs

To date, more than 1,000 of hESC lines have been derived worldwide from donated embryos. However, the overwhelming majority of these cell lines have been directly or indirectly exposed to animal materials during their derivation, propagation and cryopreservation, which hinders their clinical application, due to the possibility of contamination with xenogeneic biomolecules and pathogens. For clinical purposes in the future, several issues should be addressed during the generation and expansion of hESC lines (Fig. 3.1). To circumvent political and ethical issues, generating xeno-free hESC lines from single blastomeres removed from eight-cell embryos or from arrested embryos without destruction of embryos is a good choice. hESC lines can also be generated from naturally dead embryos. For the derivation of new hESC lines in a xeno-free, chemically defined system for future clinical applications, mechanical or laser-assisted isolation of the ICMs from the blastocysts may be applied to avoid exposure to animal materials.

Another most critical problem is the selection of optimal culture systems for derivation and expansion of clinical-grade hESC lines. In recent years, researchers have found that recombinant protein matrices (such as laminin and vitronectin) produced in animal-free systems, as well as synthetic matrices (synthetic polymer and synthetic peptide-acrylate) can

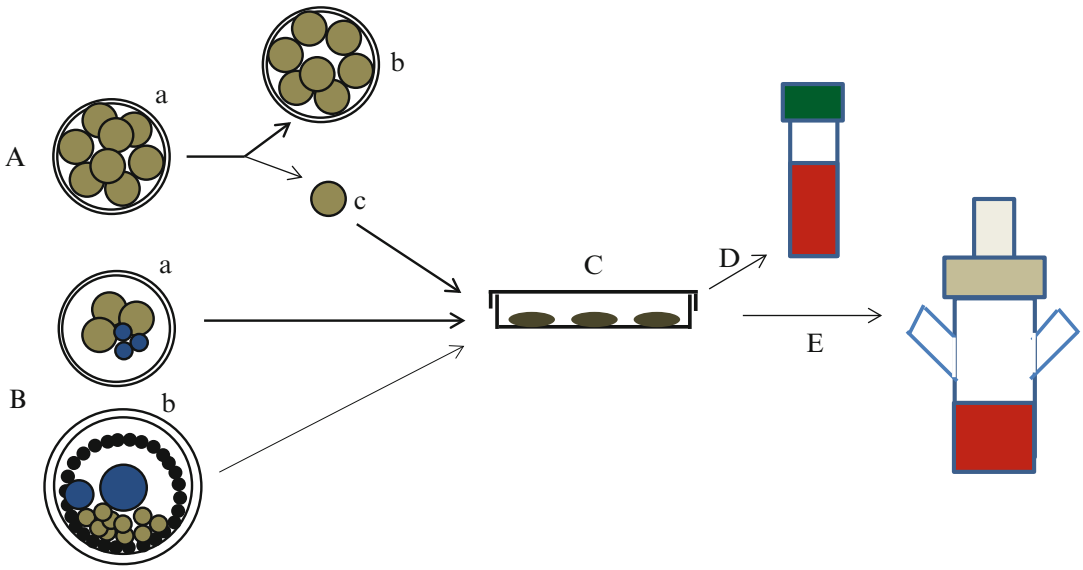

Fig. 3.1 Strategies for derivation and expansion of clinical-grade hESC lines. *A*. Derivation of hESCs from a biopsied single blastomere: blastomere (*c*) removed from eight-cell embryo (*a*) can be used to generate hESCs under xeno-free, defined conditions (*C*); *B*. Derivation of hESCs from dead embryos (*a* and *b*): dead or arrested embryos can be used to isolate hESCs under xeno-free, defined conditions (*C*); Before scale-up of hESCs, they can be froze down using xeno-free, defined cryoprotectant in liquid nitrogen. Adherent culture of hESCs can be transferred to bioreactors and expanded in large-scale (*E*). All procedures should be manipulated under current good manufacturing process (*cGMP*) standards

be used to support the long-term growth of hESCs. As for xeno-free culture media, HEScGRO (Millipore), TeSR2 (Stemcells Techniligies), Xeno-free/feeder-free (XF/FF) culture medium (Stemgent) and Knockout xeno-free serum replacement (XF-KSR) are commercially available now. Presently, more and more xeno-free matrices and culture media are being released. However, not every pair of medium and matrix can support hESC adherence and proliferation. Synergy of culture medium, matrix, and growth factors should provide an adequate supply of nourishment and signaling pathways for supporting hESC growth and self-renewal in undifferentiated state. Under xeno-free, defined culture system, hESC lines can be first generated and expanded in adherent culture at early passages, and then transferred to small bioreactors, progressively up-scaled to large stirred suspension cultures before clinical trials and applications.

Other manipulations related to the derivation, and expansion of hESCs, such as cell dissociation, freezing/thawing also should be performed under xeno-free, chemically defined conditions. Also, all procedures such as generation, expansion, freezing and thawing of hESC lines should be manipulated under current good manufacturing process (cGMP) standards. Only xeno-free hESC lines generated and expanded under cGMP conditions can be used as candidates for the clinical applications. In addition, these cell lines must meet the defining criteria for pluripotent stem cells after long-term culture, including cell and colony morphology, the expression of pluripotency markers, stable karyotype and three-germ layer differentiation in vivo and in vitro. Here, one of the most important things is to establishing a therapeutic human embryonic stem cell bank using these xeno-free, cGMP hESC lines, as enough numbers of hESC lines can meet the need of patients with different genetic backgrounds and histocompatibility.

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Self-Renewal of Naïve State Mouse Embryonic Stem Cells: Role of LacdiNAc in LIF/STAT3 Signaling

4

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Contents

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Abstract

Recent studies have indicated that mouse and human ES/iPS cells are at different developmental stages, namely the naïve state and the primed state, respectively. They therefore require different extrinsic signals for the maintenance of self-renewal and pluripotency. LIF/STAT3 signaling is one of the important factors that distinguishes the naïve and primed states. In general, signals from extrinsic factors are regulated by the glycosylation of molecules on the cell surface and the extracellular matrix, such as ligand receptor molecules. LacdiNAc carbohydrate structures on LIFR and gp130 are required for a sufficiently strong LIFR/STAT3 signal to maintain self-renewal in naïve state cells; these structures act by controlling the localization of the receptors to cell membrane rafts/caveolae. Therefore, an important factor in the differential response of naïve and primed state pluripotent cells to LIF is the level of LacdiNAc structures on LIFR and gp130, and expression of LacdiNAc is required for the induction and maintenance of naïve state pluripotent stem cells. The characterization and functional analyses of cell surface glycans on stem cells will provide further information of value for defining and characterizing stage-specific pluripotent stem cells, and this information will be vital to the development of these cells as a resource for regenerative medicine.

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Introduction

It is now known that very large numbers of cell surface proteins are glycosylated (Kaji et al. 2006); indeed, more than 50% of secreted proteins and cell surface proteins are considered to be glycoproteins. Various types of glycoprotein and glycolipid are present on the cell surface and their patterns of glycosylation (i.e., the glycan structures on the proteins and glycolipids) change dramatically during development (Fig. 4.1). Some of these glycoproteins and glycolipids have been used as markers of embryonic stem cells (ES cells) and induced pluripotent stem cells (iPS cells), for example, stage-specific embryonic antigen-1 (SSEA-1), SSEA-3, SSEA-4, TRA-1-60 antigen and TRA-1-81 antigen (Adewumi et al. 2007). Mouse ES cells express SSEA-1 (also known as Lewis X carbohydrate antigen), Galβ1,4(Fucα1,3)GlcNAc. An anti-SSEA-1 antibody gives positive staining of both ES cells and the inner cell mass (ICM), the origin of mouse ES cells. However, human ES cells do not express SSEA-1. This difference suggests that mouse and human ES cells are at different stages of development. Human ES cells express SSEA-3, TRA-1-60 antigen, and TRA-1-81 antigen (Adewumi et al. 2007). More recently, SSEA-5 has been proposed as a novel carbohydrate marker for human ES cells and iPS cells (Tang et al. 2011).

Glycan structures on the cell surface function to regulate cell-cell interactions, cell-extracellular matrix interactions, and signals from extrinsic factors, such as Wnt, Hedgehog (Hh), bone morphogenetic protein (BMP) and fibroblast growth factor (FGF). Analysis of a *Drosophila* model system showed that heparan sulfate (HS), a glycan structure, has a key role in the regulation of these basic developmental signals; these extrinsic factors are also known to act as morphogens during various stages of development (Nishihara 2010). Specific sulfated regions of HS can bind to these extrinsic factors and work as co-receptors or can stabilize the factors. In mouse ES cells, self-renewal and pluripotency are maintained by a

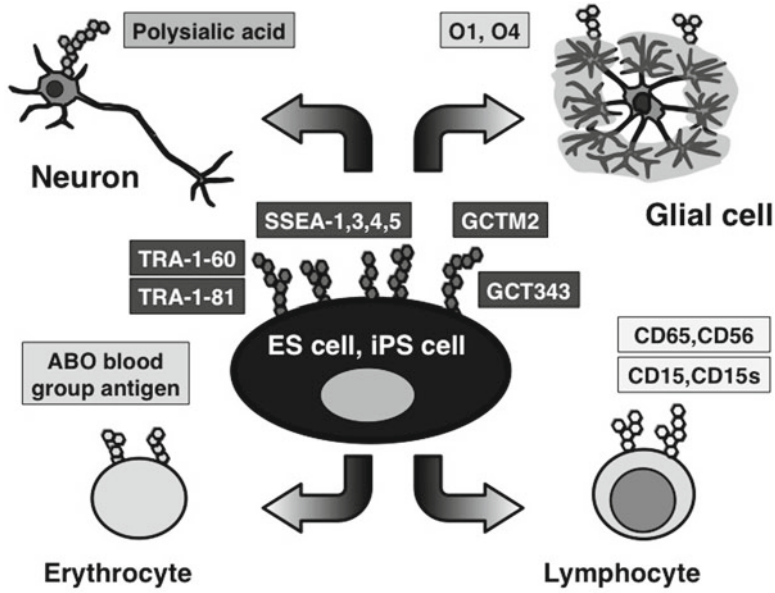

Fig. 4.1 The glycan structures on the cell surface change during differentiation. SSEA-1 is used as a marker of mouse ES cells, while SSEA-3, SSEA-4, TRA-1-60, TRA-1-81, GCTM2 and GCT343 are used as markers for human ES/iPS cells. Polysialic acid is expressed on neural cells, while O1 and O4 are expressed on glial cells. The ABO blood group antigen is expressed on erythrocytes, while CD65, CD56, CD15 and CD15s are expressed on lymphocytes

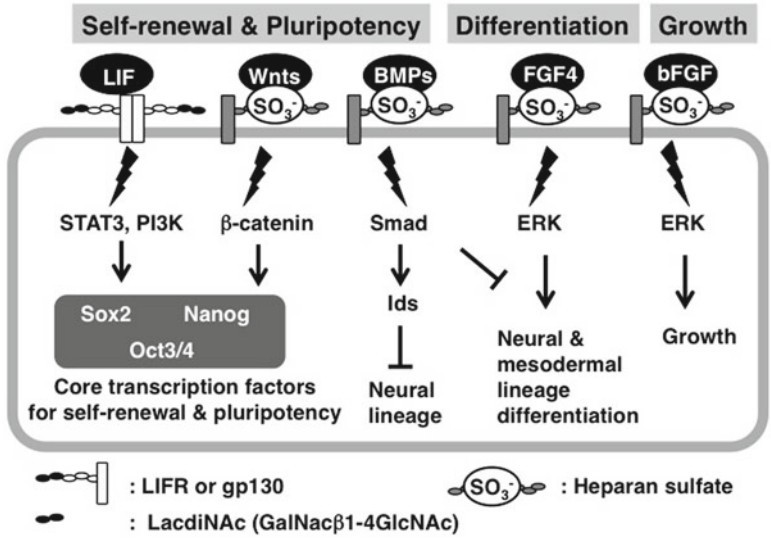

Fig. 4.2 The roles of glycan structures on mouse ES cells. LacdiNAc and heparan sulfate contribute to the self-renewal and pluripotency of mouse ES cells through LIF/STAT3 signaling, and Wnt/β-catenin and BMP/Smad signaling, respectively. Heparan sulfate also contributes to the differentiation and growth of mouse ES cells through FGF4/ERK and bFGF/ERK signaling pathways, respectively. Heparan sulfate works as the co-receptor or the stabilizer for the extrinsic factors

balance of several signaling pathways, such as Wnt, BMP, and FGF, and disruption of this balance induces differentiation of ES cells into a specific lineage. HS also contributes to the regulation of these signals and works in the maintenance and differentiation of mouse ES cells (Fig. 4.2) (Kraushaar et al. 2010; Lanner et al. 2010; Sasaki et al. 2008, 2009). However, the physiological functions of the other glycans in ES cells and iPS cells are still largely unknown. Recently, we identified a novel glycan function in naïve state mouse ES cells and primed state embryonic stem cells after an RNAi screen of more than 100 glycosyltransferases (Sasaki et al. 2011). This chapter focuses on the functions of this glycan structure, LacdiNAc, in LIF/STAT3 signaling and in self-renewal of naïve state ES cells.

LIF/STAT3 Signaling Contributes to Self-Renewal of Mouse Embryonic Stem Cells

LIF is essential for self-renewal and pluripotency of mouse ES cells, but does not have this function in human ES and iPS cells (Boeuf et al. 1997; Daheron et al. 2004; Hirai et al. 2011; Niwa et al. 2009). Therefore, LIF-responsiveness is considered to be one of the key distinguishing features of mouse and human ES cells (Fig. 4.3a).

LIF is a member of the Interleukin 6 (IL6) cytokine family and its signaling pathway is well characterized. In mouse ES cells, the binding of LIF to the LIF receptor (LIFR) induces three signaling pathways, namely, Jak/STAT3, PI3K/Akt, and SHP2/MAPK (Hirai et al. 2011; Niwa et al. 2009) (Fig. 4.2). Nanog, Sox2, and Oct3/4, which are core transcriptional factors for the maintenance of self-renewal and pluripotency, regulate each other to form the core transcriptional factors network and then collaborate to regulate downstream gene expression. LIF signaling induces the expression of these core transcriptional factors through the Jak/STAT3 and PI3K/Akt pathways to maintain this network. Thus, in mouse ES cells, LIF signaling contributes to the maintenance of self-renewal and pluripotency by inducing core transcriptional factors.

In addition to LIF, several extrinsic signaling pathways involved in self-renewal and pluripotency have been identified in mouse ES cells. Wnt/β-catenin signaling regulates the expression

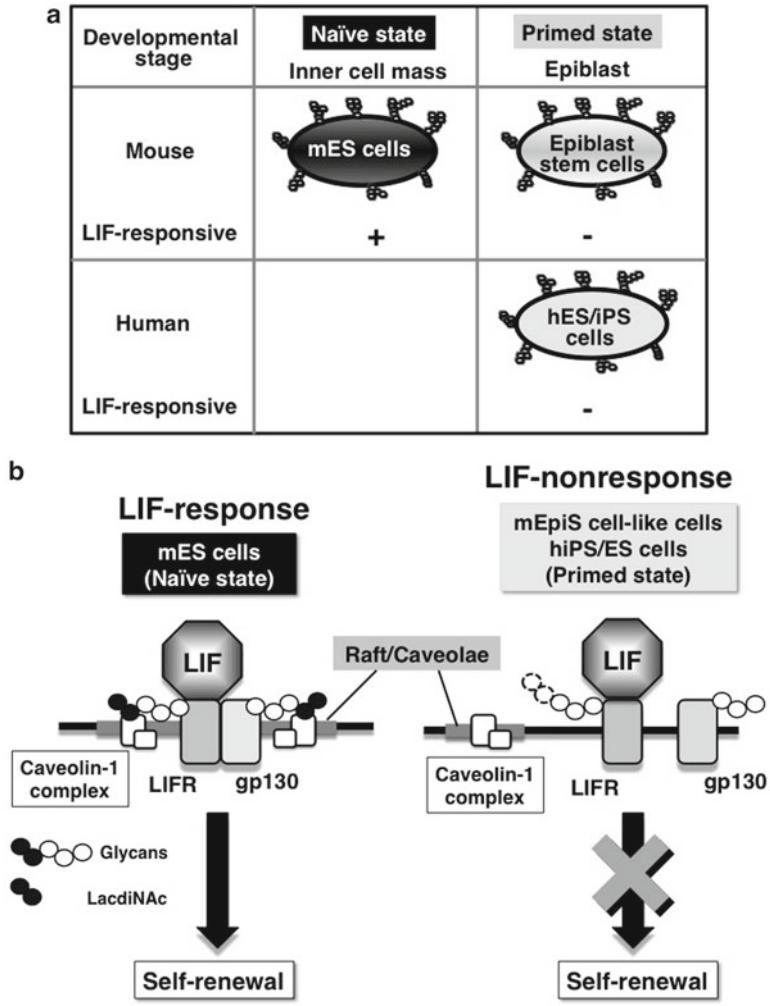

Fig. 4.3 High levels of LacdiNAc structures on LIFR and gp130 are required for maintenance of the naïve state in pluripotent stem cells. (**a**) Mouse ES cells are at a different developmental stage from human ES/iPS cells: mouse ES cells correspond to the inner cell mass (naïve state), while hES/iPS cells correspond to the epiblast (primed state). This difference is characterized by their respective levels of responsiveness to LIF. (**b**) Expression of LacdiNAc structures on LIFR and gp130 is required for the induction and maintenance of naïve state pluripotent stem cells. In naïve state mouse ES cells, the LacdiNAc structures on LIFR and gp130 contribute to the association with the caveolin-1 complex components of caveolae/rafts; this association stabilizes LIFR and gp130 localization to the rafts. This localization is necessary to induce a sufficiently strong transduction of LIF/STAT3 signaling to maintain self-renewal. In primed state pluripotent stem cells, such as mouse mEpiS cell-like cells and hiPS/ES cells, the receptors do not have LacdiNAc and cannot localize to the rafts. As a result, the cells cannot induce LIF/STAT3 signaling to maintain self-renewal

of Oct3/4 and Nanog and of their core network (Sasaki et al. 2008; Sato et al. 2004; Wray and Hartmann 2012) (Fig. 4.2). Bone morphogenic protein 4 (BMP4)/Smad signaling contributes to the maintenance of self-renewal and pluripotency in cooperation with LIF (Ying et al. 2003). By contrast, in human ES/iPS cells, BMP4 signaling induces differentiation (Xu et al. 2002), and FGF2 and activin/nodal signaling are involved in the maintenance of self-renewal and pluripotency (James et al. 2005). However, the role of Wnt/β-catenin signaling in the maintenance

of self-renewal and pluripotency in human ES cells remains uncertain, although activation of Wnt/β-catenin signaling is known to inhibit their differentiation (Sato et al. 2004; Wray and Hartmann 2012). The outcome of Wnt/β-catenin signaling might depend on other signaling factors such as FGF2 (Wray and Hartmann 2012). Thus, there is clear evidence that mouse and human ES/iPS cells maintain their pluripotency using different signaling factors.

As stated above, mouse and human ES/iPS cells are considered to be at different developmental stages (Fig. 4.3a) and, therefore, to require different extrinsic signals to maintain self-renewal and pluripotency. Pluripotent stem cells at different stages of development have been identified in the mouse, namely, epiblast stem cells (EpiS cells) and FGF/activin/BIO-stem cells (FAB-S cells) (Brons et al. 2007; Chou et al. 2008) (Fig. 4.3a). EpiS cells are derived from the post-implantation epiblast and have similar features to human ES cells. Both cell types are maintained by the same extrinsic signals, that is, activin/nodal and FGF2 signaling. Mouse EpiS cells are at a more developmentally advanced stage than mouse ES cells and can be induced to revert to mouse ES cell-like cells (Bao et al. 2009; Yang et al. 2010; Zhou et al. 2010). FAB-S cells are generated from the pre-implantation epiblast under defined culture conditions, including the presence of FGF2 and activin, and they can be induced to revert to the mouse ES cell-like state. The relative developmental stage of FAB-S cells is uncertain but they are thought to be at a developmentally more advanced stage than mouse ES cells. On the basis of the data obtained from several studies using various mouse stem cells derived from different developmental stages, human ES cells are believed to be at a later developmental stage that corresponds to the mouse post-implantation epiblast, which is now designated as the "primed state" (Fig. 4.3a). In contrast, mouse ES cells are derived from the ICM and their developmental stage is described as the "naïve state". The specific culture conditions that are required to maintain mouse ES cells have recently been shown to induce human ES cells in the primed state to revert to a naïve state corresponding to that of mouse ES cell-like cells. These naïve human ES cells can then be maintained under culture conditions containing LIF (Hanna et al. 2010; Xu et al. 2010). The findings from these studies provide support for the contention that mouse and human ES/iPS cells are at different stages of development. The difference in developmental stages of mouse and human ES/iPS cells is also reflected in their different responses to extrinsic signals. For example, LIF signaling contributes to the maintenance of self-renewal in mouse ES cells but not in human ES cells (Boeuf et al. 1997; Daheron et al. 2004; Hirai et al. 2011; Niwa et al. 2009). Therefore, LIF responsiveness is an important key factor that distinguishes the naïve and primed states of pluripotent stem cells (Fig. 4.3a).

LacdiNAc Structures Are Expressed on Mouse Embryonic Stem Cells But Not on Human Induced Pluripotent Stem Cells or Mouse Epistem-Like Cells

The LacdiNAc carbohydrate structure, GalNAcβ1-4GlcNAc, is a unique terminal structure in the outer chain moieties of *N*-glycans and *O*-glycans. It was originally identified on the human glycoprotein hormones leutropin and thyrotropin, which are secreted from the pituitary gland. Initially, LacdiNAc was thought to be restricted in vertebrates to a limited number of glycoproteins and glycolipids, such as glycoprotein hormones, although it is frequently present on glycoproteins and glycolipids in invertebrates (Manzella et al. 1996). In vertebrates, the LacNAc element Galβ1-4GlcNAc, appears to be more commonly present as a type II blood core carbohydrate structure than LacdiNAc, (Manzella et al. 1996). Various other molecules, such as glycodelins in the amnion of fetal tissue and α-dystroglycan in the extracellular matrix (Breloy et al. 2012), have recently been found to carry LacdiNAc on *N*- or *O*-glycans.

Mouse ES cells show high expression of the LacdiNAc structure on their surface (Sasaki et al. 2011). The expression of LacdiNAc decreases

during differentiation in mouse ES cells cultured without LIF. LacdiNAc expression is also reduced in mouse EpiS cell-like cells produced from mouse ES cells in EpiS cell culture conditions including activin A and FGF2 and in human iPS cells which are at developmentally more advanced stage than mouse ES cells. Thus, LacdiNAc is highly expressed in naïve state pluripotent stem cells, while it is expressed at a lower level in primed pluripotent stem cells (Fig. 4.3b) (Sasaki et al. 2011).

To date, nine types of β1,4-*N*-acetylgalactosaminyltransferase (β1,4GalNAc-T) have been cloned and their substrate specificities identified. Of these, β1,4GalNAc-T 3 and 4 show activity in the synthesis of LacdiNAc structures (Gotoh et al. 2004; Sato et al. 2003). Both recombinant enzymes have very similar substrate specificities but show very different distributions in human tissues. *β4GalNAc-T3* mRNA has been detected in the stomach, colon and testis, whereas *β4GalNAc-T4* transcripts are predominantly detected in the brain, ovary, and mammary glands. In mouse ES cells, RNAi of *β4GalNAc-T3* reduces the amount of LacdiNAc structures on the cell surface (Sasaki et al. 2011) and, therefore, β4GalNAc-T3 is considered to be the principal contributor to synthesis of the LacdiNAc structure. By comparison to mouse ES cells, expression of *β4GalNAc-T3* is lower in mouse EpiS cell-like cells and human iPS cells, reflecting the reduced expression of LacdiNAc on their cell surfaces. *β4GalNAc-T3* is expressed highly in naïve state pluripotent stem cells, but at a lower level in primed pluripotent stem cells. The role of β4GalNAc-T 4 remains under investigation.

LIFR and gp130 Localize on Lipid Rafts/Caveolae and Transmit the LIF/STAT3 Signal

Lipid rafts are defined as microdomains within the lipid bilayer of cellular membranes. They are small, heterogeneous and dynamically changeable structures enriched in glycosphingolipids, sphingomyelin and cholesterol, and subsets of transmembrane or glycosylphosphatidylinisotol-anchored proteins (Lingwood and Simons 2010). Lipid rafts are considered to be essential in cellular signaling processes because many signaling molecules, receptors and Src family kinases are assembled there. Biochemically, lipid rafts resist extraction in cold detergent and, therefore, they are described as detergent-resistant membrane fractions.

During the process of LIF binding, LIFR recruits the membrane protein gp130 to form a heterodimer (Hirai et al. 2011) (Fig. 4.3b). Heterodimerization triggers activation of associated JAK tyrosine kinases and then phosphorylation of gp130; these events result in the activation of STAT3. In mouse ES cells and embryonic neural precursor cells, gp130 and LIFR localize to one particular type of lipid raft termed a caveola (Lee et al. 2010; Sasaki et al. 2011; Yanagisawa et al. 2004). This localization is required for strong LIF/STAT3 signaling in mouse ES cells (Lee et al. 2010; Sasaki et al. 2011). However, the role of caveolin-1, a component of rafts/caveolae, in localization of the receptors is still ambiguous, although both LIFR and gp130 have caveolin binding motifs: YGTVVFAGY (amino acids 318–326) and FTFTTPKF (amino acids 604–611), respectively (Couet et al. 1997; Sasaki et al. 2011). In addition to caveolin-1, other raft/caveolar components, such as RTPF/cavin-1, caveolin-2, and glycosylphosphatidylinositol-anchored proteins might stabilize localization of the receptors.

LIFR and gp130 with LacdiNAc Structures Are Localized on Lipid Rafts/Caveolae

The LacdiNAc carbohydrate structure GalNAcβ1-4GlcNAc along with β1,4GalNAc-T 3, which synthesizes LacdiNAc (Sato et al. 2003), are highly expressed in naïve state pluripotent stem cells, such as mouse ES cells (Fig. 4.3b) (Sasaki et al. 2011). Protein analyses showed that both LIFR and gp130 are modified by LacdiNAc. Both LIFR and gp130 are localized on lipid rafts and co-immunoprecipitated by caveolin-1, a

marker for and component of lipid rafts/caveolae. LIFR and gp130 also show co-localization with caveolin-1 on the surface of mouse ES cells indicating that receptor molecules carrying LacdiNAc are localized on lipid rafts/caveolae (Sasaki et al. 2011). It has been reported that gp130 has nine *N*-glycosylated sites among the 11 potential *N*-glycosylation sites in its extracellular domain (Moritz et al. 2001). In mouse embryonic neural precursor cells, *N*-glycans on gp130 are required for heterodimerization with LIFR (Yanagisawa and Yu 2009). Potential *O*-glycosylation sites are also found in both gp130 and LIFR (Sasaki et al. 2011). However, it has not yet been determined whether there are LacdiNAc structures on *N*- or *O*-glycans that modify gp130 and LIFR on mouse ES cells.

LacdiNAc Structure Is Essential for Self-Renewal in Naïve State Pluripotent Stem Cells (Mouse Embryonic Stem Cells) Through LIF/STAT3 Signaling

Mouse ES cells were subjected to RNAi using short hairpin RNAs targeted against various glycosyltransferase genes and self-renewal of the knockdown cells was evaluated by their alkaline phosphatase activities. This analysis showed that the cell surface glycan LacdiNAc is required for self-renewal of naïve state pluripotent stem cells (Sasaki et al. 2011). Knockdown (KD) of *β4GalNAc-T3*, which synthesizes LacdiNAc, results in reduced expression of cell surface LacdiNAc, a lower proportion of alkaline phosphatase positive colonies, and a reduction in Oct3/4, Nanog and Sox2, which are markers of the undifferentiated state. In addition, the rate of proliferation of *β4GalNAc-T3*-KD cells is significantly decreased. Thus, LacdiNAc has a range of functions in undifferentiated mouse ES cells (naïve state pluripotent stem cells) and is required for self-renewal and proliferation of the cells.

LIF/STAT3 signaling is markedly reduced in *β4GalNAc-T3*-KD cells, although BMP4/Smad and FGF4/ERK signaling is not affected (Sasaki et al. 2011). Therefore, the reduction in LIF/STAT3 signaling causes the reduction in the self-renewal ability of the cells. However, no significant differences are detectable in cell surface expression of LIFR and gp130 between control and *β4GalNAc-T3*-KD cells. In contrast, in *β4GalNAc-T3*-KD cells, LIFR and gp130 are dispersed from the lipid rafts/caveolae resulting in a reduced rate of LIFR and gp130 heterodimerization in response to LIF. LacdiNAc on LIFR and gp130 is decreased in *β4GalNAc-T3*-KD cells, and the interaction and colocalization of caveolin-1 with LIFR and gp130 is also reduced. Overall, these findings demonstrate that LacdiNAc is involved in LIF/STAT3 signaling in mouse ES cells through regulation of the localization of LIFR and gp130 to rafts/caveolae and through controling their heterodimerization (Fig. 4.3b). That is, LacdiNAc structures on LIFR and gp130 are required for the localization of these receptors to the rafts/caveolae by interaction with components of the caveolin-1 complex and are necessary to maintain a sufficiently strong LIFR/STAT3 signal for maintenance of self-renewal.

Reduced Expression of LacdiNAc Structures in Primed State Pluripotent Stem Cells (Mouse Epistem-Like Cells and Human Induced Pluripotent Stem Cells) Causes the Inert State of the LIF/STAT Signal

LIF/STAT3 signaling does not function in either mouse EpiS cells or human ES cells, (Brons et al. 2007; Daheron et al. 2004), although it maintains self-renewal in mouse ES cells (Fig. 4.3a). As mentioned above, expression of LacdiNAc is reduced in primed state pluripotent stem cells such as mouse EpiS cell-like cells and human iPS cells compared to naïve state mouse ES cells (Sasaki et al. 2011). LacdiNAc structures are a key factor in determining the responsiveness to LIF/STAT3 signaling and explain the different responses of primed and naïve state pluripotent stem cells (Fig. 4.3b).

Mouse EpiS cell-like cells are similar to mouse EpiS cells and human ES/iPS cells in showing a very weak response to LIF (Sasaki et al. 2011). Expression of LacdiNAc on LIFR and gp130 is markedly lower in mouse EpiS cell-like cells and human iPS cells than mouse ES cells. Consequently the raft/caveolar localization of LIFR and gp130 is very weak in mouse EpiS cell-like cells and human iPS cells, which are primed state pluripotent stem cells. Based on these observations, the following molecular mechanism for the weak response to LIF in primed state pluripotent stem cells is proposed: LIFR and gp130 do not localize in rafts/caveolae due to the low level of LacdiNAc and, in turn, this decrease in localization causes a weak transduction of the LIF/STAT3 signal (Fig. 4.3b).

LacdiNAc Structures Are Required for the Induction of Naïve State Pluripotent Stem Cells (Mouse Embryonic Stem Cells)

Both LIF/STAT3 signaling (Bao et al. 2009; Yang et al. 2010) and LacdiNAc expression (Sasaki et al. 2011) contribute to reversion from the primed state to the naïve state.

Stable *β4GalNAc-T3*KD EpiS cell-like cells were produced from stable β*4GalNAc-T3*KD ES cells (Sasaki et al. 2011). Mouse EpiS cell-like cells can be induced to revert to mouse ES cell-like cells under defined culture conditions (Hanna et al. 2010; Zhou et al. 2010). The rate of reversion in mouse EpiS cell-like cells derived from stable *β4GalNAc-T3*KD ES cells is markedly lower than in control cells, indicating that up-regulation of LacdiNAc is required for reversion from the primed state to the naïve state (Sasaki et al. 2011).

Thus, one of the important factors in the differential response of naïve and primed state pluripotent cells to LIF is the level of LacdiNAc structures on LIFR and gp130, and the expression of LacdiNAc is required for the induction and maintenance of the naïve state in pluripotent stem cells (Fig. 4.3b).

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5 Cancer Gene Therapy Potential of Neural Stem Cells Derived from Human Embryonic Stem Cells and Induced Pluripotent Stem Cells

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Abstract

The discovery of the inherent tumour-tropic properties of neural stem/progenitor cells (NSCs) has opened new avenues for targeted cancer therapy. The significance of NSC-based cancer gene therapy strategy lies in the capability of the cells to trace not only primary tumours, but also the disseminated foci when a tumour becomes invasive and metastatic. Increasing numbers of reports have demonstrated that NSCs are capable of efficiently delivering therapeutic agents, including suicide genes and/or immune-modulatory cytokines, to a wide variety of cancers. Despite the promising clinical potential of NSC-based anticancer therapy, safe acquisition of a renewable and steady supply of human NSCs remains a great challenge. Human pluripotent stem cells, such as embryonic stem cells (ESCs) and recently discovered induced pluripotent stem cells (iPSCs), can proliferate indefinitely and differentiate into cells of all three germ layers. Therefore, pluripotent stem cells are theoretically attractive cell sources to generate tumour tropic NSCs for cancer gene therapy. In this review, we discuss the

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underlying mechanisms governing tumour tropism by NSCs, the methods for generating human NSCs from ESCs and iPSCs, the therapeutic approaches using NSC delivery system in cancer gene therapy, as well as current limitations restricting the use of less differentiated NSCs and the potential solutions to overcome these barriers. The development of NSC-based strategies holds great potential for cancer treatment. Its efficacy, safety and feasibility should be further evaluated in randomized and controlled clinical trials.

Introduction

Neural stem/progenitor cells (NSCs) are defined by their ability to self-renewal in the central nervous system and the multipotent potential to differentiate into three fundamental neural lineages: neurons, astrocytes and oligodendrocytes. NSCs have found their way into many medical applications, especially in treating brain illnesses where tissues cannot ordinarily re-grow, such as Alzheimer's and Parkinson's. Since 2000, a number of studies have reported that NSCs hold potent tumour tropic capacity. Regardless of injection location in the brain, the injected NSCs can migrate to tumour bed and chase down infiltrating tumor cells throughout the brain (Aboody et al. 2000; Benedetti et al. 2000). Further investigations demonstrated that intravenously administrated NSCs can migrate to various organs, including the brain, liver, ovary and bone marrow, to target primary and disseminated tumor cells of both neural and non-neural origin (Aboody et al. 2008; Yang et al. 2012; Zhao et al. 2012a, b). These studies suggest that administration of NSCs can be exploited as a powerful approach to target not only major tumor mass, but also disseminated tumor foci in distant. The findings are encouraging and promising, and have stimulated the rapid development of NSC-based new therapeutics for cancer treatment (Ahmed et al. 2010).

The great potential of NSCs in cancer therapy necessitates the development of a safe, renewable and steady supply of NSCs for future medical treatment. NSCs that have been tested so far in pre-clinical settings differ substantially in their sources and methods of isolation/generation. These cells include primary NSCs derived from brain tissues (Taupin 2007), oncogene-immortalized NSCs (Aboody et al. 2008), and NSCs differentiated from pluripotent stem cells, such as embryonic stem cells (ESCs) (Zhao et al. 2012b) or induced pluripotent stem cells (iPSCs) (Yang et al. 2012). While each type of NSCs has its own merits and disadvantages, the use of pluripotent stem cell-derived NSCs appear more attractive, given that potential clinical translation requires uniform cellular products in large scale. The Nobel Prize winning discovery of iPSC is a striking breakthrough in stem cell field (Takahashi et al. 2007). With this powerful technology, human somatic cells can be reprogrammed to become pluripotent. The generated iPSCs can act as renewable sources for differentiation into tumor-tropic NSCs. In this review, we will summarize the current knowledge regarding the signals and pathways that are involved in regulating the inherent tumor tropism of NSCs, the approaches for generation of human NSCs derived from ESCs and iPSCs, the application of NSCs for treating cancers, the current barriers that NSC-based cancer therapy is facing, and the possible solutions.

Molecular Mechanisms Underlying Tumour Tropism of Neural Stem Cells

First published in 2000, several research groups reported in vitro and in vivo studies using NSCs for gene therapy of glioblastomas, which demonstrated that NSCs are capable of delivering therapeutic gene to main tumor mass, tumor outgrowth, as well as metastatic tumor beds (Aboody et al. 2000; Benedetti et al. 2000). Further studies of NSCs inherent tumor tropism were performed in both primary and secondary CNS malignancies, such as intracranial medulloblastomas, melanoma brain metastases and

neuroblastomas bone metastasis (Aboody et al. 2008; Sims et al. 2009). More recent studies have shown that NSCs are able to target not only brain tumors but also tumors of nonneural origin. Kim and colleagues showed that genetically engineered NSCs expressing a suicide gene selectively migrated toward human epithelial ovarian cancer cells and inhibited ovarian cancer cell growth in vitro in the presence of prodrugs (Kim et al. 2010). Aboody's group and our group independently reported that NSCs can be successfully employed as a systemic delivery vehicle for therapeutic genes again metastatic breast cancer (Yang et al. 2012; Zhao et al. 2012a). The tumour-selective migratory capacity of NSCs is critical to their role as cellular delivery vehicles for targeted anticancer therapy.

NSCs have an intrinsic tropism for sites of brain injuries, and can spread through the existing migratory pathways as well as nontypical routes. While the precise tumor homing mechanism of NSCs still remains unclear, it is possible that tumor growth and development cause tissue damage and result in releasing numerous cytokines, chemokines, growth factors and cell trafficking regulators, which in turn act as stimulatory signals for NSC migration. Thus, NSC migration may use cytokines, chemokines, and/or growth factors released from the injury sites as candidate migration stimulatory signals. In contrast to normal healing wounds, tumor growth and development that cause damage to nearby normal tissues is not self-limiting, thus resulting in uncontrolled cell proliferation, invasion and metastasis. Furthermore, tumor cells commonly misregulate the expression of chemokines, growth factors, and extracellular matrix proteins during tumor development. These observations suggest that common cellular and molecular mechanisms might be active between tissue injury and tumor development to stimulate NSCs migration. It is also clear that this tropism is not tumour-type specific since NSCs migrate toward a large variety of different tumors. Possibly, NSCs migrate toward either intracranial or extracranial tumors when the growth of the tumors causes tissue damage and secrets the cell trafficking regulators.

Cell migration is a highly complex yet dynamic process, which includes sensing of microenvironmental cues, reconstructing the cellular cytoskeleton, regulating cell detachment and attachment to extracellular matrix, and signalling all the processes to coordinate the movements. While the signals required for recruiting and homing of NSC to tumor sites and the precise molecular mechanism of tumor-tropism of NSCs have yet to be elucidated, studies have shown that numerous cytokines, chemokines, growth factors and receptors released from injury sites and expressed by tumor microenvironment play potent chemotactic roles in stimulating NSC migration. Cytokine/receptor such as stem cell factor (SCF)/Kit, monocyte chemoattractant protein-1 (MCP-1)/CCL, annexin A2, and stromal cell-derived factor 1 (SDF-1)/CXCR4 have been reported as chemotactic agents in promoting NSC-to-tumor tropism (Ahmed et al. 2010). Growth factors, such as epidermal growth factor (EGF)/EGFR, vascular endothelial growth factor (VEGF)/VEGFR, and hepatocyte growth factor (HGF)/c-Met are potent regulators involved in activating the migration capacity of NSCs (Ahmed et al. 2010). In addition, as one of the critical features of cancer microenviroment, hypoxia caused increased NSCs migration to glioma in vitro, and NSCs preferentially distributed to hypoxic areas within intracranial glioma xenografts in vivo (Zhao et al. 2008). Hypoxia stimulating NSC-to-tumor tropism is through transcription factor hypoxia-inducible factor-1α (HIF-1α) mediated upregulation of numerous chemoattractants and receptors, including SDF-1/CXCR4, uPA/uPAR, VEGF/VEGFR2, and HGF/c-Met (Zhao et al. 2008).

To further reveal the molecular mechanism underlying NSCs tropism for tumor, we performed a cDNA expression library screening and identified a gene encoding transmembrane protein 18 (TMEM18) which regulates NSCs migration towards gliomas in vivo as well as in vitro. Overexpression of TMEM18 increased NSCs migration and correlated with augmented transcription of C-X-C chemokine receptor type 4 (CXCR4). Further study by Jurvansuu and colleagues showed that TMEM18 localises to

the nuclear membrane and binds DNA with its C-terminus in a sequence-specific manner (Jurvansuu and Goldman 2011). Once overexpressed, the TMEM18 bring the chromatin very close to nuclear membrane. This perinuclear localisation of TMEM18-bound DNA inhibits transcription of some transcription factors including Yin Yang 1 (YY1), which has previously been shown to suppress CXCR4 transcription. Although the interactions might be indirect, it is demonstrated that TMEM18 overexpression leads to increased transcription of CXCR4 through TMEM18 mediated repression of the YY1, and as a consequence stimulating NSCs migration through SDF-1/CXCR4 signalling pathway.

Moreover, we recently used a cDNA microarray analysis to compare gene expression profiles of NSCs collected from migratory versus non-migratory cells and identify the neuronal nitric oxide synthase (nNOS) as a regulator in controlling NSC migration towards both glioblastoma cells and breast cancer cells (unpublished observation). Nitric oxide (NO) is a radical molecule involved in a variety of pathophysiological processes. NO is synthesized from L-arginine by NOS, which are co-localized with the cellular cytoskeleton. The reorganization of cytoskeleton, which can be induced by extracellular signals, will indirectly affect NOS production. Previous studies have observed that a decreased expression level of nNOS in cerebral regions that the migrating cells passed through (Zhang et al. 2007), and NOS inhibition decreases NSCs differentiation while promotes proliferation (Luo et al. 2010). Our study demonstrated that nNOS was down regulated in migratory NSCs and NSCs-mediated tumor tropism was improved upon using NOS inhibitors. These findings support a hypothesis that down-regulation of nNOS decrease intracellular NO level and inhibit NSCs differentiation, which in turn might promote cytoskeletal dynamics and thus enhance NSC migration toward tumors. However, other studies by Tegenge and colleagues have indicated that in early developing human brain cells, NO/cGMP signaling stimulates human neural progenitor cells migration by activating guanylyl cyclase that produces cGMP upon activation with NO and the migration of neural progenitor cells is blocked by the use of nNOS inhibitors and enhanced by the use of an NO donor (Tegenge et al. 2011). These discrepancies are correlated with a previous observation that the regulation of neurogenesis by nNOS is bidirectional (Luo et al. 2010). The possible explanations are the different physiological and/or pathological conditions, the variable species and sources of neural precursor cells, as well as the different source of nNOS. Further in vivo studies need to be carried out to investigate whether nNOS inhibitors could increase NSCs tumour tropism.

The multiple tumor homing mechanisms and the selective targeting of malignancies utilized by NSCs support their use as an optimal delivery vehicle over other targeting strategies that primarily depending on a single factor approach. A better understanding of the molecular and cellular events that regulate NSC tumor tropism is required to optimize clinical implementation, ensure safety and maximize therapeutic efficacy of NSCs.

Generation of Human Neural Stem Cells from Embryonic Stem Cells and Induced Pluripotent Stem Cells

Various types of NSCs have been tested so far. Autologous transplantation of primary NSCs obtained from regions in the adult nervous system would circumvent problems of immunogenic potential of the cells as well as concerns over donor and host compatibility. However, currently available technologies for isolating autologous NSCs require invasive surgical procedures. The risks associated with surgeries, such as damaging healthy brain tissues, have limited its clinical applications (Taupin 2007). Furthermore, the primary NSCs that isolated from human brain tissues is generally insufficient in quantity, variable in quality and have limited passaging capacity, thus posing significant challenge in large-scale preparation of cells with stable properties.

The oncogene-immortalized human NSC lines, on the other hand, provide a solution to expand

the limited life span of NSCs with stability. These immortalized NSCs were generated by introducing of viral oncogenes, such as proto-oncogene v-myc, SV40 large T-antigen, and human telomerase reverse transcriptase (hTERT). Although most of the immortalized allogeneic NSC lines are well characterized and have been tested in preclinical cancer models, this method faces major concerns regarding the tumorigenicity from the transforming and oncogenic potential of the immortalized NSCs, as well as immunogenicity of NSCs from allogeneic sources (Aboody et al. 2008).

Pluripotent stem cells, such as human embryonic stem cells (ESCs) and induced pluripotent stem cells (iPSCs), are capable of proliferating indefinitely under ideal conditions and differentiating into cells of all three germ layers. Hence, ESCs and iPSCs are attractive cell sources to generate NSCs. To facilitate the selection of the most suitable NSC cell line for clinical translation, a comprehensive approach is necessary for the comparison of multiple different NSCs lines. In this review we focus on the methods in generation of NSCs from ESCs and iPSCs.

Generation of Human NSCs from ESCs

Human NSCs could be induced successfully from ESCs using two principally different methods. For both methods, the quality of starting ESCs is critical for the subsequent differentiation of neural derivatives. The obtained cell populations are heterogeneous and additional step, such as fluorescence-activated cell sorting (FACS), may be required for further purification.

The first approach uses formation of embryoid bodies (EBs) or neurospheres intermediates that mimic the in vivo environment by providing appropriate cell-cell interaction signals. When cultured in suspension without growth factors, ESCs spontaneously differentiate and form embryoid bodies (EBs), the three-dimensional multicellular aggregates. These EBs are subsequently seeded onto gelatine or laminin substrates in a defined medium containing fibroblast growth factor 2 (FGF-2) for further conversion to NSCs. The generated NSCs could differentiate into neurons and glia derivatives (Zhang et al. 2001). The disadvantages of this method, such as the extended time used to derive NSCs through the formation of neurospheres or embryoid bodies, the difficulty in handling cell aggregates, and the variability of final populations limit the large-scale generation of NSCs.

The second approach uses the adherent monolayer culture protocol which deprives the cell-cell interactions of ESCs and evokes a default mechanism for direct differentiation of NSCs (Shin et al. 2006). Generation of NSCs from ESCs occurs in a defined neural promoting medium containing FGF-2 and epidermal growth factor (EGF). Our recent study has shown that ESCs-derived NSCs are similar to natural NSCs in their genetic make-up, surface markers, and multipotent potential to differentiate into neurons, astrocytes and oligodendrocytes (Zhao et al. 2012b). In this less cumbersome method, embryoid body formation is dispensable, thus reducing the time to generate human NSCs from several months for cell-aggregation method to 1 month. Quantitative fluorescence-activated cell sorting analysis revealed that 98% of cells were positive for the NSC marker nestin, indicating a high purity of NSCs generated from monolayer culture condition. These NSCs could be propagated for 6 months under standard culture conditions while maintaining their neural multipotency (Zhao et al. 2012b). This long term proliferative capability is necessary to produce sufficient amounts of cell-based therapeutics in a reproducible manner, and is also useful for ex vivo genetic modification and selection of the modified cells.

Generation of Human NSCs from iPSCs

Isolation of human ESCs faces ethical issues due to destruction of human embryos, and the use of allogeneic ESCs cells might trigger immune rejection. The breakthrough in the induced pluripotent stem cells (iPSCs) technology provides an alternative stem cell source for large scale derivation of NSCs. iPSCs are generated synthetically through the expression of specific transcription factors

in differentiated adult somatic cells, creating cells that possess the ability to differentiate into any cell type in the body. Although iPSCs retain a transient epigenetic memory of their somatic cells of origin, human iPSCs are similar to ESCs with respect to pluripotency marker expression, self-renewal and multilineage differentiation potential (Takahashi et al. 2007).

Recently, we applied the adherent monolayer culture method adopted from ESCs to iPSCs and successfully generate NSCs in a defined culture condition containing bFGF and EGF (Yang et al. 2012). These iPSCs-derived NSCs were able to express specific markers associated with neural stem/precursor cells, form neurospheres when transferred to suspension culture, and displayed the functional hallmark of NSCs: differentiation into β3-tubulinpositive neurons, GFAP-positive astrocytes, and O4-positive oligodendrocytes. In vitro migration assay showed the iPSCs-derived NSCs generated by adherent monoculture method have a strong tumor tropism (Yang et al. 2012). The doubling time of iPSCs-derived NSCs was ~3–4 days and the culture can be passaged for about 15 times before their proliferation speed slowing down. We also investigated the neural aggregates formation method that mimics the in vivo environment for inducing NSCs from iPSCs, which generates NSCs with strong tumor tropism. However, the variability in the quality of the final product is observed, probably due to the multiple derivation steps.

iPSCs are more attractive than ESCs in generating NSCs as they possess similar characteristics but circumvent the bioethical controversies associated with isolating embryos. Moreover, somatic cells isolated from patients could theoretically be reprogrammed into iPSCs that can be used to generate NSCs for autologous transplantation, hence minimizing host-vs-graft reaction. Likewise, human leukocyte antigen (HLA)-typed iPSCs could be generated to make iPSC-derived cells that are more patient-specific than other cell sources (Nakatsuji 2010). The use of iPSC-derived NSCs may be favourable for generating consistent experimental outcomes, and for potentially accelerating the translation of NSC-based anticancer therapies into the clinic.

Cancer Gene Therapy Using Human Embryonic Stem Cells and Induced Pluripotent Stem Cells-Derived Neural Stem Cells

The inherent tumor tropism of NSCs to primary and metastatic tumor foci can be exploited to deliver therapeutic agents to invasive human cancer. Since Yamanaka's and Thomson's breakthrough discoveries of human iPSCs, much attention has been focused on exploring the potential of NSCs that derived from ESCs or iPSCs as cellular delivery vehicles for cancer gene therapy over the past several years (Fig. 5.1).

We have recently investigated the tumor tropic property of ESCs-derived NSCs, which targets human glioma both in vitro and in vivo (Zhao et al. 2012b). In vitro migration assays showed that ESCs-derived NSCs migrated specifically to glioma cell lines, with a tropism similar to that of a well-studied mouse NSC cell line C17.2 cells. After contralateral brain injection, 25% of the ESCs-derived NSCs were able to migrate through brain parenchyma to reach the intracranial glioma in the opposite hemisphere in 2 weeks. Upon loading the herpes simplex virus thymidine kinase (HSVtk) suicide gene through baculovirus infection, the ESCs-derived NSCs were able to inhibit the growth of human glioma xenografts and prolong the survival of tumor-bearing mice in the presence of ganciclovir (GCV). In the case of systemic injection through tail vein, the intravenously injected NSCs could migrate across the blood–brain barrier, penetrate into and distribute extensively throughout the intracranial glioma. However, the majority of systemic injected NSCs became stuck in the lung and liver due to the narrow diameters of lung capillaries and liver sinusoids. Damaging normal proliferating cells in these organs by the bystander effect of phosphorylated GCV is a major safety concern. One possible way to alleviate this problem is to develop a new regulation system that triggers transgene expression in NSCs only after they home in on tumors.

Similar results were also observed using mouse iPSC-derived NSCs in the treatment of

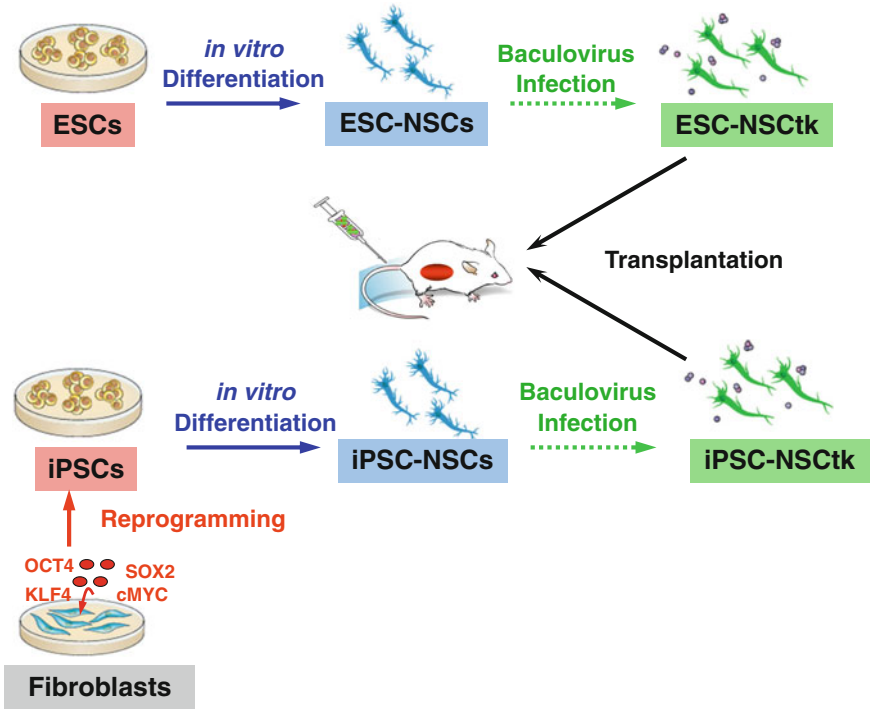

Fig. 5.1 Generation of human NSCs from ESCs or iPSCs for cancer gene therapy. NSCs can be generated in vitro through specific differentiation from either human ESCs or human iPSCs that being reprogrammed from fibroblasts. The derived NSCs are loaded with a therapeutic gene via viral transduction, for example baculoviral transduction of the suicide HSVtk gene. The transduced NSCs (named as NSCtk) are then injected into mice bearing a tumour to test the therapeutic effects of the engineered cells

disseminated brain tumors (Lee et al. 2011). When mouse iPSC-derived NSCs were transduced with a baculoviral vector containing the HSVtk suicide gene and injected into the cerebral hemisphere contralateral to a tumor inoculation site in a mouse intracranial human glioma xenograft model, with daily intraperitoneal administration of GCV, the growth of the glioma xenografts was inhibited and the survival of tumor-bearing mice was significantly extended.

Furthermore, human iPSC-derived NSCs have been demonstrated to be an effective cellular delivery vehicle for the treatment of a metastatic breast cancer in both immunodeficient and immunocompetent mice models (Yang et al. 2012). In this study, a non-invasive in vivo imaging technology was used to longitudinally monitor the distribution of DiR-labeled, tail vein-injected iPSC-NSCs at multiple time points. In normal mice without tumor, the tail vein-injected iPS-NSCs were initially distributed predominantly to the lung, and 1 day later moved out from the lung toward many other organs, including liver, spleen, and femur. The DiR signals in these regions remained stable for 14 days in immunodeficient NSG mice, but decreased significantly at days 7 and 14 in immunocompetent BALB/c mice. While in 4 T1 breast cancer bearing mice, 23% and 16%~32% of tail vein injected DiR-labeled iPS-NSCs accumulated at the tumor inoculation site within 24 h and remained for 14 days in NSG mice and in BALB/c mice, respectively. The distribution differences observed between normal versus tumor-bearing BALB/c mice suggest the tumor growth and tumor homing of the NSCs counteract immune rejection of human cell xenografts. In addition, baculoviral vector was also used in loading HSVtk suicide gene into iPSC-NSCs. Systemic injection of HSVtk-expressing iPS-NSCs coupled with GCV treatment not only

inhibited the orthotopic 4 T1 breast cancer growth, but also attenuated the cancer metastasis in various organs, thus leading to prolonged survival of tumor-bearing animals. The significance of this study lies on the following aspects. Firstly, it demonstrated the possibility of using iPSC-derived NSCs for metastatic breast cancer gene therapy through systemic administration. Secondly, it expanded the study scope to immunocompetent animal models and indicated that the nonautologous tumor-targeting iPSC-NSCs can play therapeutic roles to induce tumor regression before transplant rejection. This would further benefit the clinical studies and applications of nonautologous NSCs.

Therapeutic Approaches Using Neural Stem Cells in Cancer Gene Therapy

Two therapeutic strategies are commonly applied in NSC-based anticancer gene therapy in research and clinical medicine. The first molecular approach is based on suicide gene therapy. In this approach, NSCs that genetic engineered to express suicide genes migrate to tumor sites where the expression of suicide enzyme converts non-toxic prodrugs into toxic metabolites, thus leading to cell death in tumor sites. The second immunologic approach involves the gene transfer of immunomodulatory cytokines in NSCs.

Molecular Approach: Suicide Gene Therapy

The keys of this molecular approach lie in two aspects: Firstly, the choice of suicide enzyme/prodrug systems; Secondly, the effective and safe way for introducing specific suicide gene into NSCs. Several suicide enzyme/prodrug systems have been reported in research and clinical studies, including cytosine deaminase (CD)/5-fluorocytosine (5-FC), carboxylesterase (CE)/irinotecan (CPT-11), and herpes simplex virus thymidine kinase (HSVtk)/ganciclovir (GCV) (Aboody et al. 2000; Kim et al. 2010). In this review, we discuss several systems and focus on HSVtk/GCV system using baculovirus gene delivery vector in more detail.

HSVtk in combination with prodrug GCV has been widely used in gene therapy based experimental trials for cancer treatment, and also in clinical trials of brain tumors. The action mechanism of the HSVtk/GCV system is illustrated in Fig. 5.2. In HSVtk expressing cells, GCV is selectively phosphorylated to the monophosphate by thymidine kinase. Further phosphorylation can be accomplished by cellular enzymes. GCV triphosphate competes with the endogenous dGTP for incorporation into DNA, leading to cell death. In surrounding non-HSVtk expressing cells, GCV at the mono-, di- or triphosphate level can be transferred directly via Gap junction intercellular channels (GJIC), resulting in its incorporation into DNA with subsequent cytotoxicity. In our study (Zhao et al. 2012b), a small portion (25%) of the injected NSCs that migrated into the U87 glioma side appears already enough to display therapeutic effects. The rest of the injected cells will be eliminated by the HSVtk/GCV treatment. This is considered to be an advantage of using a suicide gene therapy regimen that removes stem cell vehicles to minimize undesired immune reactions associated with the persistent presence of allogeneic cells.

In the cytosine deaminase (CD)/5-fluorocytosine (5-FC) suicide gene system, *E. coli* CD converts the non-toxic 5-FC to a pyrimidine analogue 5-fluorouracil (5-FU), and 5-FU is then processed further by mammalian enzymes to cytotoxic metabolites that inhibit RNA processing and DNA synthesis in the cells. This strategy has been used in the treatment of hepatic metastasis of colon cancer, and was also tested in intracranial glioma, melanoma brain metastasis and breast cancer. After implantation of CD-expressing NSCs followed by systemic 5-FC administration, a significant reduction up to 70–80% in tumor burden was observed in mice models bearing invasive glioma or melanoma (Aboody et al. 2000). Currently, a pilot clinical trial study for human recurrent high grade gliomas using CD-expressing NSCs together with oral administration of 5-FC is carried out in City of Hope Medical Center, Duarte, CA (http://clinicaltrials.gov/ct2/show/nct01172964). Whether the observations

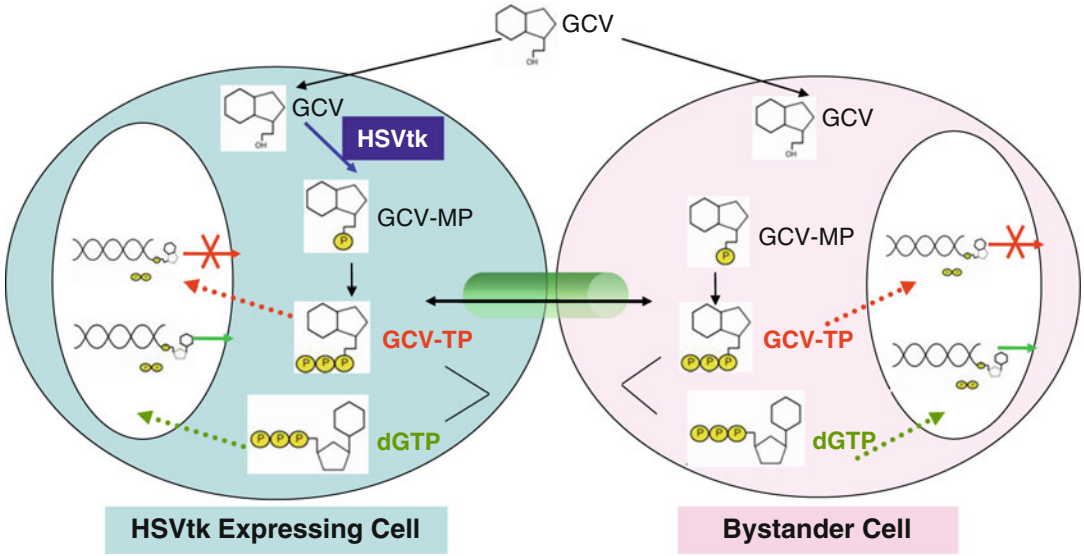

Fig. 5.2 Bystander effects of the HSVtk/ganciclovir (*GCV*) system for cancer gene therapy. In HSVtk-expressing cells, GCV is phosphorylated by HSVtk and competes with the endogenous dGTP for incorporation into DNA, leading to cell death. Phosphorylated GCV can be transferred via gap junction intercellular channels (*GJIC*) to nearby cells, resulting in subsequent bystander cytotoxic effects

that engineered NSCs targeting and treating of animal brain tumors will also hold true for human glioma is under clinical investigation.

Another enzyme/prodrug therapy strategy for cancer treatment is carboxylesterase (CE)/irinotecan (CPT-11). In this system, CPT-11 is hydrolysed by CE to 7-ethyl-10-hydroxy-camptothecin (SN-38), an inhibitor of topoisomerase I. CPT-11 is used to treat a number of cancers, in particular colorectal cancer, and is also tested in ovarian cancer (Kim et al. 2010), and metastatic neuroblastoma. Intravenous administration of CPT-11 and a rabbit CE-expressing NSCs increased the antitumor effect of CPT-11 and significantly prolonged the survival of mice bearing disseminated neuroblastoma (Aboody et al. 2000).

To successfully exploit NSCs as delivery vehicles for cancer therapy, it is crucial that these cells can be genetically modified in a safe and effective way to carry therapeutic genes over a rational period of time. Baculovirus derived from *Autographa californica multiple nucleopolyhedrovirus* is recently emerging as a potential safe class of gene delivery vectors since it can not replicate in mammalian cells and do not integrate into transcriptionally active regions of host genomes (Wang and Balasundaram 2010). Thus bypass the risks of viral replication in host mammalian cells, risks borne by conventionally used animal viruses such as adenovirus, retrovirus and adeno-associated virus. And the non-integrating feature poses much less risk of insertional mutagenesis, oncogene activation and cellular transformation. Other advantages associated with baculoviral system reside in areas such as a large cloning capacity to accommodate a DNA insert up to 38 kb, low cytotoxicity to transduced cells, rapid construction of recombinant baculovirus, and easy production of high-titer viruses. The non-replicating and non-integrating features of baculovirus, together with the absence of pre-existing immunity to baculoviruses in humans and the relative low immunogenicity of baculovirus virions, make these vectors far less harmful in mammalian genome and become an ideal delivery tool for clinical applications.

Baculoviral vectors have been proved to be capable of infecting both dividing and non-dividing cells, including human ESCs (Zeng et al. 2007), mesenchymal stem cells (Bak et al. 2010), human ESCs-derived neurons (Zeng et al. 2009), and human ESCs- and iPSCs-derived

NSCs (Yang et al. 2012; Zhao et al. 2012b), to provide transient expression of transgenes suitable for cancer therapy. Our study has shown that baculoviral vectors with a cytomegalovirus/woodchuck hepatitis virus post-translational regulatory element expression cassette can maintain transgene expression in human NSCs for at least 3 weeks (Zhao et al. 2012b). The transient gene expression mediated by baculovirus of non-integrating nature limits its efficacy in the studies that require long-term transgene expression. This limitation is not unique to baculovirus, but is also faced by adenovirus. Further genetic engineering of baculoviral expression cassette by including other elements, such as the *oriP* sequence and the *EBNA1* gene of Epstein-Barr virus (Lo et al. 2009), is a possible way to prolong transgene expression without chromosome integration.

Immunologic Approach: Immunomodulatory Gene Therapy

Immune-mediated cancer rejection is relatively rare but well-studied autoimmune response. Enhancing T cell-mediated immune responsiveness against cancer is a promising way in tumor-specific immunotherapeutic intervention. Genetic engineered NSCs expressing immunomodulatory genes, such as interleukin 4 (IL-4), IL-12, IL-23, interferon-beta (IFN-β) and tumor necrosis factor-related apoptosis-inducing ligand (TRAIL), have been proved to be effective in cancer immunotherapy. For examples, in C6 gliomas and syngeneic GL261 mouse gliomas, IL-4 expressing NSCs resulted in long-term survival of tumor-bearing mice (Benedetti et al. 2000). Similarly, intratumoral therapy with IL-12-secreting NSCs prolonged survival of glioma-bearing mice, enhanced T-cell infiltration in tumor microsatellites and produced long-term antitumor immunity (Ehtesham et al. 2002b). Bone marrow-derived NSCs that expressing another cytokine IL-23 can effectively inhibit intracranial glioma growth and induce immune-mediated antitumor effects through the activation of immune cells such as CD8(+) and CD4(+) T cells and natural killer cells (Yuan et al. 2006). In addition, a tumor growth delay of disseminated neuroblastoma was observed using IFN-β expressing NSCs, while glioma regression and significant prolonged survival were manifest when using genetically engineered NSCs encoding IFN-β together with cytosine deaminase following the application of 5-FC (Ito et al. 2010). Furthermore, when NSCs were modified to express TRAIL, apoptosis in glioma and tumor satellites was induced and associated with a reduction in tumor progression (Ehtesham et al. 2002a).

Besides above mentioned immune-stimulating cytokines, recombinant monoclonal antibodies also play important roles in NSC-based cancer immunotherapy. For example, Frank and colleagues genetically modified NSC to express Herceptin (trastuzumab), a monoclonal antibody to treat HER2-overexpressing breast cancer. These immunoglobulin-secreting NSC effectively inhibited the proliferation of breast cancer cells in vitro, and human breast cancer mice xenografts in vivo (Frank et al. 2009). The tumor-tropic NSCs could serve as an ideal cellular delivery vehicle for targeting antibodies to tumors, meanwhile the NSCs delivery platform efficiently overcome the obstacles that antibody-mediated therapy faced, such as the large molecular size of antibodies restricts their penetration into solid tumors and precludes their crossing of the blood–brain-barrier into the central nervous system.

Other cancer immunotherapy strategies, such as in vitro manipulation of dendritic cells to initiate and regulate antitumor T cell immunity (Zeng et al. 2012), and direct vaccination of cancer cell specific antigens to induce immune-mediated cancer rejection (Hanke et al. 2002), are attractive approaches currently being investigated. These promising immunologic approaches offer great potential for tumor-specific cytotoxicity.

Current Limitations and Further Improvements

In addition to their contribution in modelling and treatment of neurodegenerative disorders, NSCs have been proven to be valuable in the field of

cancer gene therapy. However, limitations and risks that associated with the use of NSCs may halt the on-going clinical translation.

Tumorigenesis Concern

Risk of tumorigenesis is the primary safety concern not unique for proto-oncogene c-myc immotalized NSC lines, but also for iPSC-derived NSCs. Classic methods using retrovirus or lentivirus delivery vectors for the generation of iPSCs also raise safety concern regarding insertional mutagenesis. This hurdle has been partially resolved by virus-free and integration-free strategies using episomal-, mRNA- or protein-based vectors (Kim et al. 2009; Yu et al. 2009; Warren et al. 2010). Although the reprogramming efficiency using these alternative methods is relative low and variable, the derived iPSCs are free of exogenous DNA, thus eliminating the possibility for insertional mutagenesis. The iPSC field has also become increasingly aware that iPSCs are not functionally true equivalents to ESCs as initially assumed, and the subtle differences between these types of cells might affect their utility for disease modelling and therapeutic applications (Yamanaka 2012).

The potential links between induced pluripotency and induced tumorigenicity raise important safety concerns for future clinical application of iPSCs. The genomic mutations are inevitable during reprogramming and later expansion processes no matter what reprogramming approaches were used. Even if the differentiated derivatives of iPSCs, such as iPSC-NSCs, instead of iPSCs themselves will be used for transplants, the risks of tumorigenesis cannot be excluded as the reprogramming and oncogenic transformations are related processes. Therefore, extensive studies and genetic screening for iPSCs and iPSCs-differentiated cells should be further carried out to ensure safety before their clinical applications.

Our in vitro and in vivo studies demonstrated that the ESC- or iPSC-derived NSCs are similar to natural NSCs in expressing specific markers and maintaining multipotent differentiation capacities (Yang et al. 2012; Zhao et al. 2012b). Although tumor formation using human ESC-NSCs or iPSC-NSCs has not been observed in our animal studies or reported in publications so far, the risk of tumorigenesis needs further long-term investigation. Nevertheless, human ESC and iPSC technologies provide opportunity for endless supply of NSC populations with homogeneous molecular and genetic properties. The derived NSCs loaded with suicide genes are destined to die following the administration of prodrugs.

Immunogenicity Concern

Studies have shown that the differentiated progeny of ESCs could elicit an enhanced immune response, implying that clinical transplantation of allogeneic ESCs or ESC derivatives might require immunosuppressive therapy. The breakthrough discovery of iPSCs technology has fuelled great hope and enthusiasm in stem cell biology field that iPSCs could act as renewable sources for transplantation purposes in treating a wide variety of human diseases. Patient specific autologous iPSCs have been initially assumed and touted as a solution to the problem of immune rejection in cell replacement therapy. This perceived notion has been challenged by a recent study demonstrating that syngeneic iPSCs, which trigger T-cell-dependent immune response in syngeneic recipients, are more immunogenic than their ESCs counterparts (Zhao et al. 2011). Two reprogramming methods were used in this study, and it was found that iPSCs generated using episomal approach are less prone to be immune rejected than those derived with retroviral vectors, suggesting that further optimization of reprogramming methodology is necessitate for mitigating iPSCs-caused immune response. While precise mechanisms for syngeneic iPSCs-elicited immunogenicity are still unclear, more evidences indicate that genetic and epigenetic factors might contribute to this phenomenon.

On the other hand, NSCs are known to display immunosuppressive properties and have been used to ameliorate experimental autoimmune encephalomyelitis (Einstein et al. 2007). Whether it also holds true for human NSCs derived from

ESCs and/or iPSCs, or on the contrary, whether these derived NSCs display enhanced immunogenicity upon direct differentiation are largely unknown and worth further investigations since only differentiated progenies from ESCs and/or iPSCs will be applied in clinical transplantation.

Timing and Cost Concern

Current technologies take several months or more for iPSC generation, expansion, validation, even before the differentiation process begins. For most acute and/or life threatening illnesses and injuries, such a slow process will not be suitable and exclude the use of iPSCs in a patient specific manner clinically. Furthermore, the cost for patient-specific therapies will be exorbitantly high, the vast majority of people will not be able to afford. Hence, for future clinical translation purposes, it may be more practical to establish human iPSC banks consisting of a wide range of iPSC lines that derived from diverse human lymphocyte antigen (HLA) haplotypes (Nakatsuji 2010). These banked iPSCs could then be alternatively selected to produce HLA-compatible tumor-targeting NSCs with reduced likelihood of immune rejection for allogeneic rather than autologous transplantation. Furthermore, significant efforts are required to develop Good Manufacturing Practice (GMP)-compliant conditions and protocols prior to large-scale mass manufacture of uniform cellular products, including iPSC- and iPSC-NSCs. Instead of collecting and expanding primary cells from individual patients, large-scale manufacture of iPSCs and their derivatives has many advantages. It will provide sufficient cells for repeated patient treatments, eliminate variability in the quality of cellular products, facilitate reliable comparative analysis of clinical outcomes, and increase cost effectiveness by reducing the laboriousness and simplifying the logistics of cell culture operations. Theoretically, these batch-prepared, off-the-shelf iPSC-NSCs can also be cryopreserved in a ready-to-go commercial products format. The potential clinical translation would be possible only when these cellular products are available freely and affordable to the public.

In conclusion, NSC-based cancer gene therapy is moving forward in spite of current challenges. Obviously, human NSCs derived from ESCs and/or iPSCs hold great potential for cancer treatment, yet much more issues needs to be resolved to overcome the technical hurdles before translating this promising research from laboratory study into clinical therapy.

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Part II

Cancer Stem Cells

6 The Role of Cancer Stem (–Like) Cells and Epithelial-to-Mesenchymal Transition in Spreading Head and Neck Squamous Cell Carcinoma

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Contents

Abstract

Head and neck squamous cell carcinoma (HNSCC) is the sixth commonest cancer worldwide. Recurrences or metastases, thought to involve cancer stem(−like) cells (CSCs), are common and survival rates remain low. Recently, multiple studies have implicated the link between CSCs and Epithelial-to-mesenchymal transition (EMT) during tumorigenesis. This review aims to summarize some of the key events that are involved in EMT, discusses their relevance in HNSCC progression and metastasis and explores the possibility of targeting EMT as a novel therapy in HNSCC.

Introduction

A majority of head and neck squamous cell carcinomas (HNSCC) treated with radio- and chemotherapy as first-line-therapy or in adjuvant or neo-adjuvant settings initially respond well. However, depending on the tumor stage, more than half of the patients suffer from local or distant relapse within the first 5 years after diagnosis. The development of metastases and recurrences with increased resistance to radio- or chemotherapy remains the main reason for mortality. To form metastases, tumor cells have to undergo changes in order to be able to leave the primary tumor environment and migrate to distant sites. Responsible for these morphological changes is the physiological process of

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epithelial-to-mesenchymal transition (EMT) which allows cells to change their shape and polarity and to become motile. In recent years, considerable attention was paid to the existence of a subpopulation of cancer cells with stem cell-like properties, so-called cancer stem(–like) cells (CSCs). They have been isolated from the majority of cancer types and are characterized by increased resistance to therapy and the potential to drive neoplastic growth in human cancers. It is now becoming increasingly apparent that links exist between the CSC concept and EMT. In addition, microRNAs regulate many important cellular functions including both EMT and CSCs.

The Role of EMT in Cancer Progression

During normal embryonic development and wound healing, EMT allows epithelial cells to undergo morphological changes, leading to the breakdown of cell–cell and cell–extracellular matrix connections. The cells are then free to migrate to other locations in the body to reconstruct and repair damaged tissue. Apart from its normal physiological involvement, EMT is also important in the migration of cancer cells (metastasis) and has been linked to progression in many types of cancer. While the correlation of EMT with progression in HNSCC currently still lacks prognostic significance, several links are emerging. In pharyngeal and hypopharyngeal carcinomas, the overexpression of EMT-associated transcription factors such as Twist and/or Snail was observed (Jouppila-Matto et al. 2011). In addition, overexpression of vimentin and suppression of E-cadherin, both characteristics of EMT, were correlated with the invasion of HNSCC (reviewed in Chen et al. 2012).

Overexpression of epidermal growth factor receptor (EGFR) is present in 90% of HNSCC and associated with poor prognosis. Involved in many oncogenic pathways, EGFR signaling can lead to increased cell motility and invasion via the induction of EMT: EGFR activation through EGF resulted in EMT and enhanced cell migration/invasion via increased MMP-9 production and MMP-9-mediated degradation of E-cadherin, involving ERK1/2 (Zuo et al. 2011). In the presence of hyaluronan (HA), EGFR can induce a migratory cell phenotype through interaction with the HA receptor CD44, often present in head and neck cancer and a putative CSC marker, also involving ERK1/2 (Wang and Bourguignon 2006). Interestingly, a recent publication suggests that the $CD44^{high}/EGFR^{low}$ population displays an even more increased EMT phenotype and resistance to radiation and several drugs, than the $CD44^{high}/EGFR^{high}$ population (La Fleur et al. 2012).

Another important inducer of EMT is the transforming growth factor (TGF)-β. It is well-known for its role in angiogenesis and has been linked to metastasis and progression in various types of cancer, including HNSCC. The closely related family members of bone morphogenetic proteins (BMPs) have also been associated with metastasis and both TGF-β and BMP can mediate EMT via Smad signaling. For example, stimulation of epithelial HNSCC tumor cells with human BMP-4 protein lead to an increase of mesenchymal characteristics, and a significantly higher expression of BMP-4 and p-Smad1 protein was found in HNSCC patients with lymph node metastases compared to those without. In turn, knockdown of Smad1 suppressed BMP-4-induced EMT and reduced invasion and migration of HNSCC tumor cell lines in vitro (Xu et al. 2011).

Genes involved in EMT and nuclear factor-κB (NF-κB) signaling were the most prominent molecular characteristics of high-risk tumors when comparing 75 genes predictive of disease with low-risk groups of HNSCC using DNA microarray analysis (Chung et al. 2006). In HNSCC, EMT was induced via overexpression of the tropomyosin-related kinase B (TrkB), resulting in up-regulation of Twist and down-regulation of E-cadherin (Kupferman et al. 2010), or via overexpression of the DNA double-strand break repair protein Nijmegen breakage syndrome (NBS) 1, resulting in up-regulation of Snail. Additionally, the T-box transcription

factor (Tbx) 3 appears to play a role and was strongly up-regulated in HNSCC cells displaying an EMT-phenotype, while Tbx3 depletion lead to a decrease in cell invasion (Humtsoe et al. 2012). Several other proteins have also been implicated in EMT and HNSCC progression and have been reviewed in Scanlon et al. (2012). Together, these data show the importance of EMT in the progression of human cancers including HNSCC, and the participation of multiple molecular pathways.

Features of EMT and Cancer Stem (–Like) Cells

In the past decade, a subpopulation of cells with self-renewing capacities, so-called tumor-initiating or cancer stem(–like) cells (CSCs), has been identified in many types of solid tumors. They have been reported to be more resistant to chemo- and radiotherapy and may be responsible for tumor maintenance, recurrence and metastasis. Various CSC models have been proposed and include the hierarchically fixed stem cell model, the stochastic and the evolutionary model. The hierarchy model is similar to the hematopoietic system and cell subpopulations with functionally different properties such as self-renewal and asymmetric division are responsible for tumor heterogeneity. According to this model, only one cell type, the CSC, can initiate tumors. The stochastic model on the other hand assumes that all cells within a tumor have similar properties and that heterogeneity or a CSC-phenotype is determined by intrinsic (e.g. activated signaling pathways) and extrinsic factors (e.g. hypoxia, stress, drug treatment). Because of the importance of the factors involved, this model indicates that these states are, at least in part, reversible. The evolutionary model proposes a dominant sub-clone with survival advantages compared to the other cells/clones. Depending on intrinsic properties and extrinsic selection pressure, one or the other clone survives and becomes dominant. More recently, an inducible CSC type that is mediated by microenvironmental factors such as hypoxia/anoxia or EMT has been suggested. However, these CSC models are not mutually exclusive and combinations may exist.

It is well known that hypoxia mediates several signaling pathways involved in cell proliferation, invasion, angiogenesis, metastasis, and drug resistance, mostly via the hypoxia-inducible factor (HIF)-1 complex consisting of the hypoxia-responsive HIF-1α and HIF-1β. It may therefore not come as a surprise that both hypoxia and HIF-signaling also play an important role in the regulation and maintenance of the CSC as well as the EMT phenotype (Bao et al. 2012). Moreover, the induction of EMT not only promotes tumor cell invasion and metastasis, but also contributes to drug resistance and enriches for cells with the phenotype and properties of CSCs. Accumulating evidence in the past few years suggests that tumor cells capable of undergoing EMT could be CSCs and that these cells may be responsible for drug resistance as well as metastasis. It is therefore important to gain a better understanding of the underlying molecular pathways of these cells to create novel therapeutic opportunities for many cancers that are currently resistant to treatment or that relapse.

Supporting this information, we previously showed that anchorage-independent cell culture of HNSCC cell lines enriched for ALDH$^+$CSCs that displayed EMT characteristics and showed enhanced colony forming ability and invasiveness (Chen et al. 2011). Further, the presence of HNSCC CSCs with the ability to switch between two phenotypes has been discovered. Migratory CD44$^+$EpCAM^{low} EMT CSCs expressed EMT markers and a mesenchymal phenotype, while proliferative CD44+EpCAM^{high} non-EMT CSCs had epithelial characteristics (Biddle et al. 2011). EMT CSCs thereby required an ALDH$^+$phenotype to metastasize successfully. In this model, HNSCC CSCs can migrate to other locations in the body using the EMT phenotype. The ALDH+cell fraction is then able to revert back into the proliferative non-EMT phenotype, using the reverse process of MET (mesenchymal-to-epithelial transition), and can establish the metastasis. HNSCC cells positive for CD133, a putative HNSCC CSC marker, were shown to express increased levels of zinc finger E-box-binding

homeobox (ZEB) 1 and ZEB2 (Chu et al. 2012), which regulate EMT via repression of E-cadherin. Overexpression of ZEB1/2 in CD133-negative cells resulted in a CSC phenotype while siRNA-mediated knockdown reduced CSC characteristics and drug resistance in vitro and decreased tumor growth and metastasis in vivo. In addition, the authors found that ZEB1/2 expression was increased in lymph node metastasis of HNSCC patients and linked to poor survival (Chu et al. 2012). Up-regulation of CD133 was also reported to induce EMT in HNSCC via increased phosphorylation of Src (Chen et al. 2011). EMT can also be mediated by S100A4. This pro-inflammatory factor is involved in the regulation of many biological functions such as cell motility, metastasis, angiogenesis, differentiation and survival. Overexpression of S100A4 in HNSCC cells resulted in an EMT phenotype, and enhanced stem cell-like properties, migration and invasion, while knockdown of S100A4 reduced their self-renewing and tumorigenic properties in vitro and in vivo (Cordenonsi et al. 2011).

A novel approach of investigating the CSC concept was undertaken by Driessens et al. The current gold standard to evaluate the existence and behavior of putative CSC populations is the transplantation into immuno-compromised mice, often involving subcutaneous injection and the use of matrigel, and therefore do not necessarily represent the actions in physiological conditions. Driessens et al. were the first to show the existence of cancer cells with stem-like cell properties in an unperturbed carcinogen-induced squamous skin cancer model. They hereby labeled individual basal tumor epithelial cells using a tamoxifen(TAM)-inducible genetic lineage tracing system that allowed the tracking of these cells over time. The experiments showed that only a fraction of tumor cell clones had the ability to long-term self-renew, while the others differentiated, and provided evidence that the induced benign papilloma was maintained by a small tumor cell population with CSC properties that gave rise to a progenitor cell population. Moreover, when observing invasive squamous cell carcinoma, the authors found signs of EMT in cells that had left the main clone population. Using clonal analysis, they concluded that the hierarchical mode of tumor growth is mediated by cell fate decisions that mimic a stochastic model (Driessens et al. 2012). Together, the data presented by the groups above and many more, create a strong link between the CSC concept and the EMT phenotype and show their importance in tumor progression.

Regulation of EMT by MicroRNAs

Both EMT and the CSC phenotype can be regulated by small non-coding single strand RNAs, so-called microRNAs (miRNAs, miRs). These 19–25 nucleotide long RNAs control gene expression on the post-transcriptional level by pairing their seed sequences (2–8 nucleotides at the 5' end) to complementary sequences of target messenger RNAs (mRNAs). This interaction can either directly inhibit mRNA translation or increases their degradation. The highly complex regulation allows a single miRNA to regulate several mRNA targets and also multiple miRNA species to control a single mRNA.

MiRNAs affect a wide range of cellular activities, including EMT, drug resistance and CSCs, and have been linked to cancer and progression by acting as tumor suppressors or oncogenes. The miR-200 family (miR-200a, miR-200b, miR-200c, miR-141, miR-429) and miR-205 have been linked to EMT via regulation of ZEB1 and ZEB2 expression, both of which are transcriptional repressors of E-cadherin (Park et al. 2008). In head and neck and oral cancers, suppressed levels of miR-200 family were reported, allowing the induction of EMT via up-regulation of ZEB1 and ZEB2 and consecutive reduction of E-cadherin (Babu et al. 2011). Reduced levels of the family member miR-200c were also found in HNSCC lymph node metastases and in ALDH+/CD44+HNSCC CSCs, accompanied by elevated Bmi-1 expression (Lo et al. 2011). Overexpression of miR-200c inhibited the metastatic potential of ALDH+/CD44+HNSCC CSCs via modulation of Bmi-1 expression that led to a reduction of

ZEB1 and a consecutive increase of E-cadherin expression. The restoration of miR-200c could therefore yield therapeutic potential in targeting HNSCC and CSCs (Lo et al. 2011).

Several additional miRNAs have also been linked to cancer progression. For head and neck cancer, the up-regulation of miR-21, miR-31 and miR-155 have been reported and the down-regulation of miR-26b, miR-34a, miR-107, miR-133b, miR-139 and miR-138 (see Chen et al. 2012). Recently, a mechanistical link between microRNAs, EMT/cell motility and the CSC phenotype has emerged in HNSCC. Yang et al. have found that the microRNA let-7i, a tumor suppressor, is directly suppressed by Twist, an important inducer of EMT (Yang et al. 2012). Twist thereby induces and cooperates with Bmi-1 to suppress let-7i. Suppression of let-7i leads to the induction of stem-like cell properties as well as the upregulation of NEDD9 and DOCK3. This signaling cascade is followed by activation of RAC1 which allows the mesenchymal-type cell motility in a three-dimensional environment, which is currently regarded as a more physiological condition than monolayers/2D (Vinci et al. 2012). Moreover, the authors were able to link the activation of this Twist – let-7i – NEDD9 signaling axis to increased tumor invasion and reduced survival in HNSCC patients (Yang et al. 2012).

Targeting EMT for Cancer Treatment

Drug resistance and treatment-escaping cells have been a major problem in the fight against cancer and many chemotherapeutic drugs have been affected. Resistance to therapy was thereby often linked to an EMT phenotype (see review by Wang et al. 2010). Links have emerged to associate EMT with CSCs, and both phenotypes with enhanced metastatic activity. It may therefore be plausible to reduce tumor progression and drug resistance by targeting the EMT/CSC phenotype. There are many ways of how this could be addressed, from targeting transcription factors or cytokines involved in the induction of EMT to reversing or blocking the EMT/CSC phenotype via regulating microRNAs, and attempts have been made to attend to this.

The EGFR tyrosine kinase inhibitors (TKi) gefitinib and erlotinib are relevant to HNSCC due to the overexpression of EGFR, but in particular gefitinib has so far been disappointing as a single agent in head and neck cancer therapy. While drug resistance is a problem, both drugs and the MEK inhibitor UO126 were able to inhibit tumor cell invasion in HNSCC cell lines (Sun et al. 2011). Interestingly, HNSCC cells undergoing EMT have also been reported to show increased resistance to gefitinib which could be overcome using the HDAC inhibitor vorinostat. Vorinostat thereby induced E-cadherin and ErbB3 expression, while vimentin, EGFR and ErbB2 were down-regulated (Bruzzese et al. 2011), leading to a more epithelial cell type. Likewise, the HDAC inhibitor trichostatin A (TSA) was able to prevent TGF-β-induced EMT in renal epithelial cells and may also prove effective in HNSCC. The combination of an HDAC inhibitor, such as vorinostat or trichostatin, and gefitinib could therefore be a promising therapeutic strategy in the treatment of head and neck cancers.

Additionally, some natural products were able to reduce migratory properties of cancer cells and CSCs. Grape seed proanthocyanidins (GSPs) reduced tumor cell invasion through potential reversal of EMT via inhibition of EGFR and NF-κB signaling (Sun et al. 2011), both of which play important roles in cancer progression, metastasis and EMT. Treatment with GSPs led to diminished activation of NF-κB/p65 and ERK1/2 (p-ERK1/2), enhanced expression of epithelial markers (E-cadherin, cytokeratins, desmoglein-2) and a decrease of mesenchymal markers (vimentin, Slug, fibronectin, N-cadherin). The polyphenol Epigallocathechin gallate was also recently reported to reduce cancer stem-like cell characteristics and invasive properties of nasopharyngeal cancer cell lines via the reduction of vimentin/inhibition of EMT (Lin et al. 2012).

Targeting transcription factors (TFs) involved in EMT, such as ZEB1, Twist, Slug or the cytokine TGF-β could be considered another therapeutic approach. However, while inhibition may

be possible, the actions of TFs are often context-dependent. Systemic inhibition may lead to unforeseeable and potentially dangerous side effects. Prior to targeting TFs, one needs to fully understand their role in various cell types and biological processes. A practical problem may be the localization of transcription factors in the cytoplasm or nucleus, which can influence their accessibility. It may therefore be easier to target molecules on the cell surface, such as the membrane receptors that transmit signals to activate the EMT program.

An additional therapeutic strategy is the reversal of EMT which also has the potential to affect a variety of CSC phenotypes. The regulation of many processes involved in the EMT/CSC phenotype by microRNAs makes them a particularly interesting strategy for therapy. A recent publication showed that miRNA expression can be altered through the use of drugs. The authors showed that the anti-inflammatory drug sulindac sulfide not only decreased cell proliferation but also inhibited invasion of human cancer cell lines via the suppression of NF-κB-mediated transcription of miR-10b, miR-17, miR-21 and miR-9 (Li et al. 2012). Kong et al. were able to revert EMT to MET in prostate cancer cells by forced re-expression of miR-200, which also significantly inhibited the self-renewal capacity (Kong et al. 2009). Although efforts have been made to develop therapies that target miRNAs (reviewed by Xia and Hui 2012), several limitations still remain. As described previously, miRNAs act sequence-specific, often influencing a number of genes. Additionally, gene silencing sometimes only requires partial complementary binding of the miRNA, resulting in an even greater number of genes affected. The use of miRNAs as therapeutic tool therefore needs to be carefully evaluated and could result in off-target effects with potentially uncontrollable and even fatal outcome. While advances have been made using tissue culture systems, miRNA administration in patients is still challenging due to a lack of appropriate delivery systems. However, one option to deliver miRNA therapy could be nanoparticles. They have previously been used to deliver drugs to in vivo tumor sites, and more recently, cationic lipid nanoparticles successfully delivered pre-miR-107 (NP/pre-miR-107) in vivo, leading to reduced tumor growth and a survival advantage (Piao et al. 2012). This method may also yield potential for the delivery of other HNSCC-relevant miRNAs.

As well as providing further insights into the miRNAs strategy, studies of the EGFR pathway were extended to elucidate the invasive mechanisms in HNSCC. In HNSCC cell lines, the EGFR tyrosine kinase inhibitors (TKi) gefitinib and erlotinib, and the MEK inhibitor UO126 were able to inhibit tumor cell invasion. Additionally, the grape seed proanthocyanidins (GSPs) reduced tumor cell invasion through potential reversal of EMT via inhibition of EGFR and NF-κB signaling, both of which play important roles in cancer progression, metastasis and EMT. Treatment with GSPs thereby resulted in reduced expression of EGFR and NF-κB-responsive proteins, accompanied by diminished activation of NF-κB/p65 and ERK1/2 (p-ERK1/2). In turn, this led to enhanced expression of epithelial markers (E-cadherin, cytokeratins, desmoglein-2) and also a decrease of mesenchymal markers (vimentin, Slug, fibronectin, N-cadherin), which may offer a putative addition to already existing therapies. Similarly, the HDAC inhibitor vorinostat was able to induce E-cadherin and ErbB3 expression in HNSCC cells, while vimentin, EGFR and ErbB2 were down-regulated. This is particularly interesting since HNSCC cells undergoing EMT showed resistance to gefitinib which could be overcome using vorinostat, making a combination of vorinostat and gefitinib a promising therapeutic strategy. Like wise, the HDAC inhibitor trichostatin A (TSA) was able to prevent TGF-β-induced EMT in renal epithelial cells and may also prove effective in HNSCC (reviewed in Chen et al. 2012).

While the ideas presented above sound promising, testing in a clinical setting can also be disappointing. Various attempts have previously been made to target the motility of cancer cells. Overexpression of some matrix metalloproteinases (MMPs) has been associated with the EMT process for example. In HNSCC, particularly MMP-9 was found to be located at the invasive front

containing putative CD44+ and ALDH1+CSCs (O-Charoenrat et al. 2001 and Sterz et al. 2010). Unfortunately, most MMP inhibitors (MMPis) exhibited poor performance in clinical trials. Some explanations for their limited success have been outlined by Gialeli et al. and include toxicity (e.g. musculoskeletal syndrome), and inhibition of MMPs at incorrect times (in particular broad-spectrum MMPis) which is difficult to calculate due to their various roles in the multiple steps of cancer progression (Gialeli et al. 2011). However, the selection of patients into groups according to cancer type and stage may lead to better predictability of clinical responses, and this may also be true for other EMT/motility-targeting drugs.

Conclusion

EMT is a complex process, involving multiple transcription factors and signaling pathways, and is associated with cancer progression, drug resistance, and cancer stem-like cells, all of which are clinically important factors. Therefore, inhibition or reversal of EMT appears to be an attractive therapeutic strategy to reduce cancer cell invasion and metastases in HNSCC and other human cancers. Although great progress has been made in the past years, a number of important questions remain to be answered before this promising theory can be translated into a clinical application. Significant challenges are the complexity of the signaling pathways involved in the induction and regulation of EMT and the effect of putative EMT-targeting drugs to normal cellular processes. A better understanding of the role of EMT in HNSCC progression and metastases is urgently needed and could help clarify which tumor types and stages should be treated and at what time, as well as the exact signaling pathways or transcription factors, to most effectively block the initiation of EMT. Together with the identification of additional regulators of EMT, this will hopefully lead to a successful application of EMT-targeting drugs in the clinic and may help to reduce HNSCC-associated morbidity and mortality.

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Glioma Propagating Cells Show Enhanced Chemoresistance and Radioresistance (an Update)

7

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Contents

Abstract

Glioblastomas (GBMs) are the most aggressive primary brain tumors and are heterogeneous at the cellular level. Current therapeutic means are limited to a largely palliative role, with modest improvement in clinical response. The cellular and molecular heterogeneity of GBMs indicates that individual mechanisms are likely to exert different levels of influences on patient treatment response. Glioma-propagating cells (GPCs) which are purportedly enriched in tumor-initiating and sustaining potential are important because they mirror primary tumors at the phenotypic and molecular level. Moreover, they display long-term self-renewal capability, possibly accounting for the frequently observed tumor recurrence and re-growth. Hence, elucidation of GPC targets that confer therapeutic resistance is essential for effective treatment outcome. Here, we highlight several key intrinsic mechanisms that contribute to the perpetuation of GPCs, including the multi-drug resistant phenotype, DNA damage repair as well as key self-renewal signaling cascades. Another integral factor is the paracrine cues from the perivascular/hypoxic milieu. Targeting these multifaceted pathways might therefore sensitize GPCs to chemotherapeutic agents and lead to long-lasting therapies. In addition, we discuss how transcriptome analysis of GPCs derived from histologically similar yet molecularly distinct primary tumors can stratify patient subtypes, each bearing

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their own unique genetic and clinical profile. These findings highlight the clinical contribution of GPCs to disease progression and survival outcome. Identification of patient subtypes may account for the heterogeneity in treatment response and thus accelerate the development of personalized therapy that can induce sustained clinical outcome. The road ahead will likely be filled with obstacles but these emerging therapeutic paradigms promise new opportunities to patient cohorts lacking in effective treatment options.

Introduction

Gliomas represent the most prevalent of primary adult malignant brain tumors, with GBM exhibiting the worst prognosis and mean survival period of 15 months post-diagnosis. The highly recurrent, infiltrative and heterogeneous nature of the disease has prompted much research into the origin of gliomas to develop more effective therapeutic targeting. Recent transgenic mouse models utilizing lineage-tracing methodologies have implicated neural stem cells and oligodendrocyte precursor cells as the tumor-initiating cells. These cells display long-term self-renewal and are able to differentiate into all neural lineages, thus recapitulating the eventual tumor phenotype. Although transgenic models allow us to study a limited number of mutations in the tumor-initiating process; moreover such models contain an intact host immune response that more accurately reflects the microenvironmental niche; they often do not reform the phenotypic and molecular heterogeneity of patient tumors. Consequently, complementary approaches encompassing patient tumor-derived GPCs are needed. Such GPCs have been shown to mirror the phenotype and molecular fingerprint of their primary tumors. Furthermore, recent evidence demonstrates that their transcriptomic pathway networks contribute to GBM disease progression and patient survival outcome. These findings emphasize the importance of GPCs as clinically relevant in vitro culture systems. Although their use precludes the identification of the true cell-of-origin; moreover no definitive marker can be ascribed to tumor-initiating capability, nevertheless, their stem-like properties are important to understand and target for an effective and long-lasting cure.

Our previous work highlighted the molecular heterogeneity of GPCs derived from histologically similar primary tumors. These findings may account for the frequently observed patient heterogeneity to treatment response. In addition, we showed that pathway predictions based on GPC transcriptomic data matched actual cellular response to small molecules targeting various oncologic pathways. This is an important conceptual advance as traditional drug screening with commercially available cell lines often utilizes short-term viability readouts as endpoints, thus compounds that target slowly-dividing, stem-like cells may inadvertently be de-prioritized. The understanding that gliomas are heterogeneous, and that an often minor, slow-growing cellular subset is responsible for tumor-propagation thus presents a paradigm in therapeutic design. Recent reports have illustrated that multiple, closely-related driver genes are amplified and activated in intermingled, clonal populations of tumor cells, further reinforcing the functionally heterogeneous nature of the disease. In addition, GPCs display enhanced resistance to common chemotherapeutic agents and radiotherapy, and has been shown to rely on surrounding vascular networks to maintain its own survival (Bao et al. 2006b). Such observations highlight that GPCs are not likely to standalone in the tumorigenic process, and that a composite of cell-autonomous and paracrine signaling is vital to consider in effective targeting.

In this review, we will focus on several primary resistance mechanisms of GPCs, including the multi-drug resistance family of proteins, DNA damage repair pathways, and self-renewal mechanisms typified in normal stem cells, as well as the influence of the microenvironmental niche. Additionally, with the advent of public efforts in glioma molecular and clinical profiling, such as The Cancer Genome Atlas and REMBRANDT, we discuss how bioinformatics analyses can play a role in identifying patient cohorts amenable to pathway inhibition predicted by their GPC content. Our review summarizes

the cellular and molecular heterogeneity of gliomas, and further highlights the limitations of relying on solely morphology-based histological methods in patient diagnosis, consequently impacting on treatment decision.

Role of the Multi-Drug Resistance Family

One of the most important mechanisms that a cancer cell can resist chemotherapeutic agents is the expression of multiple drug resistance transporters to actively efflux a broad range of anti-cancer drugs so as to maintain an intracellular drug concentration below a threshold that is detrimental to the cell. Multiple drug transporters belong to the ATP Binding Cassette (ABC) transporter superfamily of proteins. The functions of the ABC transporters include the efflux of various endogenous organic molecules (e.g. bile acids, cholesterol, glutathione, and peptides) across the cell membrane and in some instances, expelling inorganic drugs that cause toxicity to the cells. Like normal stem cells, tumor stem-like cells have been shown to express high levels of ABC transporters. Three of the ABC transporters that have been commonly implicated in the development of treatment resistance in cancers include: ABCB1 (also known as P-glycoprotein or MDR1), ABCC2 and ABCG2 (also known as Breast Cancer Resistance Protein). ABCB1 is the first member of the ABC family to be discovered and has been shown to be overexpressed in more than 50% of all drug-resistant tumors. ABCB1 transporter is a broad spectrum multidrug efflux pump that consists of 12 transmembrane domains and two ATP-binding domains. It acts as an ATP-dependent transporter to remove cytotoxic drugs and many other structurally unrelated hydrophobic compounds from the lipid bilayer. It has been demonstrated that *ABCB1* expression is higher in the fraction of cancer stem-like cells derived from spheroid cultures of the glioma cell line U87MG, compared to its non-stem-like counterparts (Nakai et al. 2009). In addition, these cancer stem-like cells possess greater drug resistance against a panel of commonly used anti-cancer drugs such as doxorubicin (Dox), etoposide (VP-16), carboplatin, and BCNU, compared to the non-stem-like fraction. The ability of stem-like cells to efflux drugs using the ABC transporters is an important property that allows for the isolation and analysis of stem-like populations. This property enables investigators to isolate stem-like cells termed the "side population" (SP) that excludes the fluorescent dye, Hoechst 33342, by fluorescence-activated cell sorting. SP cells display enhanced expression of both ABCB1 and ABCG2. Although the molecular basis of SP cells in conferring the stemness property remains unclear, the SP phenotype has been largely attributed to the compensatory activity of the ABCG2 transporter as bone marrow cells derived from *ABCB1*-knockout mice retain normal numbers of SP cells. Studies from our laboratory have shown that SP cells possess progenitor cell-like characteristics and tumor-initiating potential in an immune-compromised mouse model. More importantly, the number of SP cells increase with Temozolomide (TMZ) treatment and this corresponds to elevated progenitor-like cells with increased expression of several ABC transporters (Chua et al. 2008). Furthermore, Bleau et al. (2009) showed that the PTEN/PI3K/AKT signaling axis, a central regulatory network in GBM growth, regulates that SP phenotype and the activity of ABCG2 transporters. Importantly, they showed that TMZ treatment increases the proportion of SP in glioma cells, especially in cells with loss of PTEN.

Role of DNA Damage Repair Pathways

Radiotherapy is essential in the treatment regime of glioma patients; yet, in combination with adjuvant chemotherapy, tumor recurrence and regrowth is frequent. Such resistance can be attributed to DNA-repair proficiency, a multitude of deregulated molecular pathways, and more recently, to the particular biologic behaviour of GPCs. Indeed, resistance to TMZ has been ascribed to enhanced expression and activity of O6-methylguanine-DNA-methyltransferase

(MGMT), an enzyme that mediates repair of double-strand DNA (dsDNA) breaks, often induced by chemotherapeutic agents such as TMZ, VP-16 and carboplatin. Epigenetically silenced MGMT in particular patients has allowed significant prolongation of survival following therapy. Interestingly, the gene expression profiles of 80 glioblastoma patients has been studied for associations with resistance to therapy. An expression signature dominated by *HOX* genes (and comprising *CD133*), evocative of a self-renewal signature recently described for leukemia, emerged as a predictor for poor survival in patients treated with concomitant chemoradiotherapy. More importantly, the *HOX* signature was an independent prognostic factor in multivariate analysis, adjusted for the MGMT status. These findings highlight that treatment resistance is due to a combination of not only the MGMT status, but also specific stem cell-related pathways.

Several investigators have shown that GPCs display enhanced resistance to several chemotherapeutic agents and express elevated *MGMT* mRNA expression compared to non-stem cells (Liu et al. 2006). Moreover, recent work by Rich and colleagues elucidated the mechanism governing radioresistance of CD133+GPCs (Bao et al. 2006a). They showed that CD133+GPCs were more efficient at repairing dsDNA breaks through activated checkpoint responses including ataxia telangiectasia (ATM) mutated and the checkpoint kinases, Chk1 and Chk2. Moreover, the checkpoint activation in CD133+ cells was biologically important as pharmacologic targeting of checkpoint function reversed the cancer stem cell radioresistance, suggesting that Chk1/2 inhibitors may warrant additional clinical development, particularly in combination with cytotoxic therapies. Together, the findings support that the development or maintenance of a cancer stem cell may select for an activated checkpoint implicating a form of convergent evolution toward a cellular behavior rather than a single molecular target. Indeed, follow-up studies by this group demonstrated that L1CAM, a neural cell adhesion molecule, marks CD133+GPCs, and is responsible for mediating the DNA damage checkpoint response via the NBS1-ATM axis (Cheng et al. 2011).

Other stem cell-related pathways that may underlie cancer stem cell radioresistance include Wnt/β-catenin, Notch, sonic hedgehog, Pten, EGFR and Bmi-1. Wnts and β-catenin have clearly defined roles in normal stem cell development and cancer, but their role in cancer stem cell biology is less clear. Recent studies have suggested that Wnt and β-catenin signaling may contribute to radioresistance of mammary cancer stem cells. In addition, elevated Notch activity in response to radiation in breast cancer mammospheres is known. Hedgehog and Gli signaling can also be inhibited to attenuate cancer stem cell renewal and tumor growth (Bar et al. 2007). Loss of *PTEN* function alters Chk1 localization and function to initiate genetic instability and confers radiation resistance in glioblastoma cell lines. EGF is a primary growth cytokine used to maintain GPCs in vitro and marks a dose-dependent tumorigenic capacity of the cells in orthotopic mouse models. EGFR mediates radioresistance in glioma models that can be disrupted by EGFR tyrosine kinase inhibitors. Collectively, inhibitors of stem cell pathways may sensitize GPCs to the effects of ionizing radiation, providing a broad therapeutic paradigm.

Role of Self-Renewal Signaling Pathways

Key signaling cascades that are crucial for normal neural stem cells, such as Notch, Hedgehog, Wnt, and the PI3K-Akt axis, have been a focus of increasing interest in cancer therapy as these pathways may underlie GBM therapeutic response and manipulation of these pathways may preferentially deplete GPCs. Even though GBMs share many of these alterations, each individual patient tumor has its unique genetic profile, making it a challenge to develop effective therapeutic interventions. Thus, the Achilles heel of GBMs may not be a single major genetic alteration, but rather a secondary acquired imbalance in the aberrant regulatory signaling networks.

Notch Pathway

The four Notch proteins (Notch 1–4) mediate cellular communication via interaction with their cognate ligands, including Jagged-1, -2, and Delta-like -1, -3, and -4. Activation of the Notch signaling cascade involves proteolytic cleavage by γ-secretase and is critical for the maintenance of stem and progenitor cells in promoting self-renewal and repressing differentiation. Aberrant Notch signaling has been implicated in the pathogenesis of multiple tumors including gliomas, and the overexpression of Notch and its ligands, Delta-like-1 and Jagged-1, is commonly associated with glioma survival and proliferation. The role of Notch signaling in GBMs has been widely characterized and it has been shown that down-regulation of *NOTCH1, Delta-like-1, or Jagged-1* leads to glioma cell apoptosis and translates into a prolonged survival in a mouse orthotopic brain tumor model. Fan et al. (2010) demonstrated that specific Notch targeting of patient-derived GPCs by γ-secretase inhibitor (GSI) attenuated neurosphere-forming ability with marked decrease in the expression of stemness-related markers, increased sensitivity to chemotherapeutic agents in vitro and blocked tumor propagation in vivo, suggesting a potential dependence on Notch signaling in GPCs. Activation of Notch by upstream oncogenic stimuli and microenvironmental cues is essential for the maintenance of GPCs and the facilitation of tumor propagation, suggesting a role of Notch at the centre of key regulatory GPC signaling networks. Another study has shown that overexpression of *NOTCH1* in glioma cells leads to an increase in proliferation and formation of nestin-positive, neurosphere-forming stem cells (Hu et al. 2011). Previous reports have demonstrated that exposure to radiation modulated the activation of Notch signaling in the CD44+/CD24-/low breast cancer stem cells. In addition, Notch signaling has been implicated in the radioresistance phenotype of GPCs where knockdown of *NOTCH1* or *NOTCH2* effected radiosensitivity of GPCs but not that of differentiated glioma cells (Wang et al. 2010a), suggesting that inhibition of Notch signaling may not only deplete GPC frequency and engraftment potential but also reduce the radioresistance of GPCs. Furthermore, inhibition of the Notch cascade in irradiated GPCs brought about increased apoptotic marker caspase 3/7 and positive labeling of apoptotic marker Annexin V. Thus, these data suggest that, in the case of gliomas, Notch may be a possible target in stem-like glioma cells as GPCs express Notch family genes and tumor-derived neurospheres have elevated Notch activity and might be involved in evading apoptosis and promoting proliferation. Thus, targeting of Notch and its components underlying the radioresistance of GPCs promises to confer sustained benefit for glioma therapeutics.

Additional studies have shown that γ-secretase inhibition of Notch signaling upregulated the Wnt and Hedgehog pathways, both essential for normal neural development. The Notch target Hes1 binds Gli1 directly, blocking its transcriptional level. Concomitant inhibition of Notch and Hedgehog dramatically decreased proliferation of neurospheres and primary human GBM cells, suggesting this regulatory mechanism may contribute to resistance. These findings demonstrate that Notch-targeted therapeutics can lead to alterations in other developmental signaling cascades that promote tumor survival, and suggest that combinatorial treatment with Hedgehog pathway inhibitors may be able to increase the efficacy of γ-secretase inhibition in cancer patients.

Hedgehog-Gli pathway

The Sonic Hedgehog signaling is one of key regulatory pathways critical for the maintenance of several types of adult stem cells, including neural stem cells (Clement et al. 2007). The Hedgehog signaling cascade is commonly known to be expressed by tumor-associated endothelial cells and astrocytes in platelet-derived growth factor (PDGF)-driven mouse models of GBM. The main components of this signaling pathway are the ligands (secreted Hedgehog proteins), the Patched receptor (Ptch, a 12-pass transmembrane protein), the intracellular transducing molecules Smoothened (Smo – a second transmembrane protein) and Gliotactin (Gli, zinc-finger transcription factors).

Ligand-binding of Hedgehog to Ptch represses Smo inhibition, allowing the activation of the canonical Hedgehog pathway through Gli-dependent transcription of multiple targets, including *N-myc, cyclin D, Ptch, Gli1, and Gli2*.

Several studies have implicated the ligand-dependent activation of Hedgehog signaling in tumorigenesis for various cancers. These studies ascribe a cell-autonomous role for Hedgehog signaling and propose that even in the absence of mutations in the Hedgehog cascade, tumor cells still produced and responded to the Hedgehog ligand in an autocrine manner. However, more controversial studies on pancreatic cancer have implicated a role for paracrine Hedgehog signaling in tumor stromal cells in the maintenance of the cancer stem cell microenvironment. Hedgehog-overexpressing tumor cells induce a response in surrounding stromal cells whilst epithelial tumor cells are refractory to Hedgehog pathway modulation. The expression of Hedgehog ligands by tumor cells was not associated with their expression of target genes but rather, was in concordance with the canonical pathway activity by infiltrating tumor stromal cells. Notably, analysis of human tumor xenografts revealed that levels of Hedgehog ligand mRNA expression in tumor cells corresponded with elevated *Gli1* and *Ptch1* mRNA levels in the stroma but not the tumor compartment, indicating that the Hedgehog ligands activate Hedgehog signaling in the adjacent stroma microenvironment and not the tumor epithelium. Specific inhibition of Hedgehog signaling in the stroma with either an antagonist of Hedgehog or a neutralizing anti-Hedgehog antibody or genetic ablation of *Smo* resulted in xenograft tumor inhibition, further substantiating the role of Hedgehog-activated stroma in tumor propagation. It has also been demonstrated that a constitutively activated stromal Hedgehog signal is adequate to accelerate tumor growth even in the absence of a Hedgehog ligand. In addition, chimeric xenografts coinjected with a human prostate cancer cell line that did not respond to Hedgehog ligand and a Hedgehog-overexpressing cell line were composed of a mixed population with equal cell numbers and similar tumor proliferation rate, suggesting that paracine Hedgehog signaling exerts a non-cell autonomous effect on tumor growth. The authors then identified a transcriptional gene signature of Hedgehog signaling that is reminiscent of the fetal mesenchyme, faithfully recapitulating human prostate tumors with a reactive stroma phenotype. Thus, insights into the mechanisms driving the Hedgehog pathway will be critical since patients bearing mutation-driven Hedgehog tumors, as in the case of basal cell carcinomas and medulloblastoma, are more likely to display a more favourable clinical response to Hedgehog inhibitors whilst tumors regulated by the paracrine mode remain resistant. Hence, elucidation of Hedgehog inhibition via either the stromal microenvironment or the tumor epithelia will allow for the development of more effective targeted Hedgehog antagonists for a broad range of cancers.

Hedgehog signaling is highly deregulated in a small subpopulation of human medulloblastoma and *Gli1*, a key Hedgehog target, was highly expressed in primary GBMs and CD133+GBMs (Bar et al. 2007). Conventional sources of Hedgehog ligands include CD133+GPCs and tumor-induced vasculature in GBMs (Clement et al. 2007). Several groups have investigated the role of Hedgehog-Gli signaling in GPCs and found that this signaling pathway regulates GPC function, self-renewal and tumorigenesis (Clement et al. 2007; Ehtesham et al. 2007). Forced differentiation of GBM neurospheres reduced both stemness and Hedgehog activity expression. However, not all GBMs have activated Shh signaling as determined by Gli expression (Bar et al. 2007), indicating the presence of molecular subgroups of brain tumors in which targeting of Shh would be ineffective. Treatment of GPCs with the Hedgehog inhibitor cyclopamine or *Gli* knockdown drastically depleted the GPCs by suppressing self-renewal ability and proliferation while increasing apoptotic cell death in vitro and inhibiting tumor propagation in vivo. Importantly, cyclopamine inhibition of Hedgehog-Gli signaling enhances the efficacy of TMZ to abolish GPC proliferation and improve the effect of radiation on GPCs. Taken together, these studies indicate that the Hedgehog-Gli module is critical for GPC

maintenance and targeting this pathway with specific pharmacologic inhibitors may attenuate GPC self-renewal and offer improved therapy efficiency against gliomas.

Gli1 acts at the distal end of the Hedgehog pathway, where it regulates transcription in response to activation or inhibition of the pathway. Moreover, Gli activity correlates with tumor grade in a genetically engineered mouse model. As such, further investigation must be performed to explore its role in GPC growth, maintenance and GBM recurrence. Cui et al. (2010) investigated the role of Gli1 in primary and recurrent gliomas and its ability to confer chemosensitivity or chemoresistance of glioma cells . Overexpression of *Gli1* associated with GPC chemoresistance, resulting in glioma perpetuation. Conversely downregulation of *Gli1* enhanced the susceptibility of GPCs to the synergistic effects of cyclopamine and chemotherapeutic agents, promoting apoptotic cell death, thus suggesting that Gli1 is a key mediator of chemoresistance in GBMs with aberrant Hedgehog signaling. Moreover, the constitutive Hedgehog pathway activity contributes to the resistance of glioma cells to chemotherapeutic agents by promoting self-renewal and tumor regrowth following therapy in an autocrine and/or paracrine manner (Bar et al. 2007). In contrast, abolishment of Hedgehog pathway activity abrogates tumor growth and restricts tumor recurrence, by downregulation of the expressions of *MDR1, MRP1, LRP, MGMT, BCL2 and Survivin*, which play important roles in glioma chemoresistance and repopulation, thus providing a mechanism to explain the recurrence of some gliomas.

Mouse models of medulloblastoma CSCs localized in the perivascular vicinity express Nestin and are protected from radiation, owing to the ability to activate the PI3K/Akt pathway and undergo a transient, p53-dependent cell cycle arrest. This suggests that GPCs' therapeutic response cannot be considered in isolation from the tumor microenvironment with its potential dependence on the perivascular compartment. It is therefore imperative to understand the interactions between self-renewal pathways and signals known to be altered in cancer in the context of specific microenvironments found in the perivascular niche. Hence, the Hedgehog-Gli signaling network could provide important therapeutic implications as synergistic inhibition of the Hedgehog pathway and Gli activity in some gliomas may be a potential target for the alleviation of therapeutic resistance and enhanced the chemotherapeutic response in glioma patients.

EGFR/PI3K/AKT Axis

The presence of autocrine and paracrine growth factor loops are common in malignant gliomas and these pathways regulate numerous protumorigenic cellular functions including cellular proliferation, apoptotic resistance, invasion and angiogenesis. Epidermal growth factor (EGF), routinely used in culturing EGF-responsive neural precursors, is a key growth factor used in the maintenance of GPCs. GBMs frequently display EGFR amplification, with expression of the constitutively active variant EGFRvIII, mediated through PI3K-Akt and Ras/mitogen-activated protein kinase (MAPK) downstream signaling in GBMs and is associated with enhanced tumorigenic potential and more aggressive phenotypes, such as invasiveness and therapeutic resistance. Shinojima et al. evaluated 87 primary GBM patients and found EGFR amplification to be an independent, unfavorable predictor for overall survival. In this cohort, EGFRvIII overexpression in the presence of EGFR amplification is the strongest indicator of a poor survival prognosis.

Intratumoral heterogeneity plays a major role in contributing to GBM resistance to EGFR targeted therapy due either to pre-existing resistant clones within the tumor or the interaction of non-resistant clones with the tumor cells or the tumor niche. Mazzoleni et al. (2010) showed that despite both the molecularly and functionally distinct $EGFR^{pos}$ and $EGFR^{neg}$ GPCs being able to form tumors that phenocopy the original tumor sample, only $EGFR^{pos}$ GPCs had elevated tumorigenic proliferation and highly invasive characteristic. Conversely, the $EGFR^{neg}$ GPCs formed tumors sporadically and required enforced EGFR

gene transfer to form tumors. Hence, the presence of distinct subpopulations within the same tumor might contribute to GBM resistance and EGFR targeted therapies since $EGFR^{neg}$ GPCs are insusceptible to treatment and will survive to reform the tumor mass. Inda et al. demonstrated that EGFRvIII cells secrete IL-6 and LIF, which in turn promote the growth and proliferation of wild-type EGFR cells that form the tumor bulk. This small subset of EGFRvIII cells, driven in a paracrine manner to recruit wild type EGFR cells into accelerated proliferation, enhances the tumorigenic potential of the bulk tumor and actively maintains a heterogeneous expression of both the wild type and the mutant form (Inda et al. 2010). Mice orthotopic tumors overexpressing EGFRvIII are refractory to radiation therapy, with sustained repopulation and nondescript effect on overall survival. The efficacy of EGFR kinase inhibitors have been disappointing so far as silencing of EGFRvIII compels GBM cells to undergo selective pressure in vivo to employ alternative compensatory pathways such as upreglating receptor tyrosine kinases (PDGFR, IGF1-R and c-Met) to maintain aggressiveness. These findings suggest that tumor cells are adept at bypassing single EGFR targeted therapies, reforming the tumor after an initial period of stasis, and inhibition of EGFR alone will not be adequate for translation into a beneficial clinical response in GBM patients. An effective therapeutic strategy should take into account the role of residual $EGFR^{neg}$ GPCs or that of the secreted factors in the tumor niche, and the development of a tailored combinatorial therapy targeted at both the aggressive $EGFR^{pos}$ GPCs and the less malignant $EGFR^{neg}$ GPCs or the microenvironment will be imperative to improve the clinical response of GBM patients.

One of the main molecular changes accompanying progression of gliomas to high grade, with simultaneous elevated stem cell expression and resistance to chemotherapy, is the loss of Phosphatase and tensin homolog (PTEN) and consequent elevation of Akt pathways activity. Deficiency in PTEN modulates CHK1 localization, initiating genetic instability and thereby conferring chemoradioresistance in GBMs. A number of intracellular signaling cascades are activated upon EGFR stimulation, but the PI3K-Akt module has been predominantly linked to GPC biology and contribution to the resistant phenotype (Eyler et al. 2008). Various studies have shown that hyperactivation of the PI3K/Akt and Ras/MAPK signaling pathways in cancer cells promotes tumorigenesis, increases tumor cell survival, proliferation, invasion and is significantly associated with radiotherapy resistance, either through the modulation of cell survival signaling or, by direct regulation of the DNA repair machinery. In human gliomas, there is evidence at genomic, mRNA and protein levels showing that aberrant Akt signaling prognosticates poorer survival. Indeed, chemoresistance in hepatocarcinoma stem cells may be conferred by activation of Akt, and Akt signaling promotes survival of stem-like tumor cells in the perivascular niche of mouse medulloblastoma models. It has been recently demonstrated that GPCs are more dependent on Akt signaling than their matched non-stem counterparts (Eyler et al. 2008). It has been observed that GBMs expressed significantly higher levels of phospho-PI3K and phospho-p70s6k, but not of phospho-Akt, compared to their non-GBM counterparts, implying that GBMs display dependency on these pathways possibly for their survival, proliferation and therapeutic resistance. In addition, inverse correlation between phospho-PI3K, phospho-Akt, and phospho-p70s6k levels with cleaved caspase 3 implicates the likely mechanisms employed by the members of the PI3K family in the inhibition of apoptosis and promotion of radioresistance in GBMs. Functional inhibition of Akt with the pharmacologic inhibitors preferentially disrupts GPC neurosphere formation, reduces motility and invasion, induces apoptosis in vitro, and significantly prevents intracranial tumor formation of GPCs (Eyler et al. 2008; Bleau et al. 2009). Although in vitro targeting of the EGFR-PI3K-Akt signaling cascade may have specific effects on GPC self-renewal and tumorigenic progression, clinical trials of EGFR inhibitors, such as Imatinib, have not resulted in significant survival, suggesting that EGFR inhibition solely is

an insufficient therapeutic paradigm, prompting greater focus on PI3K inhibitors.

EGFR/EGFRvIII's cross-interaction with the oncogenic transcription factor STAT3 and receptor tyrosine kinases (c-Met and PDGFR) mediates GPC resistance to anti-EGFR therapy. JAK-STAT3 pathway is constitutively activated in the majority of GBMs and the dynamic interactions between STAT3 and EGFR underlie resistance of GBM cells to Iressa. Combinatorial inhibition of JAK and EGFR/EGFRvIII abolishes STAT3 activation and synergistically suppresses the GPC proliferation. JSI-124 acts as a highly selective inhibitor of the JAK/STAT3 signaling pathway and has been shown to sensitize malignant glioma and medulloblastoma cells to TMZ, 1,3-bis(2-chloroethyl)-1-nitrosourea, and cisplatin.

Taken together, these findings discussed here suggest that EGFR signaling, either directly through the interaction with the DNA repair machinery or indirectly through the activation of key oncogenic PI3K/Akt and JAK/STAT signaling pathways, modulates sensitivity to radiation. Therefore, elucidation of the dynamic interactive EGFR networks will enable us to identify mechanisms that circumvent therapeutic resistance in GPCs and improve the modest efficacy of current EGFR-targeted therapy in GBM patients. Given the central role of the EGFR signaling pathway in conferring the aggressive phenotype in tumors, treatment resistance, and poor prognosis, considerable effort has been invested in the development of imaging strategies to non-invasively ascertain EGFR status and therapeutic response to EGFR targeting agents. Such approaches would enable more accurate stratification of the patients who are likely to benefit from EGFR targeting therapeutics and for monitoring treatment efficacy.

Wnt Signaling

The primary role of the Wnt/β-catenin pathway in embryogenesis and normal stem cell development such as the regulation of stem cell proliferation and self-renewal, has been well established. Wnts are a family of 19 secreted glycoproteins that regulate embryonic patterning and initiate a series of signaling cascade throughout development of the nervous system. Secreted regulator proteins of the Wnt family bind to specific Frizzled (Fzd) receptor complexes on the surface of target cells to initiate Wnt signaling cascades that can be broadly classified into canonical or non-canonical, determined by the composition of the Wnt/Fzd complex. The canonical pathway, which regulates the ability of the β-catenin protein to promote the activation of downstream genes, is better characterized and is therefore more relevant in the development of Wnt therapeutic interventions.

Wnt signaling has been implicated in the control over various types of stem cells and may act as a niche factor to maintain stem cells in a self-renewing state. The Wnt signaling pathway has been shown to be the predominant driving force of stem cells of the colonic crypt, hematopoietic and central nervous system. In particular, tumor-propagating cells of the colon, breast and hematopoietic system have been shown to promote tumorigenesis through major contributions from aberrant Wnt signaling. Mutations in Wnt pathway genes such as adenomatous polyposis coli (*APC*) and *CTNNB1*, are commonly present in a majority of hepatocellular carcinoma, thyroid cancer, and ovarian cancer. The fact that Wnt signaling is also dysregulated in multiple solid cancers suggests that it may also play a crucial role in the maintenance of GPCs. It has been demonstrated that *Wnt2*, an activator of the canonical pathway, was significantly overexpressed in gliomas and their expression levels correlated positively with malignancy. Similarly, higher grade gliomas were observed to express elevated *CTNNB1* expression, which subsequently correlated to a poorer prognostic impact in GBM patients. In addition, the expression of other Wnt regulators, including Dvl2, Dvl3, FRAT-1, Pygo-2, Tcf4, and Lef-1 and of specific Wnt target genes, *Cyclin D1* and *c-myc*, also increases with glioma grades. Of note, knockdown of *Dvl2* abrogated both the self-renewal ability and proliferation of gliomas and stimulated the differentiation of patient GBM samples. Tumor propagation in immune-compromised mice was

repressed upon *Dvl2* depletion. In addition to regulation of the expression of Wnt members, antagonists of the Wnt pathway are often repressed in GBMs and their expression is mostly inversely correlated with glioma grades. Frequent aberrant promoter hypermethylation of these Wnt antagonists, such as the Wnt inhibitory factor (WIF), secreted-Frizzled-related protein (sFRP) and the LRP antagonist Dickkopf (Dkk) is significantly associated with GBMs. Furthermore, a novel proto-oncogene *PLAGL2,* which is overexpressed in GBMs and induces GPCs proliferation and tumorigenic potential, has been identified. PLAGL2 stimulates the expression of Wnt-6, Fz-9, and Fz-2, inhibits differentiation, and increases proliferation of neural progenitors. *PLAGL2* amplification also associates with elevated levels of *CTNNB1* in GBMs, suggesting a possible role of PLAGL2 in GPCs via the regulation of Wnt signaling. It has been shown that hypermethylation of paternally expressed gene 3 (PEG3) promoter in glioma mitigates expression of *PEG3* and correlates with high-grade gliomas. Upon PEG3 promoter hypermethylation, β-catenin accumulates, resulting in GPCs proliferation. A recent study by Zhang et al. (2011) showed that the interaction between the transcription factor Forkhead box M1 (FoxM1) and β-catenin promotes β-catenin nuclear localization, controls transcriptional activation of Wnt target genes expression and maintains GPC self-renewal. Together, these findings validate the role of Wnt and activated β-catenin signaling in mediating GPCs self-renewal.

The Wnt/β-catenin pathway has been imputed in conferring radioresistance in mammary progenitor cells and breast cancer cell lines expressing common cancer stem cell markers. Woodward et al. demonstrated that radiotherapy results in enrichment for the stem- and progenitor-cell–containing population in a murine mammary epithelial cell culture, and particularly augments the stem-cell antigen (Sca)–positive compartment of the side population cells. Consistent with this, tumor-initiating cells from a p53-null mouse mammary tumor had upregulation of active β-catenin and were able to undergo DNA damage repair more efficiently compared to the non-TICs. It is known that Wnt1 ectopic expression activates the DNA damage response in epithelial mammary cells. WJ2, a cancer stem-cell cell line derived from GBM, displayed higher expression of stemness markers with concomitant Wnt1 expression. Overexpression of *MDR1*, a downstream target of Wnt-1/Fz-1 signaling, confers chemoresistance in neuroblastoma cells, implicating a similar chemoresistance mechanism in gliomas. Taken together, these findings suggest a possible role for Wnt signaling in the chemoradioresistance mechanisms developed by GPCs. Elucidation of the specific genetic lesions in the Wnt signaling cascade in GBMs will allow for the design of specific therapeutic tools to decrease tumor recurrence and improve patient survival.

Role of the Tumor Microenvironment

The diffuse nature of gliomas, its anatomic complexities and the cellular heterogeneity of GBM contributes to the poor response to currently available treatment regimens as chemotherapeutics are unable to reach tumor sites. Regional variations in the tumor microvasculature resulting in the heterogeneous nature of GPCs, as well as the diversity of the cancer cell subpopulation that results from the progressive stochastic selection of genetically resistant subclones, confers GPCs their therapeutic resistance property. Intimate interaction between the tumor microenvironment and the GPCs thus presents a potential avenue for therapeutic targeting of the microenvironment components as well as the GPCs.

In the adult brain, normal neural stem cells (NSCs) were observed to be concentrated around vasculature, known to have low oxygen availability and distinct extracellular matrix (ECM) profiles, for convenient access to signaling molecules, nutrition and migration. The subversion of this normal vascular microenvironment modulates GPC function and similar to NSCs, these specialized GPC niches tightly regulate oxygen and nutrition to maintain GPC phenotype, thereby facilitating its invasive and tumor proliferating properties. This disorganized florid

neovasculature is a hallmark of GBMs and has a protective role in shielding GPCs from therapeutic insults, enabling them to reform a tumor mass following initial clinical response (Gilbertson and Rich 2007). The degree of vascularization is significantly correlated with glioma malignancy, tumor aggressiveness and clinical prognostication. Calabrese et al. (2007) identified a fraction of CD133+/Nestin+ cells enriched in areas of increased microvessel density, suggesting that tumor vasculature generates a specialized niche microenvironment in which the formation and maintenance of GPCs proceeds. In addition, co-implantation of tumor cells with endothelial cells led to rapid tumor propagation, implicating the influence of the vascular niche on tumor initiation. GPCs are enriched in functional aberrant niches, predominantly the perivascular niche, which provides microenvironmental cues for the maintenance of GPCs. Abrogation of this niche via anti-angiogenic drugs effected impaired GPC self-renewal and depleted tumor growth, further validating the importance of the perivascular niche in the regulation of tumorigenesis.

Bidirectional interplay between the supportive tumor vascular niche and GPCs was validated by Bao et al.when they demonstrated that GPCs released vascular endothelial growth factor (VEGF), modulating endothelial cell migration and subsequently, facilitating tumor growth by stimulating tumor vasculature, influencing angiogenesis and vasculogenesis through increased expression of VEGF and stromal cell-derived factor-1 (SDF-1) respectively (Bao et al. 2006b; Folkins et al. 2009). Tumor angiogenesis is an essential hallmark of GBMs, whereby vascular proliferation is a WHO histopathology indicator of GBMs, and is frequently associated with resistance to radiation. Tumor vasculogenesis, which involves the recruitment of endothelial precursors to induce the formation of new capillaries, represents a possible treatment approach to prevent tumor recurrence. Kioi et al. employed a murine intracranial GBM xenograft model and demonstrated that following radiation-induced damage, increased vasculogenesis was observed to reinitiate tumor proliferation. This cascade was triggered by upregulation of hypoxia in the tumor, leading to increased levels of SDF-1. The chemokine receptor, CXCR4, is then activated by SDF-1 to promote angiogenesis and VEGF production in gliomas. GPCs express high levels of SDF-1 and given their inherent radioresistance phenotype, it is imperative that the role of GPCs in post-irradiation vasculogenesis be further elucidated to develop more effective therapeutic strategies in order to circumvent resistance to conventional GBM therapies.

Owing to the fact that GBMs are vascular-rich tumors with high levels of VEGF production, clinical trials for the anti-VEGF antibody bevacizumab (Avastin) were conducted. However, despite its initial promise in a phase II clinical trial where GBM patients responded to the combinatorial treatment of bevacizumab and irinotecan, bevacizumab only conferred a transient respite with short term tumor burden control and patients' tumor inevitably repopulated. It has been reported that treatment with bevacizumab had marginal effect on tumor growth, coupled with significant decrease of vascular supply, elevated infiltration by tumor cells in the brain parenchyma and a more aggressive tumor phenotype. Therefore, there is a pressing need to better elucidate this insidious mechanism of failure for targeted anti-angiogenesis treatment so as to improve the clinical outcomes for GBM patients. Recent studies have shown that GPCs could differentiate to tumor endothelium and when targeted, resulted in ablated tumor burden (Wang et al. 2009; Ricci-Vitiani et al. 2010; Soda et al. 2011). Ricci-Vitiani et al. (2010), Wang et al. (2009) and Soda et al. (2011) identified endothelial cells in GBM microvasculature that possessed genomic aberrations specific for the tumor, suggesting that GPCs are involved in vasculogenesis and that these endothelial cells are of tumor origins. These GPCs could also differentiate into endothelial-like cells both in vitro and in vivo, highlighting that the positive paracrine feedback between GPCs and the vascular niche is integral to promote tumor maintenance and growth. Furthermore, Wang et al. (2009) observed that exposure to bevacizumab or *VEGF* silencing inhibits the maturation of tumor endothelial progenitors into endothelium without affecting

the differentiation of GPCs into endothelial progenitors, whereas γ-secretase inhibition or *NOTCH1* silencing blocks the transition of GPCs into endothelial progenitors, suggesting that the failure of bevacizumab could be due to inability to block this transdifferentiation process. An in-depth understanding of this lineage plasticity could provide new insights into GPC biology and enable the development of more efficacious combination therapy.

Apart from interaction with endothelial cells, GPCs residing in the perivascular regions are in contact with ECM components, including microglia, T-lymphocytes, neural precursor cells, GFAP-expressing astrocytes and fibroblastic pericytes, that are preferentially expressed within and around blood vessels. Cell-ECM interactions have been shown to contribute to the response of tumor cells to radiation by: (1) Serving as a repository for proteins that modulate therapeutic response, (2) Operating as a substratum for the activation of prosurvival integrin-mediated signaling cascades following irradiation, and (3) Creating a more favorable niche for the proliferation of cells that survive therapy. Lathia et al. (2010) observed perivascular colocalization of integrin α6 with CD31-expressing endothelial cells in GBM patient samples, underlining the significance of the interplay between GPCs and the tumor vasculature (Lathia et al. 2010). Previous studies have implicated growth factors (Bao et al. 2006b), laminins and cell-to-cell signaling (Calabrese et al. 2007) in the regulation of GPCs, demonstrating the maintenance of GPCs by the ECM present in the perivascular niche via integrin α6. Targeting integrin α6 decreases GPC survival and tumorigenic potential. Another secreted factor produced by the tumor microenvironment is the cytokine interleukin 6 (IL6). *IL6* expression is highly correlated with tumor propagation as well as poor clinical outcome in GBM (Wang et al. 2009). Knockdown of *IL6* impairs GPC proliferation, demonstrating the key role of IL6 autocrine signals in GPC phenotype. Importantly, administration of anti-IL6 antibody delayed the growth of tumors initiated with GPCs. These data strongly suggest that targeting IL6 may be useful as anti-glioma therapies.

In gliomas of higher malignancy grade, the blood brain barrier (BBB) gets leaky, because tumors can actively degrade tight junctions by secreting soluble factors. However, drug uptake remains restricted as new vessels formed by GBM-released VEGFs are leaky, resulting in accumulating interstitial pressure and restricted oxygen tension. As such, this hostile microenvironment represents a serious obstacle for drug penetration. A second niche where GPCs are concentrated is the perinecrotic hypoxic microenvironment. Low oxygen levels (i.e. hypoxia) activates pro-apoptotic and pro-angiogenic pathways, and drive tumor progression by triggering a set of adaptive transcriptional response that regulates tumor angiogenesis, metabolism, migration, response to changes in redox status in the niche and tumor survival. As hypoxia promotes stem cell maintenance, the critical role of hypoxia in defining cellular sensitivity to radiation may entwine with stem cell maintenance and therapeutic resistance. The extent of hypoxia has been shown to associate with poorer patient survival, therapeutic resistances and aggressive glioma phenotype. Resistance to the chemotherapeutic TMZ has been linked to hypomethylation and overexpression of *MGMT* in the hypoxic regions within gliomas, suggesting that restricted oxygenation levels may be critical for the GPC response to DNA-damage inducing agents and cells that reside in hypoxic niches may be better suited to evade such treatment. The complexity of vasculature and high flux of oxygen status has been observed to correlate with radiation resistance, making it highly challenging to directly disrupt the hypoxic niche.

A more effective approach would therefore be to target the canonical hypoxia responsive pathways, mediated through the hypoxia inducible factors (HIFs), which are in turn regulated by prolyl hydroxylase (PHD) family. Restricted oxygen has also been shown to play an integral role in GPC maintenance and function, contributing to the formation of both GPC and non-stem-like neurospheres, with concomitant upregulation of stemness-related genes such as *SOX2* and *OCT4*. Current studies have revealed that both HIF1α and HIF2α are critical for GPC

function and tumor propagation. HIF1α is involved in maintaining the self-renewal ability of the GPC population as stabilization of the protein expands the GPC population in a bulk tumor, mediated by central oncogenic pathways such as PI3K/Akt, epidermal growth factor receptor (EGFR) and the Extracellular signaling Related Kinase (ERK) 1/2 pathways. Recent studies have shown that HIF-1 regulates tumor radioresponses by either sensitizing tumor cells to radiation through induction of ATP metabolism, proliferation, and p53 activation or by increased survival of endothelial cells. Regrettably, HIF1α is critical for the normal function of neural progenitor cells as well as normal endothelial cells, thus limiting its therapeutic index (Li et al. 2009). In contrast, HIF2α has recently emerged as a potential therapeutic target for GPCs, with response to a broader range of oxygen tensions compared to HIF1α, enabling the capability to adapt to a highly dynamic microenvironment. A recent study revealed that HIF2α is preferentially expressed in the GPC population without expression in normal progenitors (Li et al. 2009) and self-renewal ability of GPCs was abrogated upon *HIF2α* knockdown. HIF2α can also promote a more tumorigenic phenotype in non-stem glioma cells, driving the expression of genes involved in core stem cell pathways such as *Oct4, c-myc* and *Nanog* (Heddleston et al. 2009). Further substantiation data was extrapolated from the REMBRANDT database where patients with higher expression of HIF2α mRNA resulted in poorer survival. Due to a clear reliance of GPCs on HIF proteins for their survival, there is compelling evidence for HIF2α as a therapeutic target that could impair GPC responses to the hypoxic microenvironment via disruption of the niche using drugs such as the aminoglycoside digoxin. However the caveat to digoxin administration is that they indiscriminately target both HIF1α and HIF2α, thereby resulting in off-target effects in normal neural progenitors. More effective pharmacological inhibitors must thus be developed to specifically target HIF2α for more efficient clinical treatment and improved patient survival.

The contributions of extracellular tumor states may also be critical for maintaining GPC function and mediating therapeutic resistance. Acidic stress has been shown to induce plasticity in non-stem GBM cells, making them more GPC-like (Hjelmeland et al. 2011). Following exposure to physiologic tumor pH of 6.5, expression of stemness-related genes are upregulated with concurrent increase in tumorigenic potential. Elevation of pH reverts the induction of HIF2 and other GPC markers by acidic stress, suggesting that increasing intratumoral pH may be a conceivable strategy to eradicate GPCs. Subsequent to GPC targeted therapies, intrinsic cues in the GPC niche may reform a subset of GPCs to maintain tumor cell homeostasis. Hence, targeting the acidic microenvironment, through restoration of a neutral pH, followed by GPC-specific treatment may be an effective anticancer stem cell therapy.

Escape mechanisms directing adaptation and tumor progression include migration of tumor cells or intercellular communication with tumor niche, ECM secreted factors and viable circulating endothelial cells (CECs). However, adaptation forces resulting from microenvironmental influences of hypoxia, vascularity, acidic stress, starvation, exogenous growth factors, and altered responses to endogenous growth factors as well as exogenous factors including therapeutic interventions, must be evaluated. These complexities underscore the pressing need to identify the intercellular communications between resident cell types in the perivascular niche of brain tumors, the diverse cell population and signaling factors involved in maintaining the GPC phenotype within this niche so as to unravel mechanism of therapeutic resistance.

Molecular Classification

In 2006, the National Cancer Institute initiated an effort to deep profile, as one of the first cancers, glioblastoma multiforme, because of its dismal prognosis despite advanced surgical intervention and adjuvant chemotherapy and radiation treatment. This effort is predicated on the belief that

histologically similar tumors can be molecularly heterogeneous, and that distinct pathways drive the biological phenotype. The first publication arising from The Cancer Genome Atlas (TCGA) effort showed that patients with GBM sustain mutations that can be grouped into three major signaling networks: Receptor tyrosine kinases (RTKs), p53 and Retinoblastoma tumor suppressor pathways. Importantly, GBM tumors are molecularly heterogeneous, further highlighting the limitations of relying solely on morphology-based histological methods to diagnose and subsequently treat patients. A follow-up study then showed that GBM tumors can be molecularly classified into four subgroups (Proneural, Classical, Mesenchymal, Neural), with each subgroup containing unique gene expression, genomic aberrations and clinical profile. A major inference from such studies is that GBM patients can now potentially be treated according to their molecular subclasses and pathway activation. Indeed, it is known through pharmacological targeting in a panel of GBM cell lines that co-deletion of *CDKN2A* and *CDKN2C* served as a strong predictor of sensitivity to a selective inhibitor of CDK4/6. This mapped to similar patterns of *CDKN2A* and *CDKN2C* mutations in TCGA patients, leading to hyperactivated CDK4/6. The Wiedemeyer study thus demonstrates that the integration of genomic, functional and pharmacologic data can be exploited to inform the development of targeted therapy directed against specific cancer pathways. Importantly, the TCGA effort emphasizes that gene expression drives GBM disease progression and patient survival outcome.

In assessing the contribution of GPCs to the primary tumor phenotype, several studies have focused on analyzing common GPC marker expression in tissue paraffin sections, often with ambiguous data. This may be reconciled by the fact that GPC properties that sustain the tumor phenotype may reside in more than just specific marker profiles. Consequently, pathway activation resembling those functioning in stem-like cells, represented by a set of genes, is more likely to correctly interrogate the clinical contribution of GPCs. Some information is available regarding *BRCA1* mutation-associated breast tumors. Based on this information differentially regulated genes in subsets of epithelial cells can be derived and luminal progenitors are highly represented in *BRCA1* mutation-associated basal tumors, even more than the commonly anticipated stem cell population. This suggests that luminal progenitors are more likely the cells-of-origin for *BRCA1* mutation-associated breast cancers, later confirmed in a transgenic mouse model study. Such studies underscore the predictive ability of gene expression mapping of pathway activation, rather than focus on a specific marker identity. It is known that serial tumor-propagating (and not marker-defined) acute myeloid leukemia stem cells contribute to disease progression and patient survival outcome, highlighting the importance of functionally defining the cancer stem cell. Two other more relevant studies demonstrated that GPCs contribute to GBM patient survival outcome, with preferential activation of core stem cell programs (hematopoietic, neural and embryonic stem cells). The key message from such studies is that cancer stem cells perpetuate tumors not merely in terms of their cell numbers, but more accurately reflected by their pathway activation. Consequently, the primary tumor phenotype is a manifest of cancer stem cell behavior and signaling.

In conclusion, understanding the regulatory pathways of GPCs and the influence of their microenvironmental niche is crucial and is likely to contribute to disease progression and clinical outcome. Several primary resistance mechanisms of GPCs, such as the multi-drug resistance family of proteins (e.g. ABCB1and ABCG2), DNA damage repair pathways (e.g. CD133, L1CAM and MGMT), and self-renewal mechanisms (e.g. Notch, Hedgehog-Gli, Wnt/β-catenin and EGFR/PI3K/AKT axis) typified in normal stem cells, as well as the microenvironmental niche (including VEGF/SDF-1, Integrin α6, IL-6 and HIF2) have demonstrated to play critical roles in the development of radio- and chemoresistance in GPCs (Fig. 7.1 and Table 7.1). The cancer stem cell theory presents a paradigm in therapeutic design towards a long-lasting and effective cure.

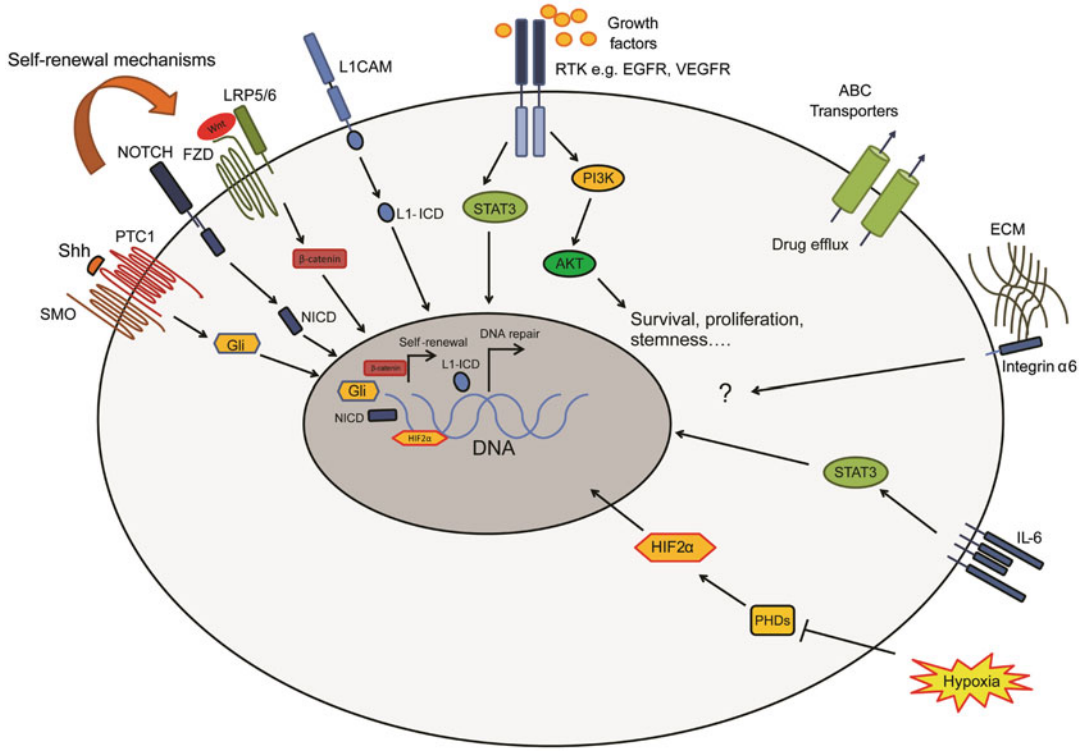

Fig. 7.1 Mechanisms implicated in the chemoresistance and radioresistance of glioma propagating cells (*GPCs*). GPCs are regulated at multiple levels by a complex network of pathways. Self-renewal signals are mainly contributed by Notch, Hedgehog, Wnt and EGF pathways. Mechanisms that mediate radio- and chemo-resistance in GPCs include ABC family of drug transporters and DNA repair pathways. Multiple environmental cues e.g. hypoxia, ECM, VEGF exert additional support to the maintenance of GPCs and provide resistance to conventional therapeutics

Table 7.1 List of mechanisms and pathways conferring glioma-propagating cells their resistance to chemo- and radio-therapies

Mechanisms/pathways	Components	Function(s)	References
Multidrug transporter family	ABCB1	Drug efflux transporter	Nakai et al. (2009)
	ABCG2	Drug efflux transporter	Chua et al. (2008)
			Bleau et al. (2009)
DNA damage repair	CD133	Normal neural and cancer stem cells marker	Bao et al. (2006a)
	L1CAM	Neuronal cell adhesion molecule	Cheng et al. (2011)
	MGMT	Repairs alkylated guanine in DNA	Liu et al. (2006)
Self-renewal	Notch	Neurosphere-forming ability and stemness related expression	Fan et al. (2010)
			Hu et al. (2011)
		Knockdown of Notch effected radiosensitivity of GPCs	Wang et al. (2010a)

(continued)

Table 7.1 (continued)

Mechanisms/pathways	Components	Function(s)	References
	Hedgehog-Gli	Regulation of GPCs function, self-renewal and tumorigenic potential	Clement et al. (2007)
			Bar et al. (2007)
		Gli1 is a key mediator of chemoresistance in GBMs	Ehtesham et al. (2007)
			Cui et al. (2010)
	EGFR/PI3K/AKT	GPCs' tumorigenicity with elevated EGFR gradient	Mazzoleni et al. (2010)
		PI3K-Akt contribute to GPC resistance phenotype	Inda et al. (2010)
			Eyler et al. (2008)
	Wnt/β-catenin	Self-renewal and proliferation of GPCs	Zhang et al. (2011)
Tumor microenvironment	Perivascular Niche	GPCs are enriched in areas of neovasculature, which shields them from therapeutic insults	Calabrese et al. (2007)
			Gilbertson and Rich (2007)
	VEGF/SDF-1	Implicated in GPC resistance to radiation therapy	Bao et al. (2006b)
			Folkins et al. (2009)
	Transdifferentiation	Confers GPC resistance to bevacizumab	Ricci-Vitiani et al. (2010)
			Wang et al. (2010b)
			Soda et al. (2011)
	Integrin α6	Involved in GPC survival	Lathia et al. (2010)
	Interleukin 6 (IL6)	Autocrine signal in GPC proliferation and tumor growth	Wang et al. (2009)
	HIF2	Integral role in GPC self-renewal, stemness and tumor propagation. Blocks cellular sensitivity to radiotherapy	Li et al. (2009)
			Heddleston et al. (2009)
	Acidic stress	Induce plasticity in non-stem GBM cells	Hjelmeland et al. (2011)

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Chemical Genomic Approaches to Eradicate Leukemia Stem Cells

8

Siddhartha Sen, Monica L. Guzman, and Duane C. Hassane

Contents

Abstract

Lead target approaches have dominated efforts in cancer developmental therapeutics. However, recent advances in genomics and computational biology have facilitated the development of chemical genomic approaches in which the transcriptional changes resulting from drug exposure are used to infer potential relationships between drugs and biological states. Recent work in the area of transcription based drug discovery has accelerated the development of pre-clinical therapies for acute myeloid leukemia (AML). AML is a heterogeneous disease that consists of a subpopulation of leukemia stem cells (LSCs). The LSC subpopulation represents a chemoresistant fraction of the overall tumor and thought to drive relapse, which produces high mortality rates in AML. LSC ablation is a complex phenotype only discerned through costly and time-consuming murine xenotransplantation assays. This chapter discusses advances in transcription based drug discovery and how it has been applied to accelerating the discovery of drugs that target LSCs.

Introduction

Despite recent advances, acute myeloid leukemia (AML) remains a mostly fatal disease wherein most patients relapse and die of their disease despite achieving an initial complete remission. Relapse is the major therapeutic

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obstacle in AML and, unfortunately, standard therapy has remained largely unchanged over the past three decades (Dohner et al. 2010). Current advances in drug discovery for cancer therapeutics have been highly focused on the development of "targeted therapy" in which small molecules are specifically synthesized to interfere with the function of aberrantly regulated proteins driving cancer. The most striking example of this success in leukemia has been imatinib, targeting dysregulated Abl kinase resulting from the *BCR-ABL* gene fusion (Capdeville et al. 2002). Current clinical trials for AML therapy are similarly targeted, centering on targets that include CD33 (Myelotarg), Flt3 inhibitors, and other inhibitors of cellular kinases (Roboz 2011).

AML is molecularly heterogeneous and this heterogeneity impacts the response to treatment and likelihood of disease survival. For example, a recent study by Patel et al. (2012) conducted extensive mutational analyses of 18 genes in a cohort of 398 AML patients to better classify patients into different prognostic groups. The analyzed mutations included *FLT3*-internal tandem duplication (*FLT3*-ITD), Mixed-lineage leukemia (*MLL*) translocations, and mutations in *DNMT3A*, *TET2*, *ASXL1*, *PHF6*, *IDH2* and *CEBPA*, among several others. Remarkably, their findings showed that more than 97% of the patients had at least one of the tested mutations, indicating the extent of heterogeneity.

Certain mutations like *FLT3*-ITD were associated with poor overall survival, while other mutations such as *CEBPA* signified a better prognosis. The mutational analysis also revealed a significant impact on the patient response to intensive chemotherapy. The authors found that patients positive for mutations in *DNMT3A* showed a significant improvement in survival when high dose daunorubicin was administered, in contrast to patients with wild-type *DNMT3A*. Importantly, dose-intensive induction therapy resulted in a significantly higher improvement in survival in patients with *DNMT3A* or *NPM1* mutations or *MLL* translocations, but not in patients with wild-type *DNMT3A* and *NPM1* and no *MLL* translocations. These findings thus indicated that genetic profiling is useful in fine-tuning the risk category of AML patients, and thus influence therapeutic strategy.

In addition to genetic heterogeneity across patients, treatment of AML is further complicated by cellular or tumor heterogeneity within an individual patient. Studies have established that an individual instance of an AML tumor is composed of different subpopulations of leukemic stem, progenitor, and blast cells resembling the structure of a normal hematopoietic hierarchy (Dick 2005). Analogous to how normal hematopoietic stem cells (HSCs) initiate and maintain blood formation, leukemia stem cells (LSCs) initiate and maintain disease. LSCs are largely quiescent (Guzman et al. 2001), which renders them relatively unresponsive to standard chemotherapeutics such as cytarabine that most efficiently target cycling AML blasts (Costello et al. 2000). Thus, LSCs that resist chemotherapy are postulated to be the likely source of disease relapse.

Supporting this concept, recent clinical reports have found that AML patients presenting with a higher LSC percentage are at greater risk of minimal residual disease (MRD) and worse outcome (van Rhenen et al. 2005). Thus, improvements in AML survival are likely to arise from the development of therapies that eradicate LSCs.

Increasing efforts have been placed on the identification of markers that can distinguish HSCs from LSCs. While some markers such as CD34 and CD38 are similar to those on the HSCs, others including CD123, CD44, CLL-1, CD47, CD25 and CD32 have been shown to be specific for the LSC (Felipe Rico et al. 2012). The identification of such markers has permitted the study of AML enriched in LSCs, enabling early efforts that sought to identify dysregulated targets in LSCs that distinguish them from HSCs. These efforts first revealed that constitutive activation of the transcription factor NF-κB, a known regulator of growth and survival, is essential for LSC maintenance (Guzman et al. 2001). This finding led to development of preclinical therapies for ablating LSCs based on NF-κB inhibition, including strategies based on proteasome inhibitors and the sesquiterpene

lactone parthenolide (PTL) (Guzman et al. 2002, 2005). Despite the well-documented efficacy of PTL in pre-clinical work, it suffers from poor bioavailability and pharmacokinetics. Thus, time-consuming medicinal chemistry efforts were initiated and successfully re-formulated PTL into a compound suitable for clinical use in phase I trials, dimethylaminoparthenolide (DMAPT) (Guzman et al. 2007).

The major challenge in the development of LSC-targeted agents lies with screening of drugs suitable for clinical use. High-throughput screens for such agents are impractical since the assay to detect impaired LSC function is murine xeno-transplantation with serial transplantation of the tumor (Dick et al. 1991). No valid LSC cell line models currently exist. While NF-κB inhibition appears to be a valid criterion for selecting small molecules that ablate LSCs, studies suggest that it is insufficient and that other properties such as induction of oxidative stress are also essential (Guzman et al. 2001; Jordan and Guzman 2004).

In contrast to single molecule or phenotype drug screens, recent developments in chemical genomics suggest that a description of biological states and drug effects in terms of transcriptional signatures can efficiently and rationally select drugs and drug combinations to improve development of cancer therapies, including therapies with complex outcomes including differentiation, drug sensitization, and LSC ablation (Hassane et al. 2008, 2010; Corsello et al. 2009; Lamb 2007; Wei et al. 2006; Hieronymus et al. 2006; Stegmaier et al. 2004). Indeed, early studies in yeast established that a compendium of gene expression profiles can be used to "connect" biological states and infer non- obvious functional relationships between genes (Hughes et al. 2000). This concept was more recently extended to human cancers in which a compendium of gene expression profiles was generated capturing transcriptional perturbations caused by FDA-approved compounds in the Connectivity Map (CMap) project (Lamb 2007).

Significant time, effort and resources are spent towards development of targeted therapies; unfortunately, in AML, few defined druggable targets actually exist. Additionally, overemphasis on a single protein or target may not be therapeutically beneficial in a complex heterogeneous disease like AML. Finally, target based screening often entails the use of in vitro assays, which often do not reflect the biological complexity of cells and tissues. Discovery approaches enabled through the CMap and related variants such as gene expression-based high throughput screening (GE-HTS) have advanced drug screens beyond simple phenotypes. By using transcriptional profiling as the main driver for drug discovery, effective therapeutic molecules can be selected without prior knowledge of a particular target or molecular mechanism of action of a drug. Moreover, drugs can be selected that exert complex cellular effects such as differentiation and LSC ablation.

Compendium-Based Screening

Hughes et al. (2000) initially described how a compendium of gene expression profiles could be used to infer biological relationships. The authors generated a compilation of transcriptional profiles in *S. cerevisiae* consisting of 300 different mutations and chemical treatments, and thus created the first "compendium" of gene expression profiles. This reference database established proof-of-principle that would later apply to gene expression-based drug discovery: (1) different mutations affecting the same cellular process had similar transcript profiles, irrespective of whether the interaction was direct or indirect; (2) low magnitude changes in transcript levels could also contribute biologically significant results; and (3) cellular functions of previously uncharacterized open reading frames (ORFs) could be predicted by simply comparing the unknown expression profile to known mutation profiles within the compendium. The authors created deletion mutants of several genes whose functions were not known, and using the compendium as a reference, found that these genes had important roles in a multitude of cellular processes, including sterol metabolism, mitochondrial respiration, cell wall function, and protein synthesis. The study thus illustrated that

it is indeed possible to use a compendium of expression profiles to understand the properties of pharmacological compounds, in a manner akin to fingerprinting, making it a powerful tool in drug discovery.

Gene Expression-Based High Throughput Screening (GE-HTS)

Other than acute promeyelocytic leukemia (APL), where all-trans retinoic acid (ATRA) has been extremely successful as a differentiation-inducing agent (de The and Chen 2010), the paucity of defined targets has greatly impeded the development of targeted therapy in AML. To overcome this predicament and identify new compounds for AML differentiation, Stegmaier and colleagues employed GE-HTS, a powerful approach that entails the use of gene expression signature as a marker for a biologic state (Stegmaier et al. 2004). In this approach, genome-wide expression profiling is first performed to define a set of genes with transcriptional changes that discern two different biological states of interest. Then, a small molecule library is screened for agents that can induce transcriptional changes on this subset of genes using a quantitative assay for these gene expression changes.

Using this technique for the first time as proof-of-principle, the authors performed microarray analyses on BM samples of AML patients as well as completely differentiated neutrophils and monocytes from the peripheral blood of normal volunteers. On comparing the gene-expression profile of the two sets (normal vs AML), they obtained with a 'differentiation signature' based on five genes that were: (1) highly predictive and representative of the myeloid differentiation state; and (2) also regulated in an AML cell line. This 5-gene signature was used to screen a small molecule library of 1,739 compounds. After eliminating false hits, the authors identified eight compounds that could induce differentiation in AML cells. Remarkably, it was observed that two of these eight hits had already been previously shown to induce myeloid differentiation, using standard biological assays. Furthermore the authors confirmed that these agents could actually induce a genome-wide alteration in the differentiation profile of the AML cells, and not merely a change in the expression of the five signature genes used for the screen. It was revealed that all eight compounds identified in the screen could induce at least one of the several parameters used by the authors to define the "myeloid differentiation" state, and four of the compounds could induce changes satisfying all the parameters, further validating the robustness and reliability of this technique.

The advantages of this technique are multiple; a key major advantage being that presumptive knowledge of a specific target is not required. Furthermore, transcriptional signature consisting of relatively few genes is enough to capture the differentiation profile. False hits can be easily eliminated by repeat measurement of the signature. Although used primarily for differentiation induction in AML, this technique has the advantage of being applied to any other biological "state of interest", for example identifying compounds that can target common translocations in AML, traditionally considered to be extremely difficult to chemically modulate. Using the GE-HTS technique, Corsello et al. (2009) showed that corticosteroids and dihydofolate reductase (DHFR) inhibitors like methotrexate could very effectively block the effects of the AML-ETO translocation, one of the most common structural aberrations in AML. Very recently, using the GE-HTS approach, Banerji et al. (2012) demonstrated that inhibition of GSK3α could induce differentiation, inhibit proliferation, induce apoptosis, and also ablate AML activity in vivo, indicating that GSK3α inhibition can be used as a new strategy in AML therapy.

Connectivity Map (CMap)

Similar to the compendium of expression profiles in *S. cerevisiae* described by Hughes et al. (2000), Lamb (2007) developed the Connectivity Map (CMap) database as a publicly available resource facilitating the interference of relationships between compounds as well as the relationship between compounds and biological states via

their gene expression signatures. The CMap is a publicly available collection of transcriptome data of several human cancer cell lines (MCF7, PC3, HL60 and SKMEL5) profiled using the Affymetrix platform following treatment with a number of small molecules or "perturbagens". Each particular treatment is defined relative to its vehicle- treated control, comprising an "instance". Two versions have been released thus far. CMap (build 01) consisted of 164 unique compounds. The current version (build 02) consists of over 7,000 profiles representing more than 1,300 compounds.

Access to the CMap database is facilitated by a convenient web-based interface, although raw data are available to permit analyses of greater complexity. Relationships are interrogated by supplying the web interface with a "query signature" consisting of two gene sets: the up-regulated and down- regulated genes associated with a condition of interest. The up-regulated and down-regulated gene sets are individually compared against each treatment- control instance of the CMap using the signed Kolmogorov-Smirnov-like "enrichment score" (ES) statistic employed in gene set enrichment analysis (GSEA) (Subramanian et al. 2007). Therefore, the ES for the up-regulated and down-regulated genes against each CMap instance are combined to generate a positive, negative, or zero "connectivity score" that indicates whether a CMap instance is most similar, opposite, or unrelated to the "query signature", respectively. Thus, positive connectivity scores suggest a likelihood of similar functions whereas negative connectivity scores suggest opposite or antagonistic functions.

Several studies have demonstrated the utility of CMap. For example, Hieronymus et al. (2006) sought to identify small molecule inhibitors of androgen receptor (AR) signaling in prostate cancer. Using an AR-activation gene signature in the LnCaP prostate cancer cell line, two new compounds, celastrol and gedunin, were revealed to be inhibitors of AR signaling. Using connections between transcriptional states in the CMap, they discovered that both celastrol and gedunin modulate AR-signaling via Hsp90 inhibition. Thus, not only were new AR signaling inhibitors identified, but celastrol and gedunin were revealed to exhibit Hsp90 inhibitory function.

Wei et al. (2006) used the CMap successfully to identify compounds that could reverse glucocorticoid (GC) resistance phenotype in acute lymphoblastic leukemia (ALL). The authors performed gene expression analysis on several pre-treatment ALL samples that were classified GC-sensitive or resistant based on the IC50 values, and thus defined a GC sensitivity signature. On querying the CMap database with this signature, the authors found that the mTOR inhibitor sirolimus (rapamycin) exhibited the most positive connectivity score with respect to the signature of GC sensitivity, indicating similarity. Of further note, gene set enrichment analysis (GSEA) identified that the Akt pathway was overexpressed in resistant samples and treatment with rapamycin sensitized the ALL cells to GC-induced apoptosis, thereby further implicating this pathway in GC resistance in ALL. Rapamycin indeed sensitized GC resistant ALL and this effect was achieved via the modulation of the anti-apoptotic protein MCL1. This approach facilitated the rational selection of rapamycin and glucocorticoids in combination to overcome GC resistance in ALL, demonstrating the utility of the CMap in translational medicine. It is important to note that the query signature generated by the authors was obtained by treating patient ALL samples, and not any of the cell lines used in the CMap database (primarily MCF7 breast cancer cells in build 01). This study indicated the feasibility of using this approach across different cell types when conserved pathways are present, further strengthening the effectiveness of gene expression-based drug discovery.

Gene Expression-Based in Silico Strategies to Ablate Leukemia Stem Cells

Subsequent studies in AML sought to use gene expression-based drug discovery to find new drugs for ablating LSCs. At the time the study commenced, the only single agents known to ablate LSCs were parthenolide (PTL) and its

pharmacologically optimized derivative, DMAPT (Guzman et al. 2005, 2007). Toward generating a more structurally diverse repertoire of small molecules for targeting LSCs, transcriptional perturbations resulting from exposure of CD34+AML cells to PTL for 6 h were captured on the Affymetrix microarrays across 12 patients alongside untreated controls. A 150 gene PTL response signature was generated and compared to 16,000+microarray profiles in the Gene Expression Omnibus (GEO) (Edgar et al. 2002) using weighted correlation and machine learning approaches (Hassane et al. 2010). This search revealed compounds previously demonstrated to have efficacy against LSCs including the proteasome inhibitor MG-132 and prostaglandin J2 (Guzman et al. 2001, 2005). The screen additionally revealed two new compounds, celastrol and 4-hydroxy-2-nonenal. Celastrol is a triterpene derived from the Chinese "Thunder of God" vine with demonstrated inhibitory activity against NF-κB (Jin et al. 2002) and Hsp90 (Hieronymus et al. 2006). 4-hydroxy-2-nonenal is a lipid peroxidation product with the documented ability under various conditions to inhibit the proteasome (Conconi and Friguet 1997), inhibit NF-κB via disruption of IκB phosphorylation (Page et al. 1999), and induce Nrf2- controlled antioxidant genes including heme oxygenase I (Siow et al. 2007). To determine whether these compounds were PTL-like as predicted by the gene expression based screen, hallmarks of PTL exposure in AML such as NF-κB inhibition and Nrf2 activation were confirmed for celastrol and 4-hydroxy-2- nonenal. Next, the ability of these small molecules to mimic the anti-AML effects of PTL was ascertained at the bulk, stem and progenitor level. Both celastrol and 4-hydroxy-2-nonenal eradicated CD34+CD38- AML derived from primary patient specimens upon ex vivo exposure within 24 h. Moreover, AML progenitor function was impaired in colony forming unit assays performed in methylcellulose culture medium without significant effects on normal myeloid and erythroid colony formation. Finally, the ability of these compounds to impair engraftment of AML into NOD/SCID mice was determined to ascertain impaired LSC function. This screen effectively expanded the repertoire of agents for targeting LSCs in a manner unrelated to the generation of structural derivatives of PTL. Transcriptome level descriptions of drug effects clearly allow the drug discovery process to overcome restrictions to particular chemical structures or families of that may also share in undesirable pharmacological attributes, thereby vastly increasing the likelihood of identifying a structure suitable for clinical use. The transcriptome can also serve as a blueprint, revealing unappreciated drug effects that may be undesirable, allowing for the selection of rational drug combinations that suppress undesirable attributes exerted by drugs.

Subsequent studies revealed undesirable attributes of PTL exposure and used connections established by transcriptome profiling to suppress these attributes (Hassane et al. 2010). The PTL signature used to discover celastrol and 4-hydroxy-2-nonenal was submitted to analysis in CMap (build 02). The PTL signature was found to demonstrate negative connectivity scores to compounds inhibiting PI3K/mTOR pathway, signifying that PTL exerts opposite transcriptional effects relative these inhibitors, which included wortmannin, LY-294002, and sirolimus (rapamycin). This finding generated the hypothesis that the effect of PTL may be to activate the PI3K/mTOR pathway on the basis that PTL exerts opposite transcriptional effects relative to PI3K/mTOR inhibitors. This finding was notable in that activation of PI3K is known to lead to activation of Nrf2 (Nakaso et al. 2003) and its downstream antioxidant genes. Nrf2 activation is a well-established consequence of PTL exposure (Umemura et al. 2008). To test this hypothesis, activation of mTOR was verified through analysis of mTOR substrates including its immediate downstream phoshorylation target, p70S6K. This finding suggested that combination of PTL with mTOR inhibitors would suppress Nrf2 cytoprotective responses. Targeting of LSCs by the combination of PTL or its derivative of DMAPT was significantly enhanced in the presence of the rapalogue temsiroliumus in both ex vivo assays and in NOD/SCID mice with preestablished AML xenotransplants. Moreover,

the combination of PTL/DMAPT with rapalogues impaired PTL-mediated Nrf2 activation as well as the activation of Nrf2-controlled antioxidant genes such as heme oxygenase I. Thus, in addition to identifying similar compounds, gene expression based drug discovery demonstrated utility in making existing compounds more effective at lower doses through rational selection of drug combinations.

Selection of Drugs that Antagonize Transcriptional Programs Operating in Leukemia Stem Cells

Ultimately, the leukemic state is differentiated from the normal state via a complex interplay of mutations and/or epigenetic alterations that modify cell signaling and transcriptional programs. Thus, if gene expression based screening could be used to directly ascertain the drugs that maximally reverse these programs, the drug discovery process would be greatly expedited. A pair of recent studies has explored this concept (Ashton et al. 2012; Sampson et al. 2012.) based on the notion of defining small molecules that result reverse "cooperation response genes" (CRGs) defined by the recent work of McMurray et al. (2008). These investigators discovered that a subset of genes is synergistically up-regulated or down-regulated given the presence of two cooperating oncogenes. However, these same genes may only be subtly altered in the presence of single oncogene. RNA interference studies demonstrated the functional relevance of these CRGs. Indeed, 14/21 CRGs contributed to tumor formation, but only 1/14 genes responding in a non-synergistic manner contributed to tumor formation. These findings suggested that CRGs hold special importance in malignancy and that their genetic and perhaps chemical suppression is most likely to disrupt tumor formation. In follow up to the original RNA interference work, a CMap-based gene expression screen was performed in which small molecules were sought that maximally antagonized CRG expression changes. This screen revealed the efficacy of HDAC inhibitors as antagonists of CRG expression profile and their efficacy in reducing tumor in a colon cancer system (Sampson et al. 2012).

The concept of directly discovering small molecules that reverse the expression of genes crucial for tumor maintenance was then applied to the selective eradication of LSCs. To this end, investigators employed a previously described mouse model of blast crisis CML (bcCML) involving the cooperation between *BCR-ABL* and *NUP98-HOXA9* that yields a defined LSC population with the specific immunophenotype of Lin^- c-$Kit^{+/-}$ $Flt3^+$ $Sca\text{-}1^+$ $CD34^+$ $CD150^-$. To define CRGs in this system, primitive hematopoietic bone marrow cells (Lin^- $Sca\text{-}1^+$ c-Kit^+) were transduced with a retroviral vector expressing *BCR-ABL* marked with green fluorescent protein (GFP) and a retroviral vector expressing *NUP98-HOXA9* marked with yellow fluorescent protein (YFP). Transduced cells were transplanted in congenic mice (CD45.1 marker) and primitive Lin- cells were sorted by flow cytometry, separating untransduced cells from cells transduced with *BCR-ABL* and/or *NUP98-HOXA9* on the basis of fluorescent protein expression. Gene expression profiling was performed on each cell population and CRGs identified according to the procedure of McMurray et al. (2008). Gene expression based screens against a CMap-based database (build 01) was used to reveal small molecules that reverse the CRG signature of primitive bcCML cells, revealing 4-hydroxy-2-nonenal and tyrphostin AG825, a known ErbB2 inhibitor (Osherov et al. 1993), that was selectively toxic to primitive LSC-enriched bcCML, without significant effects on normal mouse HSCs.

Conclusions

Considerable advances mark the last decade in cancer research with respect to molecular understanding of disease. However, translating these advances for therapeutic benefit in the clinic has posed a major challenge. Therefore, alternative approaches that go beyond target-based drug discovery are essential. One novel approach that has proven fruitful is the use of gene expression based drug screens. By using the entire cellular

transcriptome as the main reference/driver for drug discovery, prior knowledge and characterization of a defined target is not needed. Thus, databases driven by gene expression based drug discovery such as the CMap serve as a powerful platform for discovering FDA approved compounds that recapitulate the effects experimental small molecules, drugs that interact favorably with existing compounds in clinical trials, and drugs that "normalize" the transcriptional programs entire disease states without necessarily first understanding the details of the mechanisms underlying these states. As databases such as the CMap are poised to expand and include more small molecule and RNA interference-based perturbations in more cellular contexts, the power of gene expression-based drug discovery and its routine integration into more discovery efforts is likely.

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Isolation of Cancer Stem Cells Showing Drug Resistance in the Human Epithelia Ovarian Cancer

9

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Contents

Abstract

Epithelial ovarian cancer remains the most lethal gynecologic malignancy and high recurrence rates due to resistance to further treatment with chemotherapy. Evidences showed that a pool of self-renewing malignant progenitors, cancer stem cells (CSCs) may contribute to chemotherapy resistance. Thus, targeting of CSC fraction may be critical for eliminating cancers cells. Here we will discuss the current knowledge regarding isolation of ovarian CSCs, EMT and the role of regulation by miRNAs in the context of drug resistance. Furthermore, we discuss direct CSC targeting approaches, for which therapeutic proof of principle has recently been established.

Introduction

Epithelial ovarian cancer remains the most lethal gynecologic malignancy despite recent advances in adjuvant chemotherapy. Although the majority of patients with Stage III/IV ovarian cancer initially respond to platinum-based therapies, recurrence rates are high due to resistance to further treatment with chemotherapy. The overall 5-year survival rate has not been significantly improved dispite advances in treatment. The mechanisms underlying chemoresistance in cancer are not clear. One hypothesis is that certain cells in the cancerous

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population had acquired changes that conferred drug resistance and hence a selection advantage, eventually generating a new tumor population resistant to chemotherapy. These cells compose a pool of self-renewing malignant progenitors known as cancer stem cells (CSCs) or cancer-initiating cells (CIC) (Dalerba et al. 2007). Such CSCs have been thought to contribute to chemotherapy resistance (Croker and Allan 2008). Thus, treatment of the drug-resistant CSC fraction may be critical for ultimate cure rates. Here we will focus on the newly discovered mechanisms of drug-resistance of CSCs and the potential therapeutic strategy targeting CSCs.

Isolation of Ovarian Cancer Stem Cells

Recent reports have demonstrated that CSCs from epithelial organs can be expanded as sphere-like cellular aggregates in serum-free medium (SFM) containing epidermal growth factor (EGF) and basic fibroblast growth factor (bFGF) (Uchida et al. 2000), a method derived from the culturing of neural stem/progenitor cells (NSCs) (Kondo et al. 2004). In ovarian cancer, Bapat et al. (2005) isolated two clones from patient ascites that took on anchorage-independent, spherical structures (spheroids) in culture. These clones formed xenografts in nude mice, with a histopathology similar to the parental human tumors, underwent serial propagation in animals, and expressed the stem cell factor receptor CD117. Further, Zhang et al. (2008) reported that CD44+ & CD117+ cells derived from primary human ovarian tumors are associated with CSCs characteristics. Our lab has previously obtained self-renewing and anchorage-independent spheroids by culturing patient-derived ovarian cancer cells or SKOV3 cell line in stem cell-selective conditions. The spheroid cells expressed stem cell markers including Oct-4, nanog, nestin, Sox-2, ABCG2, CD133 and CD117 surface markers and had the characteristics of drug resistance (Liu et al. 2010; Ma et al. 2010).

Drug Resistance of Cancer Stem Cells

The proliferating and differentiated cells that form the bulk of the tumor can be destroyed by chemotherapy intervention. However, cancer stem cells which represent approximately 0.1–10% of all tumor cells are relatively quiescent/dormant and have protective mechanisms for repairing DNA and counteracting cytotoxic drugs, thus CSCs can escape chemotherapy-induced cell death after treatment but eventually re-enter the cell cycle which leads to tumor re-growth and tumor resistance (Eyler and Rich 2008).

Moreover, the specific membrane proteins shared by both CSCs and normal stem cells have been associated with CSCs drug resistance. For example, ATP-binding cassette (ABC) drug transporters, which are highly expressed in normal stem cells, are membrane efflux and also highly expressed in many tumors. Researchers considered ABCG2 transporter to be a protective protien for stem cells to maintain progenitor cells in an undifferentiated state. They thought ABCG2 may serve as the primary line of defense against the cytotoxic effects of drug, which is one of the most important biological characteristics of cancer stem cells (Krishnamurthy and Schuetz 2006). We also demonstrated that the cancer stem cells isolated from ovarian cancer cell line SKOV3 highly express ABCG2 and might contribute to more drug resistance (Ma et al. 2010).

One of the main hallmarks of neoplastic transformation is deregulation of cell proliferation. The cell cycle checkpoints are activated after DNA damage and induce cell cycle arrest allowing repair of damaged DNA. P53 and the checkpoint protein kinases (Chk1 and Chk2) are the most critical molecules in the DNA damage machinery after chemotherapy or ionizing radiations. In particular, p53 induces growth arrest by holding the cell cycle at both the G1/S and G2/M regulation points (Vogelstein et al. 2000) whereas Chk1 contributes to DNA damage repair by affecting S-phase and G2/M phase arrest (Xiao et al. 2003). Compared to Chk2, Chk1 possesses an essential role in the maintenance of DNA integrity. Bartucci

et al. (2012) investigated the DNA damage response in cancer stem cells derived from non-small-cell lung cancer (NSCLC) patients. They found the cancer stem cells undergo cell cycle arrest for DNA damage repair after exposure to chemotherapeutic drugs. And they demonstrated that Chk1 was the earliest and most significant activated independently of p53 status. Furthermore, the use of Chk1 inhibitor in combination with chemotherapy reduced cancer stem cells growth either in vitro or in vivo. The increased expression of DNA repair genes such as MEOX2, SULF1, $IL_{17}RD$ etc. has been seen in $CD133^+$ glioblastoma cells which were radio-resistant and $CD44^+/CD24^-$ breast cancer cells (Phillips et al. 2006). Small molecule inhibition of Chk1 or Chk2 kinases could make CSCs more sensitive to radiation.

Additionally, CSCs express increased levels of proteins to improve survival. We focused on analyzing the altered gene profile in ovarian CSCs and differentiated cells using cDNA microarray analysis followed by functional clustering. A large proportion of the classified genes were found to be related to angiogenesis, extracellular matrix, integrinmediated-signaling pathway, cell adhesion and cell proliferation (Ma et al. 2010). Evidences show that aldehyde dehydrogenase (ALDH-1) was amplified in leukaemic CSCs enabling them to resist cyclophosphamide and indicated poor prognosis (Ginestier et al. 2007). Serrano et al. (2011) discovered that one subset of lung cancer cells enriched in markers of stemness and endowed with stem cell properties were positive for ALDH. $ALDH^+$ CSCs display longer telomeres than the non-CSC population. In ovarian CSCs, the results showed that the expression and activity of ALDH enzyme was dependent on specific cell types (Saw et al. 2012).

Researchers generally accepted that differentiated cells will acquire a multipotent stem cell like phenotype in the conditions such as tissue injury or tumorigenesis. These transdifferentiation programs are identified as epithelial to mesenchymal transitions (EMT) which are required for tissue morphogenesis during embryonic development. Cancers are currently considered as abnormal tissue injury and never healing. Thus cancer cells may also undergo adaptive changes following therapy. Furthermore, Wnt, Notch and Hdegehog, known to regulate self-renewal and stem cell maintenance of both normal stem cells and CSCs are also involved in the tight regulation of EMT (Malanchi et al. 2008). Intriguingly, EMT indeed can trigger reversion to a CSC-like phenotype (Polyak and Weinberg 2009), which providing a potential mechanistic explanations for the association between EMT, CSCs and drug resistance. AL-Hajj et al. (2003) found that disseminated breast cancer cells derived from pleural effusions were enriched for a $CD44^{high}/CD24^{low}$ CSC-like population, which indicate that metastatic cancer cells had presumably undergone EMT and exhibit CSC phenotype. Additionally, stem cells isolated from normal breast tissue or breast cancers express a number of canonical EMT markers. According to the report by Yauch et al. (2005), microarray analysis of non-small cell lung carcinoma (NSCLC) tumour cell lines indicated that epithelial but not mesenchymal gene signature sensitized resistant cancer cells to erlotinib, the small molecule-EGFR (the epidermal growth factor receptor)-inhibitor, and induced growth inhibition. Further clinical trials also confirmed that erlotinib provides more benefit to the NSCLC patients with high E-cadherin levels compare to the E-cadherin negative patients (Thomson et al. 2005). The expression of intact HER-4, E-cadherin and β-catenin and loss of expression of platelet-derived growth factor receptor β are associated with response to cetuximab (an inhibitory monoclonal antibody to EGFR) therapy. Among these proteins, E-cadherin seems to play a central role in modulation of EGFR response in urothelial carcinoma. Silencing E-cadherin in two sensitive cell lines reduced sencibility to cetuximab in both.

Target CSCS by Molecular Interventions Against Molecular Markers of CSCS and Tumor Microenvironment

Because CSCs are molecularly distinct from differentiated tumor cells, these difference molecules can be used to target CSCs. For instance, the specific cell surface marker such as CD133, CD44, CD117 could be used for antibody-directed therapy to target proteins. The ATP-dependent drug efflux pumps (ABC transporter), could be targeted

by ATP-competitive agents (Kuhnle et al. 2009). Schatton et al. (2008) isolated a subpopulation from human malignant-melanoma-initiating cells (MMIC) based on the expression of the chemoresistance mediator ABCB5. Subsequently a monoclonal antibody directed at ABCB5 was used in melanoma xenotransplantation experiment and studies showed tumour-inhibitory effects.

Nevertheless, the using of antibodies to target CSCs is still challenging, since CSCs are just sub-fraction in tumor cells. Moreover, CSC markers overlap with other CSCs and normal stem cells. And there are still many CSC specific markers not identified by us. There are many other markers which are not strictly CSC specific antigens but are important for maintaining CSCs within the tumor microenvironment. A specialized microenvironment consisting of cells, matrix proteins and growth factors is known as a 'niche'. Many molecules such as Notch, Wnt, Hedgehog, BMPs and FGF have been identified to be important for CSCs formation. These molecules can be an indirect approach to target CSCs. For instance, targeting brain CSCs by anti-angiogenic therapy can reduce CSCs stemness and increase CSCs sensitivity to chemotherapy, since vascular endothelial cells are critical components of the neural stem cell niche (Folkins et al. 2007). However, targeting CSCs by antibodies and other molecular interventions can only increase survival rate and improve quality of life but not cure cancers.

Target CSCS by Repressing the EMT

Since EMT is involved in triggering the reversion to a CSC-like phenotype, preventing the EMT progress of cancer cells may encourage the elimination of cancer cells. Recently, a proof of principle study showed that poorly differentiated cancers with a high CSC content can be induced to a differentiated state. It was reported by Gupta et al. (2009) that one compound, salinomycin, sensitize the breast CSCs to paclitaxel and it also inhibited the tumor growth in animal model. The global gene expression profiling showed that CSC genes obviously reduce in breast cancer treated with salinomycin. Interestingly, salinomycin induced the differentiation of mesenchymal-like cancers in vivo, accompanied by increased expression of E-cadherin and reduced expression of vimentin. Further evidences come from the studies about histone deacetylase (HDAC) corepressor proteins which repress expression of E-cadherin during EMT. Researchers also found that HDAC was associated with degradation of HIF-1α and repression of NF-κB expression, both which drive EMT and drug resistance (Kong et al. 2006).

Recently, a role for microRNAs in TGF-β signaling has been appreciated, since several reports indicate that TGF-β induced EMT in cancer cells is dependent on microRNA function. Members of TGF-β family perform importantly in embryonic development and tissue homeostasis in the adult. They exert their cellular effects via binding to serine/threonine kinase receptors(TβRI & TβRII) followed activation of downstream effector proteins in which the Smads transcription factors are most notably. Comijn et al. (2001) reported that Smads recruit ZEB proteins to downregulate mammalian E-cadherin transcription. Wellner et al. (2009) demonstrated that ZEB1 could upregulate expression of stem cell factor such as Sox2 and Klf4 in cancer cells and mouse embryonic stem cells through suppress expression of stemness-inhibiting miR-200c, miR-203 and miR-183, which also was a promoter of mobile, migrating cancer stem cells. Consistently, Burk et al. (2008) also reported that microRNA-200 family member miR-141 and miR-200c strongly activated epithelial differentiation in various cancer cells, and ZEB1 could regulate their transcription. Interestingly, they found these microRNAs could reversely target and downregulate the EMT activators TGFβ2 and ZEB1. They proposed that ZEB1 triggers a microRNA-mediated feedforward loop that stabilizes EMT and promotes invasion of cancer cells.

Target CSCS by Immunotherapy

Recent clinical studies have shown that chemotherapy combined with immunotherapy has survival benefits in comparison with chemotherapy alone.

Moreover, immunotherapy can sensitize tumors to chemotherapeutic agents mediated killing. For instance, sensitivity of tumor cells to subsequent drug cytotoxicity is increased by T cells via up-regulation of death receptors DR5 and Fas, to which TRAIL and CD95L/FasL ligands bind, respectively (Galon et al. 2006). Most current immunotherapeutic approaches aim at inducing antitumor response via stimulating the adaptive immune system, which is dependent on MHC-restricted T cells. However, loss of MHC molecules is often observed in cancer cells, rendering tumor cells resistant to T-cell-mediated cytotoxicity. γδT cells exhibit potent MHC-unrestricted lytic activity against different tumor cells in vitro, suggesting their potential utility as anticancer therapy. Our group reported (Lai et al. 2011) that γδT cells suppressed the proliferation of ovarian CSCs either in vitro and in vivo, followed with down-regulation of CSCs mark genes. Furthermore, γδT cells increased the sensitivity of ovarian CSCs to chemotherapeutic drugs and induced G2/M phase cell cycle arrest and subsequent apoptosis in ovarian CSCs. Interestingly, we found the level of IL-17 production significantly increased after coculturing γδT cells with ovarian CSCs. This indicated that γδT cells may efficiently kill ovarian CSCs mediated with IL-17 and represent a promising immunotherapy for ovarian cancer. Koehn et al. (2012) indicated that neuroblastoma (NBL) cells can be destroyed via antibody-dependent cell-mediated cytotoxicity (ADCC) by using monoclonal antibodies which bind to the high expression protein of disialoganglioside (GD2) in NBL. The clinical studies also proved that patients' cancer-free survival has been improved after treated with anti-GD2 mAb in combination with cytokines such as IL-2 and GM-CSF which enhance ADCC. According the report of Vatakis et al. (2011), immune-based therapies such as the adoptive transfer of tumor-specific autologous T cells have been used to target cancers. These autologous T cells are peripheral T cells which are transduced ex vivo with a vector expressing a T-cell receptor (TCR) specific for a certain cancer-associated antigen and reintroduced them into patients. But it seems limited to use this approach, since a lot of manipulation before transduction may make T cells reduce their potency. Moreover, the genetically engineered peripheral T cells do not undergo negative selection in thymus, which may induce autoimmunity. Finally, peripheral T cells are not long lived and may not support a lasting therapy. Further, researchers considered that antigen-specific T cells generated from human hematopoietic stem cells (hHSC) would overcome the disadvantages above. They used a modified version of the bone marrow/liver/thymus (BLT) humanized mouse model in the study, which allows long-term peripheral immune reconstitution. The specific T cells generated from human hematopoietic progenitors, which were transduced with an HLA-A*0201–restricted, melanoma specific TCR, were transplanted into the model. The T cells immunity protected the mouse challenged subsequently with the HLA-A*0201–matched human melanoma. Importantly, the transduced cells were detected 4 months later after implantation, which indicated long-term immune reconstitution in treated mice.

In conclusion, the current findings initiate an understanding of CSCs in ovarian epithelial cancer. These results indicate that CSCs may contribute to the failure of existing therapies to consistently eradicate ovarian cancer cells. Therefore, CSCs represent novel and translationally relevant targets for clinical cancer therapy. Importantly, proof-of-principle experiments have strengthened the rationale for developing CSC-targeted therapeutic modalities that might complement more conventional cancer therapies. Indeed, CSC-targeted approaches have shown promise in preclinical models. These approaches include direct strategies, such as ablation by targeting molecular markers of CSCs or CSC-specific pathways, reversal of resistance mechanisms, and differentiation therapy, and indirect strategies, such as antiangiogenic therapy, immunotherapeutic approaches, and disruption of protumorigenic interactions between CSCs and their microenvironment. Continuing efforts along divergent lines to elucidate the existence, association, and contribution of these cells are necessary to ultimately make the promise of specific targeting of these cells in cancer therapy a reality.

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Cancer Stem Cells in Pediatric Sarcomas

10

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Contents

Abstract

Sarcomas are the third most common group of malignancies in children. The current treatment strategies for sarcoma patients are outdated, have severe side-effects and increase the probability of secondary neoplasms. Clinical benefit from therapy has reached a plateau and local recurrence is still a major problem. Altering the traditionally held view of sarcomagenesis as a purely genetic disease to accommodate the more recent cancer stem cell paradigm could offer not only fresh insight into sarcoma initiation and progression but also reveal new avenues for therapeutic interventions. Here we critically appraise the evidence for the presence of sarcoma stem cells in various pediatric sarcoma entities such as Rhabdomyosarcoma, Ewing sarcoma, Osteosarcoma, Undifferentiated Pleomorphic Sarcoma and Synovial sarcoma. The functional and molecular characterization of such stem cell-like cells has provided some hints as to the possible origins of sarcoma and it is likely that the sarcoma stem cell phenotype could be dependent on the sarcoma cell of origin and its genetic background. Finally the impact of sarcoma stem cell research in various aspects of patient care is explored. As of yet, the clinically relevant drugs targeting sarcoma stem cells have been sparse and their identification represents a clear need for further research.

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Introduction

Sarcomas comprise a heterogeneous group of rare malignancies (5.9 cases per 100,000 inhabitants) with features of mesodermal or neural crest origin (Ferrari et al. 2011). They consist of a large number of histological variants with their own etiologies and consequently different clinical outcomes. They pose a unique challenge to researchers and clinicians alike as they cross the age spectrum. Although in total numbers more adults are diagnosed with sarcoma its relative incidence is higher in the pediatric populations, accounting for 7.4% of all pediatric malignancies. Significant differences have been noted in the occurrence pattern and clinical outcome of sarcoma with age. Rhabdomyosarcoma (RMS) is the most common sarcoma in the first two decades of life (median age of 15 years), followed by malignancies of fibroblastic histologies such as Undifferentiated Pleomorphic Sarcoma (UPS; previously referred to as Malignant Fibrous Histiocytoma) and fibrosarcoma (Ferrari et al. 2011). Synovial sarcoma and Ewing sarcoma could be considered as transitional tumor types as they overlap the pediatric and young adult age groups. Within a sarcoma histotype predilection to site of occurrence also changes with age, for example, Ewing sarcoma in children is predominantly diagnosed in the bone while in adults it manifests itself in the soft tissues. Survival of sarcoma patients generally decreases with increasing age at diagnosis which could be due to differences in clinical assessment and management of adult and pediatric sarcoma. There may additionally be inherent differences in the tumor biology but the available data is too limited to draw conclusions. Therefore this review will focus on sarcoma histotypes and subhistotypes which are most common in the pediatric population or are in the transitional zone between children and young adults.

Over the past two decades molecular genetics has played an increasingly important role in the diagnosis of sarcomas, as a significant number of them possess characteristic chromosomal translocations encoding for either aberrant transcription factors, for example alveolar RMS (aRMS), Ewing sarcoma and synovial sarcoma, or leading to constitutive growth factor signaling as in Dermatofibrosarcoma Pertuberans. The translocation-positive sarcoma have a near-diploid karyotype which is in mark contrast to the translocation-negative sarcoma subtypes such as embryonal RMS (eRMS), osteosarcoma, and UPS, which have rather complex karyotypes.

Translocation-negative soft tissue sarcomas have been associated with congenital syndromes and birth defects. Patients of Li-Fraumeni and Retinoblastoma Syndrome are predisposed to eRMS and osteosarcoma. There is an increased incidence of osteosarcoma in patients with germline mutations in helicase genes such as in Bloom Syndrome (mutant BLM), Rothmund Thomson Syndrome (mutant RECQL4) and Werner Syndrome (mutant RECQL2). Embryonal RMS is also known to have increased incidence in patients with Neurofibromatosis (NF1) mutations; it has also been diagnosed in patients with Gorlin Syndrome, Rubinstein-Taybi Syndrome, Beckwith-Wiedmann Syndrome, Costello Syndrome and its associated syndromes (Noonan and LEOPARD).

The etiology of sarcomas remains largely unknown. The sarcoma fusion proteins have been shown to be necessary for the survival of the cancer cell but they seem not be sufficient for tumorigenicity in most cases. Genetic instability is a hallmark of fusion negative sarcoma. Mouse models and data from epidemiological and molecular studies suggest that genetic mutations affecting chromosome mechanics and cell cycle checkpoints can lead to sarcoma formation. Loss of cell cycle checkpoints seems to be a recurrent theme in sarcomagenesis, including mutation of Retinoblastoma protein (Rb1), deletion of CDKN2A (INK4A locus), PTEN mutations and amplification of CDK4. Loss of p53 activity has been widely reported in various sarcoma subtypes through different mechanisms. Commonly found activated oncogenic pathways include MYC, HRAS and FOS. Various growth factor receptors are either amplified or the signaling pathways have been shown to be activated in different sarcoma subtypes (Broadhead et al. 2011; De Giovanni et al. 2009; Haldar et al. 2008; Lessnick and Ladanyi 2012). Lately the

importance of developmental pathways such as Wnt (canonical and non-canonical), Hedgehog, Notch and Transforming Growth Factor β (TGFβ) has been shown in diverse sarcomas (Belyea et al. 2012; Broadhead et al. 2011). Although the causative role of the above mentioned molecular aberrations is questionable, their importance in tumor progression is undeniable. There could also be as of yet unidentified functionally significant molecular changes. Interestingly it was recently noted that 29% fusion negative RMS were independent of p53, Rb1, Hedgehog or RAS mutant signatures (Rubin et al. 2011).

Treatment approach to sarcomas is multimodal which includes surgery, chemotherapy and optional radiotherapy. Pediatric sarcomas are generally considered as chemosensitive and radiosensitive tumors. However there is a likelihood of late metastasis and even though the tumor entity is considered to be chemosensitive the early benefits from therapy have been seen to dissipate over time. The treatment regimen for pediatric patients includes high dose systemic multi-drug chemotherapy that is not well tolerated by adult patients. Even so, local control still remains the major challenge in patients. It has been reported that 30–40% of children and young adults with sarcoma, irrespective of histology, will develop recurrent or metastatic disease and less than 25% of them will survive in the recurrent setting. The minority of patients that fall under the Intergroup Rhabdomyosarcoma Staging Group II (IRSG II) are known to not respond to chemotherapy. The IRSG IV patients (diagnosed metastatic disease at onset) have a 20% survival rate and the disease is highly refractory. Osteosarcoma tumors also consist of chemoresistant population with only 20% survival rate noted when treated with chemotherapy. Forty percent of osteosarcoma patients do not survive the disease and there has been no significant improvement in the treatment of metastatic disease. The scenario is similar for Ewing sarcoma where about 50% of patients survive and the survival rate has remained unchanged for decades. Surgery with negative margins is the only way to achieve complete remission. However, complete resection is not possible in most cases; therefore modeling of sarcoma initiation and progression based on biological data and clinical experience can help delineate key nodes for therapeutic intervention.

Models of Sarcomagenesis

Once a causative hit has initiated a lesion the question remains as to how this progresses to a heterogeneous malignant tumor mass. Different models of oncogenesis have been postulated and could be applied to sarcoma progression.

Clonal Evolution

The widely accepted model of oncogenesis was posited by Peter C. Nowell in 1976 where he described the evolution of a tumor at clonal level in response to selective pressure, making it more aggressive with time (Fig. 10.1a). The most telling evidence of clonal evolution would involve cytogenetic analysis at different stages of the disease. Such longitudinal view of clonal aberrations has rarely been done for pediatric sarcomas and, the few studies that do so, fail to show clonal evolution due to extensive intratumor heterogeneity. As discussed above, sarcomas cross the age spectrum with infants developing malignant lesions within the first year of their lives, leaving a very narrow window for clonal evolution. However, an increase in karyotypic complexity has been attributed to progressive disease in human sarcomas but it is unclear if this is an etiological feature. Therefore, to date, apart from the characteristic chromosomal translocations no specific clonal change has been shown to be causative or even necessary for tumorigenesis, recurrence or relapse. Overall the evidence for clonal evolution in *pediatric* sarcomas is scant but these tumors are genetically unstable and the importance of genetic changes is undeniable.

Cancer Stem Cell Models

The Cancer Stem Cell (CSC) model has seen a revival recently. The hypothesis postulates that analogous to normal regenerating organs tumors

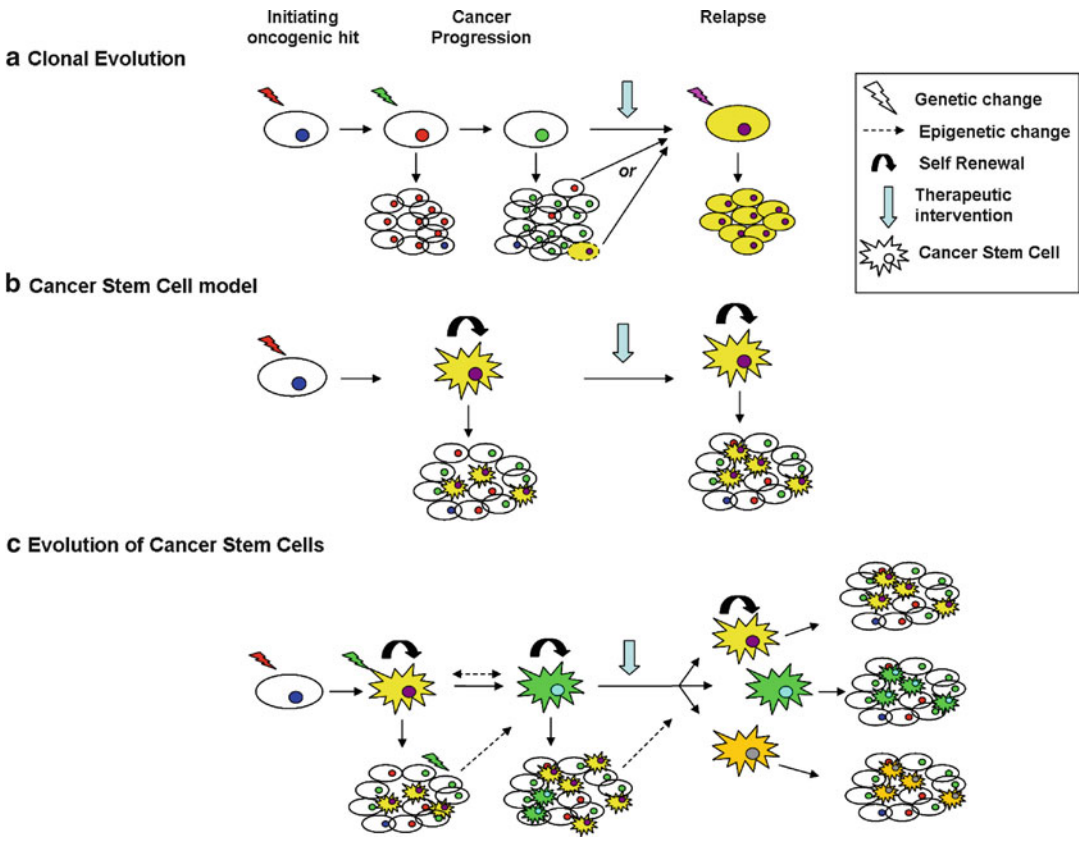

Fig. 10.1 Models of sarcomagenesis. (**a**) Sarcoma formation could be modeled based on selection of the 'fittest' clone. Upon the primary genetic disruption the 'cell of origin' becomes neoplastic and forms a homogeneous tumor mass which increases in heterogeneity, albeit with the persistence of a dominant clone. Therapeutic intervention leads to a selective sweep of a drug resistant mutant that causes tumor re-growth. (**b**) Sarcomas could also be hierarchically arranged where the initiating oncogenic change causes the 'cell of origin' to assume a stem cell-like phenotype that is able to divide asymmetrically to give rise to the heterogeneous tumor bulk. Upon therapeutic intervention the cancer stem cells (*CSCs*) persist and reform a heterogeneous tumor which is identical to the primary tumor. (**c**) The genetic and functional heterogeneity of sarcoma cells could be explained by considering an evolutionary model of CSCs where genetic and epigenetic changes lead to a co-existence of different CSC populations that respond to selective pressures

contain a subset of undifferentiated multipotent self-renewing stem-like cells while the bulk of the tumor consists of differentiated cells incapable of long-term sustenance of tumor growth (Fig. 10.1b). Although currently CSCs are considered only immunophenotypically different from their non-CSC counterparts, investigations into the genetic or epigenetic differences between these populations and even more interestingly the stability of the CSC phenotype have begun recently.

The origin of CSCs is still under speculation. Initially they were implicitly understood as cells resembling the 'cell of origin' of the tumor which in most cases has been thought to be a transformed stem cell that has continued to persist in limited numbers in a progressing tumor. However recent evidence suggests that differentiated cells could reacquire properties of stem cells due to epigenetic changes or oncogenic hits. It is also possible that CSCs emerge as a tumor progresses from a benign lesion to a malignant one. Also there might not be just one CSC phenotype but multiple which are either selected depending on the need of the tumor or are generated de novo (Fig. 10.1c). The CSC phenotypes need not be stable and

could be a purely epigenetic phenomenon based on niche factors. This latest, more complex notion, is an amalgamation of the clonal evolution and CSC models where CSCs obey Darwinian rules of evolution. This model confers versatility to a progressing tumor, explaining many of the clinical phenomena observed and additionally accounting for the functional heterogeneity within tumors (Valent et al. 2012).

Sarcomas have an extensively heterogeneous cellular milieu. The presence of undifferentiated multipotent cells in sarcoma was highlighted a couple of decades ago (Katenkamp and Raikhlin 1985). Osteosarcoma tumor cells show similarities to primitive osteoblasts with less aggressive osteosarcoma tumors being more similar to osteogenic MSCs while the more aggressive phenotypes often resemble early progenitors. Osteosarcomas have been known to possess areas of 'dedifferentiation' that can emerge de novo with an abrupt transition from the differentiated areas. It has been noted that Ewing sarcoma cells have a potential for multidirectional differentiation (Pinto et al. 2011). Even though sarcomas are generally considered to have a mesodermal origin some tumor cells can also exhibit positive immunostaining for epithelial markers as in synovial sarcoma. Recently it was shown in a zebrafish model of eRMS that the in vivo cellular heterogeneity was of functional significance for tumor migration (Ignatius et al. 2012). In case of the translocation positive tumors almost all cells express the chimeric protein. However the phenotypic effect of the fusion protein depends on its expression levels which could be varied within a heterogeneous tumor mass. Therefore one could envision a subset of cells within even an apparent clonal tumor which could be more tumorigenic than the bulk of the tumor. Clinical observations in pediatric sarcomas, as discussed previously, hint at the presence of chemoresistant cellular populations within sarcoma tumor entities that are capable of relapse giving rise to heterogeneous tumors. Interestingly the recent success of an oral maintenance therapy trial against metastatic childhood sarcoma raised the possibility of stem cell-like cells being present within sarcomas (Cripe 2008). Therefore, over the past few years, efforts have been made towards identifying and characterizing sarcoma stem cells.

Identification of Pediatric Sarcoma Stem Cells

Sarcoma stem cells have been isolated using different methods that either employ a phenotypic trait such as cell surface antigen or a functional property such as self renewal. Specifically, the CSC model has been most widely studied in osteosarcoma. In order to limit extensive referencing the reader is requested to obtain the detailed information of the primary studies summarized below in previously published reviews (Basu-Roy et al. 2012; Riggi et al. 2011; Trucco and Loeb 2012).

Cell Surface Markers

CD133

Prominin 1 or CD133 is the most promiscuous cancer stem cell marker. It has been used to isolate CSCs in colon neoplasia, brain cancer and many other epithelial cancers. Similarly it has also been shown to be expressed in many pediatric sarcomas such as eRMS, aRMS, osteosarcoma, Ewing sarcoma and synovial sarcoma. CD133 expression was found to be variable between patients diagnosed with the same tumor type and there was no specific correlation with pediatric or adult origin of the cell line or tumor.

CD133 is known to exist in different glycosylated forms of unknown functional significance. The most commonly used glycosylated epitope of CD133 in epithelial cancers is CD133/1 or AC133. Accordingly in Ewing sarcoma AC133 has been used to isolate a self renewing tumorigenic subpopulation from primary patient tumors. In eRMS CD133/2 or AC141 was found to be enriched in the sphere cultures and was subsequently used for isolating eRMS stem cells from human cell lines (Walter et al. 2011). In studies where only polyclonal CD133 antibodies were used the reconciliation with previous publications is difficult. Interestingly it was noted by

Sana et al. (2011) that the total CD133 expression was rare in primary embryonal and alveolar RMS tumors but increased in the corresponding tumor derived cell lines. Importantly the proportion of total CD133 did not change during the progression of aRMS from primary lesion to recurrence and relapse, even under the influence of chemotherapeutics. The CD133/2 epitope was expressed in synovial sarcoma cell lines and tumor samples, although most of the samples were from adult patients (Terry and Nielsen 2010; Tirino et al. 2011). Naka et al. (2010) studied primary synovial sarcoma derived cell lines that possessed a high degree of self renewing capacity in vivo but sparse CD133/1 expression. The expression level of CD133/2 was not evaluated. In many studies involving osteosarcoma the epitope status of CD133 has not been mentioned. Tirino et al. (2011), however, have specifically shown that the CD133/2 epitope is expressed in pediatric osteosarcoma cell lines and in pediatric and adult osteosarcoma tumor biopsies.

Others

CD117 (c-kit), in combination with Stro-1, was found to enrich for CSCs in osteosarcoma mouse and human cell lines. However, other studies have found primary bone sarcomas and osteosarcoma cell lines to be negative for CD117 expression. Interestingly Stro-1 was initially found to be expressed by osteosarcoma spheres. More recently, work by Zhang et al. (2013) has shown that osteosarcoma spheres were in fact CD117 and Stro-1 positive.

Another potential surface marker that needs further assessment in osteosarcoma is the primitive mesenchymal marker Sca-1. It was observed in a transgenic mouse model that a small population of tumor cells expressed Sca-1 which correlated with increased tumorigenicity. The self renewal property of the Sca-1$^+$ population was not addressed and it is yet to be studied in human samples.

In the case of eRMS it was shown that FGFR3 could be a candidate marker to isolate CSCs. FGFR3 positive cells were capable of tumor formation at lower cell numbers than the FGFR3 negative cells. But FGFR3 expression was not enriched in eRMS sphere cultures which were shown to have increased tumorigenicity. Additionally, Pressey et al. (2013) have reported that most of the FGFR3$^+$ cells from RMS cell lines of eRMS and aRMS origin were also positive for CD133.

It has also been recently highlighted that the somatic stem cell marker LGR5 is heterogeneously expressed among different Ewing sarcoma cell lines and patient samples but it is still not clear if it marks a self-renewing population (Scannell et al. 2013). It is a very interesting marker for further investigation since the functional role of LGR5 in development and in the field of CSC research has been firmly established.

Assays Based on Functional Properties

Sphere Formation and Self Renewal

Sphere formation was developed as a method to isolate neural stem cells. Similarly, the neurosphere assay has also been used to enrich for primitive long-term self renewing cells from solid cancers. Tumor cells are allowed to form clonal spherical colonies in suspension in serum-deprived media containing specific growth factors and maintained in long-term cultures. Spheres are thought to arise from primitive cells and consist of cells with varying potential for differentiation. Therefore they cannot yield pure CSC populations. The sphere assay can be used for identification of cell surface markers that could be used to prospectively sort cellular subpopulations from heterogeneous cell lines and primary tumor material as was done for eRMS. Conversely, cellular sub-populations sorted using surface antigens can be cultured as spheres to evaluate and compare the self-renewing capacity in vitro and consequently validating the utility of the surface antigen as a CSC marker. In sarcoma stem cell research sphere assays have been primarily used to indicate the presence of primitive cells in osteosarcoma, eRMS, Ewing sarcoma, synovial sarcoma and UPS cell lines of human and animal origin (Fujii et al. 2009; Naka et al. 2010; Walter et al. 2011; Zhang et al. 2013). Sphere formation efficiency in osteosarcoma cell lines correlated with tumorigenicity in vivo and it was noted that osteosarcoma patient tumor cells

from pulmonary metastasis formed larger spheres than primary tumor cells (Rainusso et al. 2011).

Tumor cells with high self renewal capacity can also be isolated based on their tumorigenic capacity in vivo or expression of genes important for conferring 'stemness'. Recent work in synovial sarcoma showed that primary tumor derived cell lines (of unspecified patient age) had a great capacity for tumorigenesis under serial transplantation. Heterogeneous expression of the embryonic transcription factors Oct4 and Nanog was seen within the tumor. However no definitive surface marker was found for prospective isolation of the CSC populations (Naka et al. 2010). In a zebrafish model of eRMS a subpopulation of tumor cells resembling activated satellite cells was identified based on increased self renewal capacity in vivo (Langenau et al. 2007). In all reports that studied CSC populations in osteosarcoma it became evident that the pluripotency genes Nanog, Oct4 and Sox2 were enriched in the self renewing tumorigenic subpopulations (Basu-Roy et al. 2012; Zhang et al. 2013). Functionally Oct4 and Sox2 have been validated as maintaining tumor-initiating cells in osteosarcoma by different studies. Interestingly, Sox2 expression correlated with the previously described Sca-1 marker. Whether the Sox2 expressing cells are also Oct4 positive however has not been investigated. Also, the proportion of cells which were Sox2-Sca-1^{high} was large (45–75%). Since serial transplantation in vivo was not performed one cannot be sure if the Sox2-Sca-1^{high} population is the true CSC compartment which is considered to be rare.

Quiescence

Analogous to most normal stem cells, CSCs could be quiescent and hence not affected by drugs that target actively proliferating cells. Quiescent cells can be identified by their property of retaining lipophilic membrane dyes such as PKH26. It was observed in osteosarcoma cell lines that 8–25% of the total population consisted of quiescent cells that were capable of long-term sphere formation and in vivo tumor formation at lower numbers. However drug resistance was not directly addressed (Rainusso et al. 2011).

Drug Resistance

Clinically relevant cancer stem cells could express membrane proteins that are able to efflux drugs or enzymes that are capable of drug detoxification. Therefore the property of drug resistance could be used to identify CSC populations.

Side Population Analysis

Cells that express the drug efflux protein family including ABCG2 and MDR1 can be isolated by their property to efflux the DNA binding dye Hoechst 3342 using flow cytometry. These cells are labeled as 'Side Population' (SP) and have been shown to have stem cell-like properties. Although isolation of SP cells has been carried out in various sarcoma entities of pediatric and adult origin their stem cell characteristics have not been proven in all cases. This could be due to the various technical aspects of SP analysis which makes reproducibility difficult. Recently, using primary osteosarcoma cells from an undefined age group Yang et al. (2011) showed that the isolated SP cells showed higher tumorigenicity in vivo. The SP cells have been reported to be heterogeneous in their tumor initiating capacity and therefore there is room for further enrichment of sarcoma stem cells by combining with other CSC markers and/or features. However none of the previously identified CSC markers, such as CD133 and CD117, segregated with the SP cells (Yang et al. 2011).

Aldehyde Dehydrogenase I Activity

A reliable method to identify cellular populations that would be resistant to alkylating drugs measures the activity of a detoxification enzyme Aldehyde Dehydrogenase 1 (ALDH1) using flow cytometry. Recently, a population with high activity of ALDH1 (ALDH1^{high}) was identified in pediatric OS99-1 osteosarcoma cell line which was tumorigenic at lower numbers than the low ALDH1 activity cells and could additionally self renew in vivo. Interestingly, it was shown that the proportion of ALDH1^{high} cells increased in xenotransplanted tumors in comparison to cell lines which highlights the importance of niche-dependent effects.

In Ewing sarcoma cell lines and early passage primary xenografts, a small population of

ALDH1^{high} cells was noted. When the cells with high and low ALDH1 activity were isolated and injected in immunocompromised mice only 0.6% of the ALDH1^{high} cells were capable of tumor formation (Awad et al. 2010). Similar to SP analysis not all ALDH1^{high} cells were capable of tumor initiation. The authors also used CD133/2 to isolate tumorigenic population and concluded that ALDH1 activity was a better marker. However, the CD133/1 epitope has been established as a Ewing sarcoma stem cell marker and hence it is still unclear whether CD133/1 expression or ALDH1 activity marks stem cell-like cells. Interestingly, the ALDH1^{high} cells were shown to be resistant to doxorubicin, a characteristic which has not yet been proven for CD133/1^{+} cells.

Selection Under Drug Treatment

Drug treatment could also be used to enrich for tumor cells with resistance capacity, and which are consequently responsible for tumor relapse. Therefore to select for cells with drug resistance properties osteosarcoma cell line MG63 was treated with a PARP inhibitor (Di Fiore et al. 2012) or grown as spheres in media containing vincristine, a commonly used drug in clinics (Lou et al. 2010). Treatment with PARP inhibitor led to the formation of a heterogeneous cell line with high sphere forming capacity and differentiation potential. The spheres formed under vincristine selection were enriched for total CD133 and drug efflux proteins. A non-tumorigenic pediatric osteosarcoma cell line HOS and aRMS cell line Rh4 were selected for cisplatin resistance and it was found that the resistant cells had an increased SP profile. Interestingly cisplatin treated HOS SP cells were capable of tumor formation in vivo (Tsuchida et al. 2008). Similarly, the SP proportion within UPS xenotransplanted tumors increased considerably when treated with conventional chemotherapeutics in vivo (Wang et al. 2012).

Characterization of Pediatric Sarcoma Stem Cells

The primitive cellular subpopulations, once identified and isolated, must fulfill certain functional criteria before being designated as bona fide CSC population. One of the key properties that a CSC population must possess is that of long-term self renewal. Although the in vitro sphere formation assay is indicative of a self renewing population it is not always definitive. Therefore higher propensity for tumor initiation must be shown in vivo by limiting dilution and preferably the self renewing capacity of the subpopulation elucidated by serial transplantation. Earlier studies on osteosarcoma stem cells did not include in vivo validation; either the experiments were not attempted or the tumor cells were deemed to be graft resistant. However recent work has shown that it is indeed possible to grow osteosarcoma cell lines and even primary cells in immunocompromised mice. Importantly some of these studies also showed that the subpopulations could self renew in vivo. Similar results have been obtained for eRMS, aRMS, Ewing sarcoma and UPS. A stem cell also possesses the ability to differentiate into various lineages. Consequently many reports have shown that sarcospheres are capable of differentiation. The multipotential characteristic of sarcoma stem cells could explain the observed heterogeneous cellular populations within the tumors that display immunophenotypes of different germ layers. Some sarcoma stem cells have also been known to possess a more lineage restricted stem cell phenotype. In eRMS, the sarcospheres were enriched for lineage specific stem cell genes such as Pax3 and Pax7 while downregulating genes important in myogenic differentiation (Walter et al. 2011), with similar results observed for the CD133^{+} eRMS cells (Pressey et al. 2013). The eRMS stem cells identified from RAS-dependent sarcomas were shown to have an activated satellite cell (adult muscle stem cell) gene expression profile. Osteosarcoma and Ewing sarcoma stem cells have been shown to possess mesenchymal stem cell features.

Sarcoma stem cells have been shown to be enriched for various genes that are important for embryonic stem cell self renewal including Oct4, Nanog and Sox2. These transcription factors are enriched in sarcospheres and in sorted sarcoma stem cell sub-populations isolated from various sarcoma entities. Although Oct4 expressing cells were specifically shown to have CSC properties in osteosarcoma the role of Oct4 in sarcoma biology

has not been established due to experimental and biological limitations. Sox2 expression in osteosarcoma seems to maintain Sca-1 expression and the undifferentiated state of the tumor-initiating population. In Ewing sarcoma it was shown that expression of Sox2 had a functional consequence for the tumorigenicity of cell lines and that Sox2 is a target gene of EWS-FLI1. Other stem cell associated genes used to characterize sarcoma stem cells are Bmi-1, Stat3, Nucleostemin, Msx1 and Nestin.

Apart from expressing 'stemness'-associated proteins sarcoma stem cells could also possess activated developmental pathways, such as Wnt, Hedgehog, Notch and TGFβ. TGFβ signaling has been shown to be an important mediator of self renewal in osteosarcoma (Basu-Roy et al. 2012). Furthermore TGFβ1 induced dedifferentiation of osteosarcoma cells led to the upregulation of various components of the Notch, IGF and PDGF pathways, making it a very interesting signaling node for future studies (Zhang et al. 2013). The gene expression profiling of osteosarcoma stem cells isolated based on their property of quiescence showed that IGF1 and IHH were expressed at high levels. Interestingly IGF, Hedgehog and TGFβ pathways have been previously implicated in osteosarcomagenesis. Curiously RECQL expression was downregulated in the quiescent osteosarcoma stem cell population. Patients with loss of function mutations in RECQL have a higher incidence of osteosarcoma than the general population and so far the role of RECQL in sporadic osteosarcoma has not been established. Recently, activation of canonical Wnt pathway across the sarcoma spectrum was reported; the study concluded that the effect of Wnt signaling in initiating a stem cell-like phenotype was overshadowed by other mutations within the cells (Vijayakumar et al. 2011). Specifically in Ewing sarcoma it was recently shown that canonical Wnt signaling pathway can be activated using Wnt ligands and further potentiated by R-Spondin which is known to bind LGR5. Activation of Wnt signaling or LGR5 overexpression had no effect on proliferation; however it remains to be seen if it could enhance self-renewal (Scannell et al. 2013). On the contrary, activation of non-canonical Wnt signaling was necessary for derivation of UPS from MSCs and in osteosarcoma stem cells (Basu-Roy et al. 2012; Matushansky et al. 2009). Therefore it is still an open question whether *just* canonical Wnt signaling plays a role in sarcomagenesis. Embryonal RMS stem cells isolated from transgenic zebrafish RAS model possessed higher levels of Id2, Notch2 and Gli3. A transcription factor essential for myogenic differentiation, MyoD, is inhibited by Id2. Interestingly Notch signaling is important in muscle stem cell division and Hedgehog signaling has been implicated in eRMS sarcomagenesis. Wang et al. (2012) have observed that SP cells from primary UPS samples expressed higher levels of Hedgehog and Notch pathway components. Inhibition of these pathways in vivo decreased the tumor growth and also markedly reduced the tumor initiation capacity of these cells upon serial transplantation in secondary and tertiary recipients. Interestingly, while conventional chemotherapy decreased tumor growth it did not affect the ability of the cells to form tumors upon serial transplantation. Overall, the function of developmental pathways in sarcoma stem cells is slowly being elucidated and is a prime avenue for future research.

Oncogenic pathways that provide the sarcoma stem cells with their increased tumorigenic capacity are also unresolved. Cisplatin resistant osteosarcoma and aRMS SP cells displayed autocrine VEGF signaling which was dependent on increased MAPK-ERK pathway activity and therefore sensitive to MAPK or VEGF inhibition (Tsuchida et al. 2008). Also RAS-dependent eRMS stem cells were shown to be c-MET/HGFR positive. Interestingly mice that express HGF develop eRMS with a high penetrance, perhaps hinting towards an HGF autocrine signaling in a sarcoma stem cell compartment.

Clinically relevant property of CSCs is their ability to evade conventional drug onslaught. To this end it has been shown that osteosarcoma, Ewing sarcoma and eRMS stem cells isolated using different techniques are resistant to commonly used therapeutics such as cisplatin, doxorubicin, daunorubicin, methotrexate, etoposide, vincristine and chlorambucil. Stro-1^+ human osteosarcoma cells were specifically found to be enriched for the drug efflux protein ABCG2. The theoretical consideration earlier was that a stem cell nature

implied quiescence and hence only a minimal effect of a DNA damaging agent. However most reports of sarcoma stem cells have not specifically highlighted a slower cell cycle kinetic for the CSC compartment and in some cases even a higher proliferation index was reported. An alternative explanation for resistance to replication stress inducing drugs could be efficient DNA repair mechanisms. It was shown that osteosarcoma and Ewing sarcoma sphere cells overexpress DNA mismatch repair genes MLH1 and MSH2 (Fujii et al. 2009). Gene ontology analysis of eRMS spheres showed that DNA mismatch repair pathway was significantly overrepresented (Satheesha S., unpublished data). Anti-apoptotic pathways have also been shown to be activated in osteosarcoma cell line selected for resistance to PARP inhibition.

From studies in carcinoma it is becoming evident that genetic background has a bearing on CSC phenotype but its impact on the sarcoma stem cell phenotype has not yet been evaluated. There are indications that the p53 and Rb pathways could play important roles in determining cell plasticity as their functions in differentiation, de-differentiation and pluripotency induction is being revealed. In osteosarcoma and Ewing sarcoma sphere cells it was noted that the expression of p14INK4a/p19ARF was reduced (Fujii et al. 2009). Also it was noted that pediatric MSCs had a decreased expression of p14INK4a which could account for the higher cellular proliferation and possibly the permissiveness for EWS-FLI1 expression. Therefore the p53 and Rb pathway status of sarcoma cells could have an impact on the phenotype of sarcoma stem cells. In case of the translocation-positive sarcomas it was shown that the expression levels of the fusion protein determined tumorigenic capacity in aRMS (Xia et al. 2009) and in synovial sarcoma the fusion protein was necessary to form spheres (Naka et al. 2010). In Ewing sarcoma there was no difference in the expression of EWS-FLI1 between adherent and sphere cultures but ALDH1^{high} cells were sensitive to inhibition of the fusion protein activity (Awad et al. 2010; Fujii et al. 2009). However, expression of EWS-FLI1 in pediatric MSCs led to reprogramming of the cells to assume a primary Ewing sarcoma phenotype by repressing the expression of miRNA-145 (Riggi et al. 2011). Further investigations have revealed that the CD133/1^{+} fraction of Ewing sarcoma cells show disrupted TARBP2-depedent miRNA processing which seems to be important for the CSC-like nature of the cells (De Vito et al. 2012).

There are of course limitations and caveats to the studies discussed above. Since pediatric sarcomas are rare malignancies most of the data collected have been from cell lines or their xenografts in immunocompromised mouse models. However, wherever possible the existence of sarcoma stem cells should be directly validated using primary patient material and/or transgenic animal models. Alternatively the identified marker or pathway could also be validated retrospectively on a large cohort of patient samples as was done for CD133 in eRMS where high CD133 expression significantly associated with lower overall survival (Walter et al. 2011) and in osteosarcoma where CD133 expression positively correlated with lung metastasis and proved to be an independent prognostic marker (He et al. 2012). Technical caveats such as limited reproducibility of the SP analysis or lack of adherence to the CD133 epitope status across studies makes generalizations difficult at the moment. Also, care must be taken as to the use of the most stringent mouse model. It is interesting to note that the site and media composition of tumor cell engraftment had a critical role in not only primary tumor generation but also formation of metastasis, especially in osteosarcoma (Di Fiore et al. 2012; Rainusso et al. 2011). This highlights the importance of niche factors in the tumorigenicity of cells and consequently the significance of orthotopic engraftment routes. Technical aspects of primary tumor tissue handling, such as enzymatic digestion and engraftment media composition, could have profound effects on tumor formation in vivo and therefore affect the robustness and reproducibility of the CSC marker.

Overall there seems to be a subpopulation of highly tumorigenic cells in many pediatric sarcoma entities that are multipotent and capable of self renewal in vitro and in vivo. It is possible that the property of quiescence cannot be

universally applied to all sarcoma stem cell populations and the proportion of cells within a tumor that possess stem cell-like properties is unclear as this appears to depend on the isolation technique used. Along with intra-tumoral, inter-tumoral heterogeneity will play an important role in dissecting the sarcoma stem cell biology and also establish its clinical significance. Also, one of the key questions to answer would be the origin of sarcoma stem cells.

Origin of Sarcoma Stem Cells

The histogenesis of sarcomas is usually obscured by cytological features. Using tissue specific expression of oncogenes or loss of tumor suppressors along different lineages in mice it has been shown that cells at various stages of differentiation can be transformed to give rise to sarcomas. However, the origin of sarcoma stem cells has not been addressed directly in most cases. Following the classical CSC model, they might be persistent clones of the 'cell of origin' within a tumor or in all likelihood some cells might have de-differentiated to assume a 'cell of origin' phenotype (Fig. 10.2). The stem cell origin of mesenchymal neoplasms was postulated decades ago (Katenkamp and Raikhlin 1985). Recent efforts have indicated that MSCs could be likely candidates for 'cell of origin' in many of the pediatric sarcoma entities. Ewing sarcoma tumors show features of mesenchymal stem cells. The $CD133^{+}$ cells from primary Ewing sarcoma could differentiate into mesenchymal lineages and interestingly upon EWS-FLI1 expression pediatric MSCs assumed a CSC phenotype. It has been possible to generate osteosarcoma from various points in the osteogenic differentiation lineage in vivo, including from early mesenchymal progenitors.

Based on gene expression profiling it has been hypothesized that eRMS stem cells might be the same as the 'cell of origin' which could be an activated satellite cell. Analogous to osteosarcoma, eRMS 'cell of origin' has been investigated by targeted ablation of Ptch1, p53 and Rb1 activities in a range of cells in the myogenic lineage and it appears that eRMS cells have an activated satellite cell profile irrespective of the cell of origin (Rubin et al. 2011). Since even mature myoblasts can be transformed to give rise to eRMS it is likely that eRMS stem cells are de-differentiated cells that resemble a stem cell within the lineage hierarchy. Alternatively it was shown that eRMS sarcospheres resembled the gene expression of neurospheres and glioma patient samples although the cells expressed higher levels of primitive myogenic transcription factors than the adherent eRMS cells (Walter et al. 2011). Therefore the CSCs in this case point towards a possible origin from a neuroectodermal derivative with myogenic potential. A neural crest origin has been previously posited for eRMS as evinced by eRMS development in mice with NF1 mutation. Embryonal RMS is known to occur in body parts where there are no known skeletal muscle cells present. Also the rare sarcoma entity, Ectomesenchymoma, intriguingly and most commonly, presents with intermixed areas of neuronal and rhabdomyosarcomatous differentiation and is believed to arise from a multipotent neural crest cell. Recently it has been shown using transgenic mouse models that eRMS could arise from a non-skeletal muscle, albeit mesenchymal, lineage (Hatley et al. 2012). It is apparent that fusion-negative RMS could arise from multiple lineages and therefore the phenotype of its CSC could also be varied.

Synovial sarcoma was shown to arise from myoblasts in vivo (Haldar et al. 2008). However tumor derived cell lines when grown as spheres were capable of recapitulating an early mesenchymal cell phenotype that still retained the ability to differentiate into the hematopoietic lineage (Naka et al. 2010). Therefore it is also possible for a tumor cell to acquire properties of CSCs that does not resemble either the cell of origin or a progenitor cell but a pluripotent stem cell. Based on chromatin profiling UPS cells were shown to resemble MSCs most closely (Matushansky et al. 2009) but recent in vivo modeling showed that eRMS and UPS could be a continuum of the same tumor type when arising from muscle cell lineage (Rubin et al. 2011). There has been no evidence yet regarding the lineage status of the UPS stem cells. Similarly the origin of the aRMS

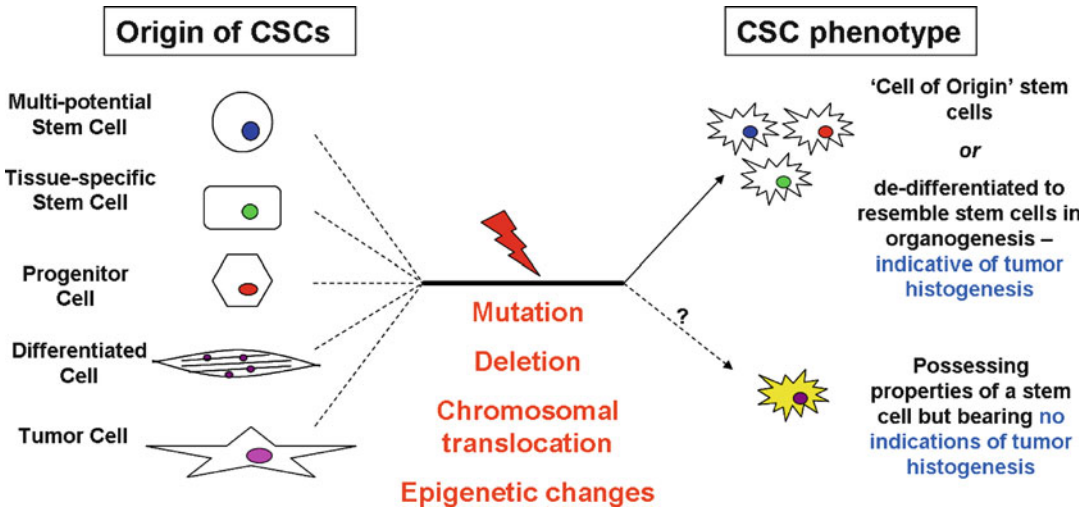

Fig. 10.2 Origin of sarcoma stem cells. Sarcoma stem cells could be persistent clones of the 'cell of origin' which could be a neoplastic stem cell or a de-differentiated mature cell. Sarcoma stem cells could also be tumor cells that have acquired the stem cell properties to either resemble the 'cell of origin' or other stem cells within the tumor histogenic lineage. Molecular characterization of these sarcoma stem cells could shed light on the developmental background of the tumor. However it is also possible that the sarcoma stem cell phenotype resembles a stem cell that has no direct bearing on the lineage of tumor development but is acquired due to advanced genetic changes or niche pressures

stem cell is also unconfirmed although an MSC origin for aRMS has been postulated (De Giovanni et al. 2009).

It should be noted that the characterization of sarcomas modeled in vivo has mainly included histological analysis and gene expression profiling; relevance to the human situation in other aspects such as latency and tumor site predilection is difficult to ascertain. Additionally, single sarcoma histological entities have been shown to have different 'cell of origin' but it is as of yet unknown if the sarcomas generated from different cellular compartments are also similar at the molecular level. It is also evident that depending on the genetic change a cell could give rise to a different spectrum of sarcomas. Therefore if one assumes that in most cases the sarcoma stem cell compartment will resemble the 'cell of origin' or cells within the lineage of tumor histogenesis then the phenotype of sarcoma stem cells could vary widely. Also, there may be multiple sarcoma stem cell populations within the same tumor with varying self-renewal potential.

Based on these concepts a new model of sarcomagenesis could be envisioned. It is likely that unique sarcoma histology can arise from multiple cells with different genetic and epigenetic backgrounds that need not be even within the same developmental lineage. It is also evident that the co-operative effects of physiological status of the sarcoma initiating cell and the oncogenic lesion determine the tumor phenotype (Rubin et al. 2011). The initiating or most dominant oncogenic lesion could behave as a lineage determinant as in the case of Ewing sarcoma where EWS-FLI1 causes expression of neural proteins and its inhibition makes the cells more mesenchymal (Pinto et al. 2011). Therefore the final phenotype of the tumor may not be indicative of tumor origin. Once a sarcoma lesion has been initiated the tumor progression need not just involve accumulation of further genetic lesions but also the gain of functional heterogeneity in the form of sarcoma stem cells in order to aggressively progress as a refractory tumor. Whether the functional cellular heterogeneity in sarcoma arises from genetic or epigenetic variation is still uncertain. If sarcoma stem cells arise from epigenetic mechanisms then the phenotype would be labile and the population much more difficult

to target. Furthermore, the sarcoma stem cell population could possibly be susceptible to selective pressure. Therefore in order to optimally design clinical trials and monitor treatment effectiveness it would be important to determine the origin and stability of the stem cell phenotype and its role in minimal residual disease.

Translational Relevance

Current treatment strategies for childhood sarcomas have reached a plateau (Norris and Adamson 2012). Research in the field of sarcoma stem cells will have an impact in all aspects of patient care (Fig. 10.3). The current design rationale of clinical trials would need to be altered to accommodate the sarcoma stem cell model. Sarcoma stem cell marker expression levels would be important guidelines for patient recruitment and also would be used to assess the efficacy of the treatment. As of now the promiscuity of the sarcoma stem cell markers make them difficult to use in the diagnosis of a sarcoma entity. However, immunophenotyping for CSC marker could facilitate prognostic conclusions and risk stratification. Possibly the most important outcome of investing in sarcoma stem cell research would be the identification of novel targets for drug intervention. Local recurrence and its refractoriness is still a major problem for sarcoma patients. Therefore the need of the hour in the research field is not only identification of tumor sustaining subpopulations but also the mechanisms that are necessary for the survival of these cells, be it cell intrinsic or extrinsic, in order to target them efficiently. In all likelihood the pathways that need to be inhibited could be important for normal developmental processes also. This poses a unique problem in the childhood cancer setting as children would be much more vulnerable to side effects of these drugs than adult patients. Therefore it will be crucial to design clinical trials to accommodate for not only short term benefit but also long term monitoring of the health status of patients.

In order to prevent the 'generation' of new CSCs with their own oncogene addiction it would be important to combine drugs that target CSCs with other targeted chemotherapeutics which have shown benefit against bulk tumor growth as the non-CSC population could also have functional

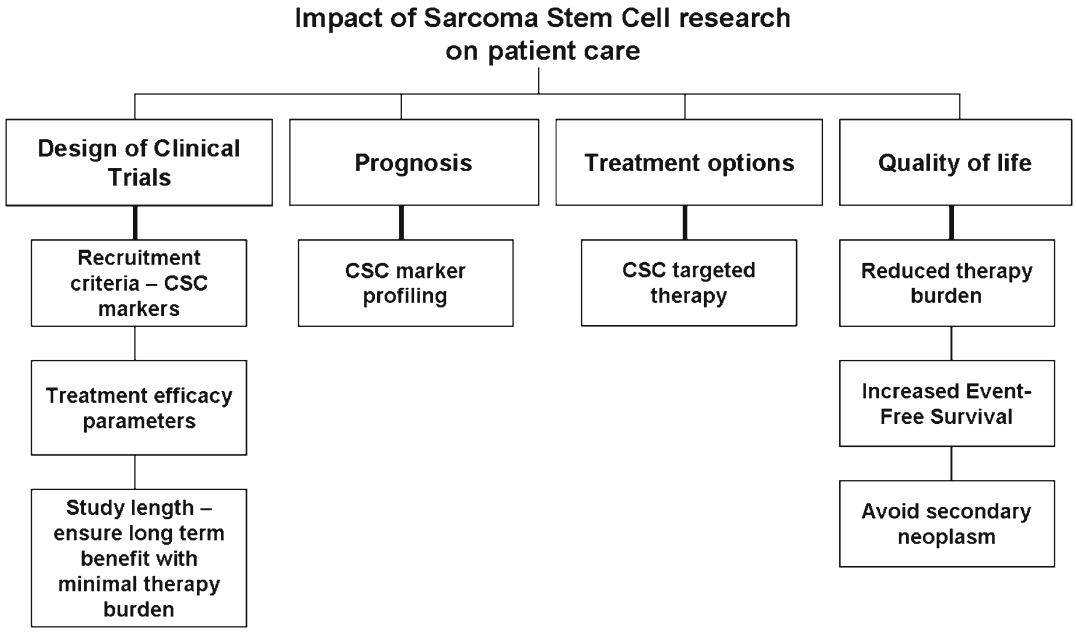

Fig. 10.3 Translational relevance of sarcoma stem cell research (*See text for details*)

significance within the tumor, as was highlighted for eRMS (Ignatius et al. 2012). It was heartening to note that Ewing sarcoma stem cells were sensitive to treatment with a small molecule inhibitor of EWS-FLI1 activity (Awad et al. 2010) and also to the restoration of TARBP2-dependent miRNA processing (De Vito et al. 2012). In the former case there would be no need for any extraneous drug and additionally since the fusion protein is peculiar to tumor cells the treatment regimen poses the least threat of undesirable effects improving the quality of life for the patients.

Future Directions

The sarcoma stem cell research field is relatively new and the technical nuances are now becoming obvious. Therefore future studies dealing with validating and targeting sarcoma stem cells should address these caveats and acknowledge the evolutionary aspect of the model (Valent et al. 2012). The researchers need to make certain that the markers and pathways observed as being important are not technical artifacts. In order to firmly establish the sarcoma CSC model and also to clarify the phenotype stability and drug resistance it would be important to do lineage tracing in transgenic mouse models with varied oncogenic hits in different cellular compartments used as origins. Furthermore, to validate the sarcoma stem cell model as a more general phenomenon different sarcoma histotypes would need to be studied and importantly specific and functionally important markers need to be identified.

It is also important to find differences, if any, between the biology of pediatric and adult sarcoma since it is likely that adult sarcomas have gathered a larger number of genetic aberrations. It is possible that the 'cell of origin' of adult sarcomas is different from their pediatric counterparts since different cells could possess permissive states for oncogenesis in the pediatric and adult variants of the same sarcoma. This would imply that the sarcoma stem cell phenotype could be different between the two age groups as well. Therefore it is crucial to note the origin of the commercially available cell lines and also account for the latency period and site of tumor formation in transgenic mouse models to ensure that the cancer studied is of pediatric origin with a niche similar to the one expected in a developing child. This aspect becomes even more important when it comes to validating the use of drugs targeting developmental pathways. After all a child is not a small adult.

In addition it would be productive to dissect the biology of the sarcoma stem cell population, namely, niche dependency and key signaling mechanisms which could offer drug targets. It is evident from in vivo experiments using sarcoma stem cells that cellular niche has a significant effect on tumor initiation capacity. Thus far hypoxia seems to be important for sarcoma stem cells. It is imperative for any cancer research to have clinical relevance and therefore whenever possible translational significance must be elucidated. It would be important to assess drug resistance and relapse of sarcoma in vivo in order to prove the refractoriness of a sarcoma stem cell population. It is clear also that sarcomas have genetic instability which is likely to also affect the cellular milieu in the tumor. Therefore the sarcoma stem cell paradigm must incorporate genetic and maybe even epigenetic background in order to achieve what any cancer model seeks to finally accomplish: a path to a cure.

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Cancer Stem Cells Derived from Mouse Induced Pluripotent Stem Cells

11

Hiroshi Murakami, Akifumi Mizutani, Chen Ling, Tomonari Kasai, Takayuki Kudoh, Li Fu, and Masaharu Seno

Contents

Abstract

The malignancy of tumors is often explained by the presence of cancer stem cells, which are considered resistant to anti-cancer agents and radiation. Cancer stem cells are typically characterized by continuous proliferation and self-renewal as well as by potential of differentiation. Stem cells are considered to differentiate into tissue specific phenotype of mature cells being influenced by the microenvironment. In this context, cancer stem cells should be derived from the stem cells under the influence of a microenvironment, which induces malignant tumors. In this review, we propose this microenvironment as a 'cancerous niche' and discuss its importance on the formation and maintenance of cancer stem cells with the recent experimental results to establish cancer stem cell models from induced pluripotent stem cells. These models of cancer stem cell will provide the great advantages in cancer research and its applications in the future.

Introduction

Cancer is a group of diseases, in which regulation of cell proliferation and growth is disrupted and cells are growing uncontrollably. The uncontrolled cell growth in cancer is caused commonly by genetic damages, including mutations of oncogenes and tumor suppressor genes. Thereby cancer cells are historically

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considered driven from a single cell, that is, they are clonal. However, the individual cells that make up the cancer exhibit significant heterogeneity in their morphology, cell surface antigens, genetic alterations, pattern of gene expression profiles, epigenetic modifications and so on. One possible explanation of their heterogeneity is that cancer is a cellular hierarchy with cancer stem cells (CSC) at the apex, just like normal tissue development with their tissue stem cells (Wang and Dick 2005). The CSC concept derives from the fact that the unlimited growth of cancer tissues depends on a small number of distinct cells of which proliferation is unlimited. Nowadays, a 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. CSCs can thus only be defined experimentally by their ability to recapitulate the generation of a continuously growing tumor as reached to the consensus at the AACR Cancer Stem Cells Workshop (Clarke et al. 2006). However, characterization and analysis of these cells are limited due to the small number of CSCs in a tumor, and technical difficulty of isolation as a homogenous population of cancer stem cells from clinical samples. If it is possible to establish appropriate CSC lines, the recapitulation of CSC properties would be more precise and at the same time the development of new clinical cancer therapy would be accelerated. In this chapter, we introduce our recent work to establish cell lines with CSC properties in vitro. And also we will discuss the concept of CSC with the results obtained from our original cancer stem-like cells.

Microenvironment to Develop Malignant Tumors

In the field of regeneration therapy, the pluripotent stem cells such as embryonic stem cells (ESCs) and induced pluripotent stem cells (iPSCs) are promising sources of differentiated cells for transplantation. When exposed to appropriate environment, the stem cells should be directed to the progenitor cells such as hematopoietic cells, neural stem cells and so on, which are destined to differentiate into each mature cell such as macrophage, monocyte, neural cell, cardiac cell, and pancreatic β-cell. The behavior of stem cells is tightly regulated by the signals from surrounding microenvironment, so called 'niche' which supports the self-renewal of stem cells controlling stem cell number. Simultaneously, a niche regulates the differentiation, in turn, maintains tissue homeostasis (Moore and Lemischka 2006). Thus the cell fate is determined by the events and factors present in the range of a niche.

Taking the pluripotency of stem cells into consideration, it should be hypothesized that malignant neoplasm is one of the tissue types differentiated from stem cells. In this context, a CSC could be described as a progenitor cell that is destined to differentiate into a cancer cell. This might be called 'canceration' rather than tumor initiation. In this context, we propose the niche that directs stem cells into CSCs as 'cancerous niche' (Fig. 11.1). But cancerous niche could hardly be defined because normal tissue or body should have normal niche. How and where can we find the cancerous niche?

A Cancer Stem Cell Model

We designed unique experiments to convert pluripotent stem cells into the cells, which have the characteristic properties of CSCs, using established cancer cell lines (Fig. 11.2). As the result, we have reported that mouse iPSCs (miPSCs) could acquire characters of CSCs when miPSCs were cultured in the presence of conditioned medium prepared from various cancer cell lines (Chen et al. 2012). The established model of CSCs derived from miPSCs (miPS-CSCs) formed spheroids when they were cultured under non-adherent condition, implying they have capacity of self-renewal. The genes associated with stem cell properties and an undifferentiated state such as *Nanog*, *Rex1*, *Eras*, *Esg1* and *Cripto1*, were expressing in miPS-CSCs. Most importantly, miPS-CSCs

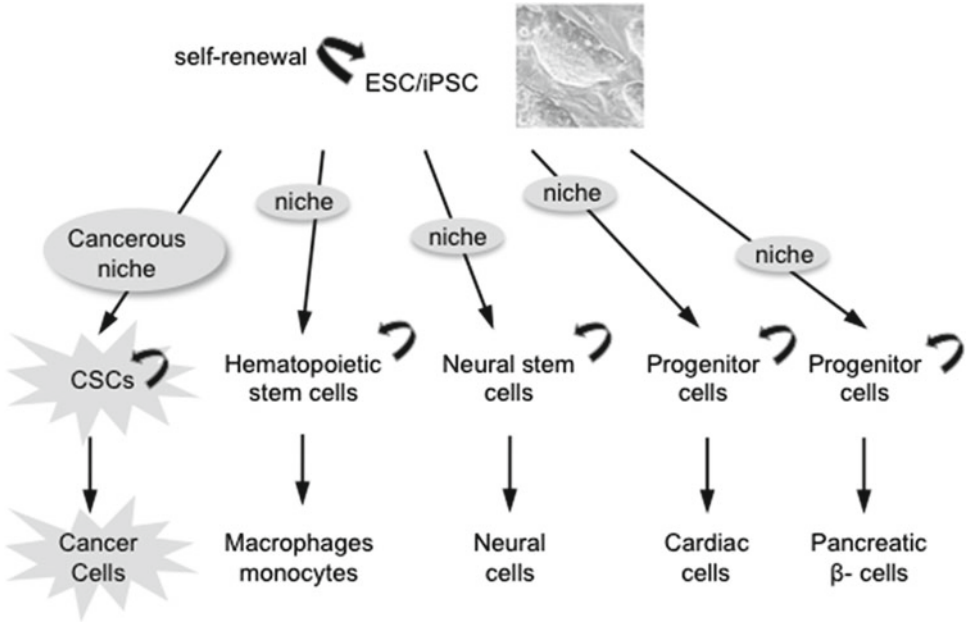

Fig. 11.1 'Cancerous niche' developing malignancy. In the appropriate niche, the stem cells/iPSCs should differentiate into progenitor cells and terminally into each mature cell. When a malignant neoplasm is considered to be a tissue differentiated from a stem cell, a cancer stem cell could be a progenitor cells giving rise to cancer cells. The niche affecting conversion of stem cells into cancer stem cells is hypothesized 'cancerous niche' in this review

exhibited high tumorigenicity with rapid growth in nude mice while parental miPSCs provided only benign tumors, teratomas. Among established miPS-CSCs, miPS-LLCcm cells, which were derived from miPSCs cultured in the media containing conditioned medium of Lewis lung carcinoma (LLC) cells, showed highly angiogenic and typically malignant phenotype after transplantation into nude mice. The procedure of establishment and assignment of this cell to a CSC is briefed below.

Based on our hypothesis that the 'cancerous niche' could generate CSCs by transforming or differentiating normal stem cells (Fig. 11.1), we cultured miPSCs with conditioned medium of LLC cells in the absence of mouse embryonic fibroblasts (MEF) as feeder cells and leukocyte inhibitory factor (LIF). After 4 weeks of culture, survived cells were expanded in the normal medium without MEF and LIF. Interestingly, in this condition, approximately 30–50% of cells retained GFP expression, which was under the control of *Nanog* promoter (Okita et al. 2007), indicating those cells should be undifferentiated. This implies that the mechanism(s) for maintenance of undifferentiated state of miPS-LLCcm cells should not depend on MEF or exogenous LIF. To evaluate the self-renewal capacity of the cells, we examined the growth of the cells in suspension culture. The formation of spheroids pressing GFP was observed. When the spheroids were dissociated, individual cells formed new spheroids during serial passage in the suspension culture.

miPS-LLCcm cells in either adherent or suspension culture formed adenocarcinomas in nude mice exhibiting cells with high nuclear to cytoplasmic ratio, nuclear pleomorphism, aberrantly high mitotic rates, and multiple pathological mitotic figures. Furthermore, CD31 positive staining in the tumor showed multiple vascular vessels, indicative of angiogenesis (Fig. 11.2). Totally, the histology revealed that the tumors formed by miPS-LLCcm cells were malignant. It is noteworthy that 30–50% of the cells were GFP-positive in the tumors derived from miPS-LLCcm cells.

The characters of self-renewal capacity and tumorigenicity observed in miPS-LLCcm cells

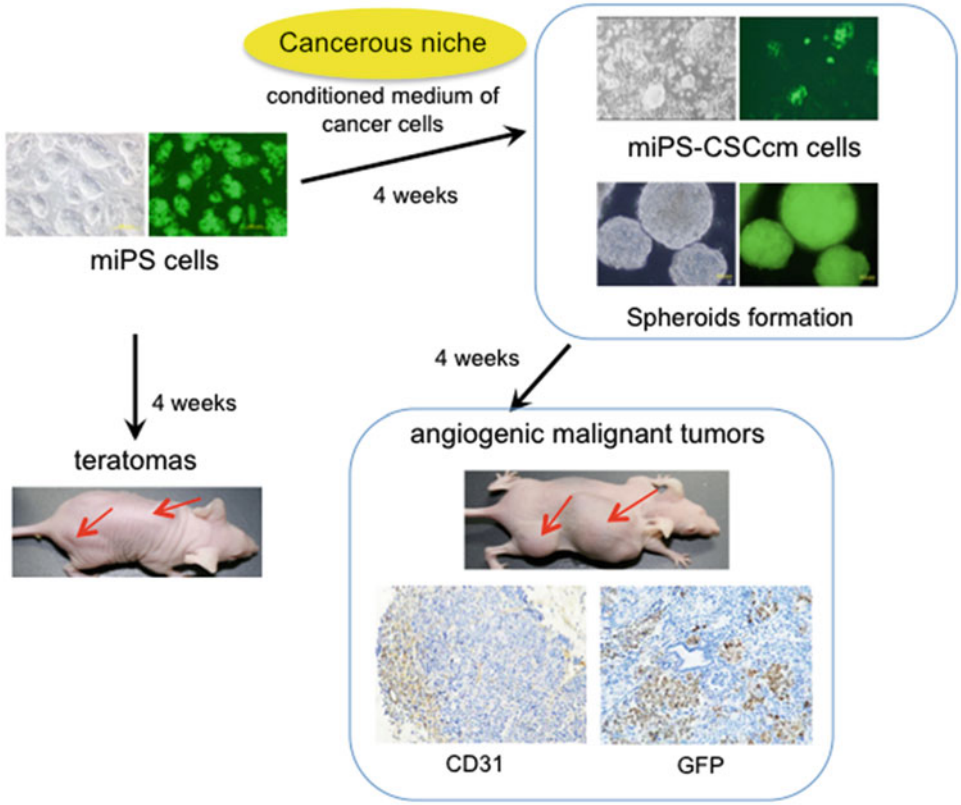

Fig. 11.2 Establishment of CSC model from miPS cells. miPS cells (Okita et al. 2007) were cultured in the media containing the conditioned medium of various mouse cancer cell lines as the cancerous niche. Resultant cells showed CSC properties such as sphere formation and malignant tumorigenicity with extensive angiogenesis in vivo (Chen et al. 2012)

are consistent with the definition of CSCs (Clarke et al. 2006). Thus, we are proposing miPS-LLCcm cells as a model of CSC. Furthermore, the section from the tumor derived from miPS-LLCcm showed that more than half of the mesenchymal cells in the stroma of tumor turned out to be GFP negative. Also the gland-like structure was extensively stained with anti-cytokeratin antibodies (Chen et al. 2012). From these observations, we concluded miPS-LLCcm cells have the potential of differentiation, showing the heterogeneous linage in the tumor. As for the differentiation potential of miPS-LLCcm cells, we will discuss again in the following section together with the previous reports on the differentiation of CSCs in glioblastomas (Ricci-Vitiani et al. 2010; Wang et al. 2010; Soda et al. 2011).

Potential of Differentiation and Induction of Angiogenesis

The capacity of differentiation in CSC should be considered with tumor angiogenesis. One of the definitive characters of CSC is the multipotency to create heterogeneous linages in tumors. Three independent groups have recently reported the origin of blood vessels in tumors (Ricci-Vitiani et al. 2010; Wang et al. 2010; Soda et al. 2011). In the glioblastoma, a subpopulation of endothelial cells was found to carry the same somatic mutation as found in tumor cells, indicating the endothelial cells arose from the neoplastic origin. A series of analyses indicated that glioblastoma stem-like cells could differentiate into vascular endothelial cells in tumors. Because of the extensive

angiogenesis in the tumor derived from our miPS-LLCcm cells, the CSC model should be a critically important source to investigate precise mechanism of tumor angiogenesis. Our preliminary data show miPS-LLCcm cells could differentiate into endothelial cells forming tubular structure in vitro. The results from our study with miPS-CSCs will shed light on the molecular mechanisms of both differentiation of CSC and angiogenesis in tumors. Through the analyses, the physiological significance of the differentiation potency and self-renewal capacity in CSCs could be further clarified because endothelial cells are considered to create a stem cell niche promoting self-renewal of CSCs (Krishnamurthy et al. 2010; Zhu et al. 2011).

Future Applications of Cancer Stem Cell Model: Anti-Cancer Stem Cell Agents

It is widely known that CSCs show resistance toward the conventional chemo- and radiation-therapy. The characters of the resistance are considered to be one of the reasons for recurrence in patients after clinical treatments. The mechanisms involved in this resistance include expression of ABC drug pumps, expression of anti-apoptotic proteins, resistance to DNA damage, and so on (Zhou et al. 2009). The CSC model generated in vitro could be useful as tools to ask why and how they acquired those resistances and what kinds of molecules are critically responsible for them. They also should be useful to screen new anti-cancer agents that would eliminate CSCs by restricting their survival and/or differentiate CSCs to make them more sensitive to traditional drugs, because a large number of CSCs should be required to perform drug-screening in a high-throughput manner.

Future Applications of Cancer Stem Cell Model: Cancer Stem Cell Vaccine

Despite an attractive theory, cancer vaccination in clinical trials has not been satisfactory or successful. A reason of failure might be unexpected presence of CSCs in tumors, which could not be characterized in detail at the diagnosis. Very recently, it has been shown that cancer vaccination induced the expression of *Nanog* in the tumor cells, and raised the relative quantity of immune-resistant stem-like cells in the tumor mass (Noh et al. 2012). Although further investigation is necessary to elicit the molecular mechanisms for selection of *Nanog* expressing cells, this report implies the contribution of CSCs to the acquisition of immuno-tolerance/escape of tumor. In the mean time, enriched CSCs were described immunogenically more effective than the whole cells in the tumor to induce protective antitumor immunity (Ning et al. 2012). Their results proposed the novel type of cancer immunotherapy against CSCs. Enough amounts of CSCs will be required as much as the drug screening process to generate vaccine in good quality and quantity. Thus, CSC models generated in vitro would have a great advantage as good sources of antigen.

Hypothetical Balance Between Stem Cells and Cancer Cells

Several studies have recently demonstrated that the ESC niche could have significant influence on the phenotype of aggressive cancer cells (Tzukerman et al. 2006; Postovit et al. 2008; Costa et al. 2009). These results indicate that the malignant phenotype of cancer cells could be suppressed in embryonic niche, accompanied by alternative expression of miRNAs and by change in epigenesis such as DNA methylation. The tumor microenvironment is supposed to play important roles in the initiation, progression and metastasis of cancer (Hu and Polyak 2008; Laconi 2007). It has been reported that tumor cells can inhibit p53 induction, one of the most famous tumor suppressor, in the fibroblasts adjacent to the tumor tissue. This suppression was considered to be dependent on the factor secreted from tumor cells (Bar et al. 2009), which raised the possibility that the factors secreted from the cancer cells might confer cancerous properties to the adjacent stem cells.

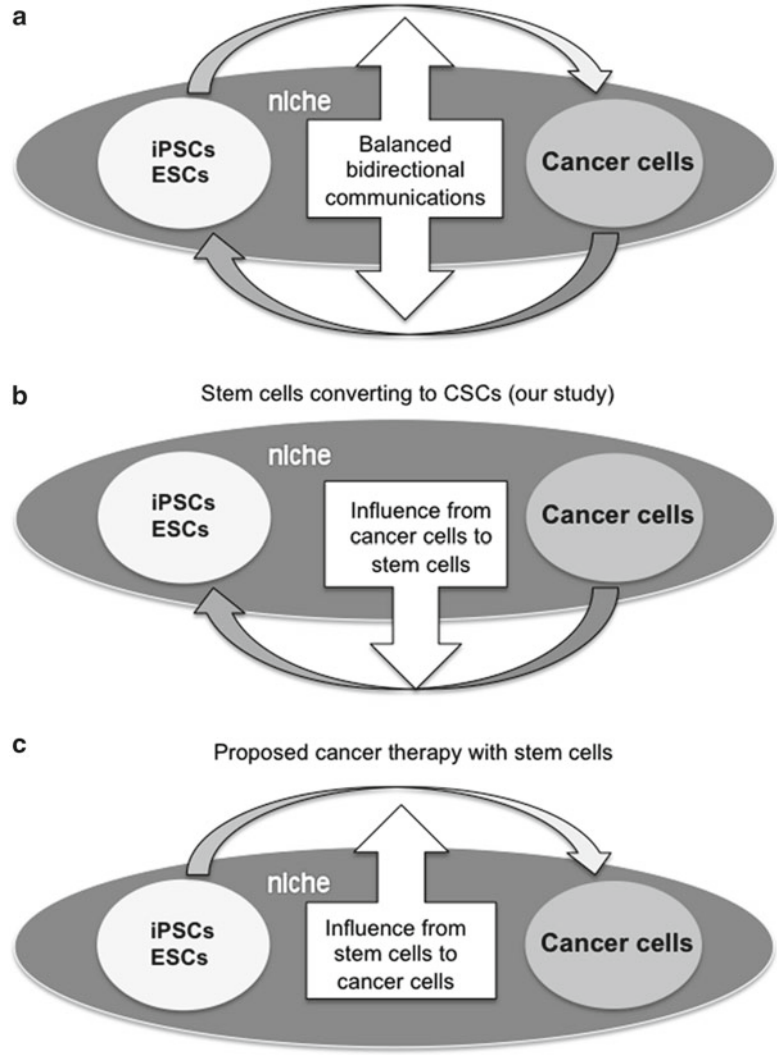

Fig. 11.3 Bidirectional communications should be present between stem cells and cancer cells. (**a**) Communications are balanced between stem cells and cancer cells. (**b**) Excessive communication from cancer cells to stem cells converts the stem cells into cancer stem cells as shown in our study. (**c**) Excessive communication from stem cells to cancer cells suppresses the growth of cancer cells (Tzukerman et al. 2006; Postovit et al. 2008; Costa et al. 2009)

Our miPS-CSCs were obtained from the culture in the conditioned medium of cancer-derived cells but were hardly obtained in the co-culture with cancer cells (Chen et al. 2012). Collectively, there appears bidirectional communications between cancer cells and ESCs (Fig. 11.3). Both communications should be regulating the activities of cancer cells and ESCs each other, so that the loss of either communication should make unbalanced regulations that would result in converting stem cells into cancer stem cells, vice versa.

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Stemness Regulation of Somatic Cancer Cells

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Contents

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Abstract

Embryonic stem (ES) cells derive from the inner cell mass (ICM) of the blastocyst in early embryo stage, which lasts only a few days during the life. Soon after the blastocyst implants in endometrium, ICM cells differentiate. Several decades' efforts have provided important information about how to dissect, maintain and propagate the ICM cells in vitro so that ICM cells can always keep their original totipotential. Extensive ES studies have revealed an important stemness regulation net work consisting of Sox2, Oct3/4 and Nanog. These factors are also expressed in solid tumor cells, both in vitro and in vivo. Although there are negative expression reports of these factors in solid tumor cell lines and tissues, the debating results may be due to technical issues. In vitro and in vivo gain and loss of function studies have proved that functional expressions of these factors will upregulate the stemness of solid cancer cells. It has been found that chemotherapeutic reagents kill mainly bulk tumor cells in vivo or most tumor cells in vitro, leaving cancer stem (CS)-like cells. In vitro studies demonstrate that ovarian and prostate cancer cells, if pretreated in hypoxia (1% O_2), express higher levels of stemness-related factors, resulting in greater abilities in colony and sphere formation. In addition, cytokines like stem cell factor (SCF), granulocyte colony-stimulating factor (G-CSF) and TGF-β etc. are also potential factors upregulating stemness of cancer cells.

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Clinically, higher levels of expressions of the typical net work factors Oct3/4, Sox2 and Nanog in numerous solid tumors are correlated with poor clinical outcome, strongly indicating their function in upregulating stemness and dormancy of cancer cells in vivo which endorse these cells therapeutic resistance. It is envisaged that fundamental studies of cancer cell stemness will disclose solutions for isolating, maintaining and propagating CS cell in vitro, an important issue in CS cell line establishment.

Introduction

Embryonic stem (ES) cells derive from the inner cell mass (ICM) of the blastocyst in early embryo stage. These cells have two distinctive properties: (1) totipotency and (2) self-renewal capability. The totipotency of ES cells is reflected in their capability to differentiate into all derivatives of the three primary germ layers: ectoderm, endoderm and mesoderm. The self-renewal feature explains their ability to replicate indefinitely. One of the central issues in ES cell study is how to maintain the cell stemness and propagate these ES cells in vitro. In vivo the cells in the ICM will gradually differentiate into more than 200 cell types in the human body. Fortunately ES studies from the past decades have decoded most of the secrets of stem cells in general. The first mouse ES cell lines were established by two groups from mouse blastocysts, and the common strategy to successfully maintain their cell lines was to grow these cells on a feeder layer of division-incompetent mouse fibroblasts. Great progress has been made by using defined chemicals, cytokines and cell stemness-friendly matrices in maintenance and propagation of ES cells without feeder cells (Solter 2006). Extensive studies on ES cells have revealed critical stemness factors Sox2, Oct3/4 and Nanog, and their proper net working upregulates and maintains the stemness of ES cells in vitro (Loh et al. 2006).

It is established that the transcription factors Oct3/4, Nanog and Sox2 play essential roles in regulating and maintaining totipotency in both human and mouse ES cells. All these factors have a special property in common, and each functions as a molecular rheostat in their regulation of the self-renewal and totipotencyof ES cells. Down-regulation of these factors or inhibition of their crosstalk result in reduced cell stemness, and the cells undergo differentiation. Thus, the factors are also named stemness factors. Furthermore, these factors have been used in generating induced pluripotent stem cells (iPSCs), a strong evidence of their stemness function. The fundamental roles of these factors in ES cells encourage extensive exploration of their function and expression patterns in tumors, in particular cancer stem (CS) cells. The common characteristics for CS and ES cells are theirs stemness features, i.e. self-renewal and differentiation abilities. The stemness features of CS cells are believed to provide these cells unlimited proliferation potential, a driving force for tumor development. Therefore stemness regulation of CS cells has already been the focus in the field of CS cell research.

CS Cells and Stemness Regulation

CS cell is defined as a cell within a tumor that possesses the capacity to self-renewal and to cause heterogeneous lineages of cancer cells that comprise the tumor. CS cells are rare cell population in tumors. According to this concept, CS cells have the normal SCs properties such as self-renewal and differentiation, in addition to the capability to initiate new tumors. The concept of CS cell initially was characterized in hematological malignancies. The clearly defined and classified CS cells were originally identified in acute myeloid leukemia showing that isolated subpopulations of CD34+/CD38- in acute myeloid leukemia were able to initiate tumors in NOD/SCID mice with the similar histology to donor (Bonnet and Dick 1997). Various solid tumor CS cells have also been reported during the past few years. CS cells are proposed to persist in tumors as distinct populations resistant to conventional chemotherapies and radiotherapies. The current cancer treatment regimens kill mainly differentiated tumor cell populations, and leave the CS cell

populations viable somewhere inside the body, for example bone marrow. Except for really early tumors treated successfully with surgery, most patients may experience a disease-free period, or so called "cancer dormancy period" before facing cancer relapse and metastasis. Increasing evidence suggests the bone marrow micrometastatic tumor cells to be CS cells.

Extensive stemness regulation studies are performed with ES cells where markers for different cell lineages have been published. Cancer cell stemness studies are still in the early phase, due to lack of reliable and universal CS markers and lack of consensus cancer cell stemness evaluation criteria for solid tumors. In the field of hematological malignancies we have for a long time had well characterized cell lineage markers. However, rapid progress has been made in the cancer cell stemness regulation field. We do know that Oct3/4 and Nanog are typically expressed in the cancer stem-like CD133+ subpopulation of lung adenocarcinoma cells, and exogenously expressed Oct3/4 and Nanog in some lung adenocarcinoma cell line enhances stem-like properties in the lung adenocarcinoma cells (Chiou et al. 2010), indicating a link between the expression of these stemness factors and CS cells. By an inducible Nanog-overexpression lentiviral system, Jeter and coworkers (Jeter et al. 2011) successfully examined the function of Nanog in prostate and breast cancer cell lines. The Nanog overexpressing cancer cell lines exhibited greater CS-like properties like drug resistance (in MCF-7 cells), tumor regeneration (in DU-i45 cells) and castration -resistant tumor development (in LNCaP cells). All these experiments suggest upregulation of the stemness of tumor cells to be associated with maintenance of CS cells and possibly correlated to unfavourable clinical outcome.

Chemotherapy and Stemness Factors

The CS cell theory highlights a feature of chemotherapeutic resistance of CS cells. The conventional way to register a treatment effect has been by observation of a reduction in tumor volume. The more differentiated cells are killed by conventional chemotherapies, but CS cells survive and continuously reside in some places in the body where the niche favors their stemness maintenance. Drug resistance of cancer stem/initiating cells is now considered to be one of the main reasons for tumor relapse. In breast cancer cell lines cancer-stem like cells can be enriched by mitoxantrone treatment, and the mitoxantrone-selected cells expressed higher levels of Oct3/4 and ABCG2 (Prud'homme et al. 2010). Similarly, chemotherapeutic reagent carboplatin induces the expression of the stemness factors Sox2 and Oct3/4 in hepatocellular carcinoma cells in culture, and knockdown of Sox2 and Oct3/4 gene expression in these cells reduces the carboplatin-induced sphere formation (Hu et al. 2012). Another liver cancer cell study conducted by Wang et al. (2010) shows that Oct3/4 may mediate chemoresistance through a potential Oct3/4-AKT-ABCG2 pathway (Wang et al. 2010). One of the essential clinical observations concerning possible CS cells and chemotherapy resistance is the presence of bone marrow micrometastasis. These bone marrow micrometastatic cells usually survive the conventional chemotherapy and reside inside this place for many years or decades before these cells develop new tumors. Although technically it is difficult to test this hypothesis, Pezzolo et al. (2011) have recently shown that all the bone marrow metastatic neuroblastoma cells (verified by anti-neuroblastoma 84 mAB) from ten patients are Oct3/4 positive, and the Oct3/4 positive tumor cells with positive surface Tenascin (TNC) behavior as tumor progenitor/stem cells.

Cancer cell dormancy is a typical stage in which tumors cells are kept occult and asymptomatic for a period of time, sometimes for decades as bone marrow micrometastasis. Cancer dormancy is a common feature for the following stages in tumor development: early stage in tumor development, micro-metastasis in distant organs and minimal residual disease left after surgical removal and/or treatment of primary tumors. However, the most understandable dormancy of tumors is the bone marrow micrometastasis after surgical removal of primary tumor with

conventional treatments including chemotherapy and radiotherapy. Theoretically, dormancy of cancer cells is depending upon their niche factors in the microenvironment and surrounding stroma. It is known that specialized niches in the bone marrow are able to mediate hematopoietic stem cell dormancy as a means to preserve the "stemness" of this population. It is also reasonable to believe that the specialized bone morrow niche in patients with bone marrow micrometatastases favors the maintenance of CS cell dormancy, or stemness. Thus, it merits systemic studies of the tumor cell dormancy in order for better understanding tumor cell stemness regulation. In particular, it is important to focus on the mechanisms involved in activation of dormant cancer cells in the bone marrow.

Hypoxia and Stemness Factors

Due to tumor cell overgrowth, most tumors suffer from disordered, impaired and intermittent blood flow, which result in low oxygen level (hypoxia) within tumor. Increasing evidence indicates that hypoxia regulates various aspects of cancer biology like promotion of drug-resistant characteristics, apoptosis-resistant clone selection, facilitation of tumor invasion and metastasis, and enhanced genomic instability. Clinical studies suggest that hypoxia may be served as an independent prognostic factor for predicting advanced progression and poor clinical outcome in cancers (Nordsmark and Overgaard 2004), likely due to the increased resistance of hypoxic tumors to conventional therapies. It is well documented that hypoxia, combined with reduced oxidative DNA damage, benefits for retaining undifferentiated status of embryonic, hematopoietic and neural stem cell phenotypes, and also influences proliferation and survival of these cells (Keith and Simon 2007).

In response to hypoxia, the key regulators hypoxia inducible factors (HIFs) are detected in mammalian cells. HIFs, the heterodimers molecules, are composed of an alpha subunit tightly regulated by oxygen level and a beta subunit which is constitutively expressed in most types of cells regardless of oxygen tension. HIF-1α and HIF-2α are the major isoforms of the α-subunit with a high degree of sequence identity and they are mostly transcriptional activators to mediate cellular and systemic homeostatic responses by regulating a number of downstream genes including those in glucose metabolism, cell survival, erythropoiesis, resistance to chemotherapy and radiation therapy in tumors and stem cell maintenance in addition to angiogenesis. HIF-1α and HIF-2α share some target genes such as VEGF, GLUT1, and ADM-1. HIF-1α also regulates the adoptive signaling including Notch, Wnt, c-myc and p53 factor. HIF-2α has an interaction with Oct3/4, c-myc, KIF4 and Sox2. HIF-1α and HIF-2α are also identified in tumor tissues or tumor cells cultivated under normoxia (Khandrika et al. 2009). In addition, these two factors have been detected increased in several cancer cells like lung, gastric, colorectal, breast, ovarian and prostate cancers.

In line with the above reports, prostate cancer cell lines PC-3 and DU145 in 7% O_2 tension expressed higher levels of Oct3/4 and Nanog, and even higher expressions of these two factors were observed in 1% O_2 tension. The upregulation of the expression of these two factors was in parallel with the increasing HIF-1α and HIF-2α expressions upon hypoxia treatment. In addition, the results revealed that the induced *Nanog* gene in the cells exposed to hypoxia was primarily derived from the retrogene *NanogP8* with relatively higher expression level than *Nanog1*. At the same time, G_0/G_1 stage was extended, side population cells were increased and CD44 and ABCG2 expressions were upregulated. Furthermore, the hypoxia induced ABCG2 positive cells were predominantly $CD44^{bright}$ and the $CD44^{bright}$ cells expressed significantly higher levels of Oct3/4, and Nanog. Compared to the $CD44^{dim}$ cells, the $CD44^{bright}$ cells demonstrated higher colony formation and sphere formation abilities (Ma et al. 2011).

It is also discovered that both ES-2 and OVCAR-3 ovarian cancer cell lines cultivated in hypoxic condition grew relatively slowly with extended G0/G1 phase. If the cells were pre-treated

in 1% O_2 tension for 48 h before brought back to normoxia, the cells showed significantly higher proliferation rate with higher infiltration capability, and significantly more clones and spheres, in comparison to the cells always cultivated under normoxia. Hypoxic treatment of the cells resulted in stronger CD44 expression in both cell lines, in addition to significantly increased number of side population (SP) cells and up-regulated expression of OCT3/4 and Sox2 in these cells (Liang et al. 2012).

Collectively, laboratory findings suggest that tumor cells in vivo may also respond with upregulation of these transcription factors, although very direct prove is still lacking, mostly due to lacking proper technologies. It is well known that tumor mass contains poor tissue perfusion, diffusion and microvessel abnormality attributing hypoxia in tumors. Actually, not only hypoxia, but also anoxia exists in tumor tissues due to the tumor overgrowth. Hypoxia in tumors has been considered as a treatment response predictor and prognostic marker. Hypoxia triggers an array of cellular defense mechanisms which protect hypoxic tumor cells from conventional therapies. Therefore targeting tumor cells surviving hypoxia/anoxia, in another word, targeting tumor cells with greater stemness will be of practical value in designing cancer treatment regimens.

Cytokines and Cell Stemness

Cytokines are small cell-signaling proteins, peptides, or glycoprotein molecules with immunomodulating functions. The most therapeutic application of cytokines in solid tumor management is cytokine-induced killer (CIK) cell treatment, developed based on lymphokine-activated killer (LAK) cell treatment procedure (Ma et al. 2012b). LAK cells are prepared by culturing natural killer (NK) cells in the presence of interleukin-2 (IL-2), while CIK cells are primarily prepared by culturing peripheral blood lymphocytes in the presence of IFN-gamma, IL-2, monoclonal CD-3 antibody, and IL-1a (Schmidt-Wolf et al. 1997). However, cytokines may also positively or negatively influence the stemness of cancer cells.

Bone marrow is the major source of hematopoietic cytokines including stem cell factor (SCF), Granulocyte colony-stimulating factor (G-CSF), and TGF-β. SCF is an early-acting hematopoietic cytokine that plays a constitutively important role in the proliferation and survival of pluripotent progenitor cells, together with its receptor CD117. The G-CSF receptor is present on precursor cells in bone marrow, and can initiate proliferation and differentiation into mature granulocytes, in response to stimulation by G-CSF. SCF has also been identified in supernatant of some non-hematopoietic tumor cell lines including breast and small cell lung carcinoma cell lines, suggesting a cell growth stimulation role by autocrine production of SCF in CD117 receptor-bearing tumor cells. The serum level of SCF is significant increased in lung cancer patients, compared to those in the control group. The data from Wiesner et al. (2008) show that prostate cancer cells could release SCF to the extracellular milieu and also indicate the SCF-CD117 signaling system has potential contribution to prostate cancer bone metastasis. G-CSF and its receptor could also be found in somatic cancer cells such as head and neck, bladder and ovarian cancer cells associated with growth of these tumor cells. G-CSF productions by lung and bladder cancers cells have been reported to be associated with invasion behavior of cancer cells. Matsuoka et al. (2009) reported a case of prostate cancer cells producing G-SCF by immunohistochemical analyses, indicating its prognostic roles in the prostate cancer. It has been reported that either SCF or G-CSF could upregulate the expressions of stemness-related factors including CD117, ABCG2, CD44, Oct3/4 and Nanog in prostate cancer cell lines in vitro. In addition, SCF and G-CSF have synergistic effect on the induction of these stemness genes. In the presence of either SCF or G-CSF, the cells' colony formation and sphere formation potentials were significantly elevated and such effect of SCF and G-CSF on self-renewal in these cells was synergistic as well, indicating that there is a favourable bone marrow niche for prostate

cancer cells in which higher levels of cell stemness are maintained at least partly by the cytokines SCF and G-CSF (Ma et al. 2012a).

TGF-β is a multifunctional cytokine regulating multiple important biological processes, among which epithelial-mesenchymal transition (EMT) is the most CS-related issue. It is known that various extracellular molecules including cytokines may trigger EMT, but TGF-β is the most powerful EMT player. EMT during carcinogenesis generates cells with stem-like properties. It is also known that TGF-β signaling, mediated through the transcription factors Smad2 and Smad3, directs cell type specific responses. It is interesting to know whether there is any CS cell type specific EMT or mesenchymal-epithelial transition (MET). Depending on niche situation of cells, TGF-β may function as a suppressor or oncogene, so called double edged sword. Proper TGF-β signaling is crucial in maintaining stem cell property, but aberrant TGF-β signaling will be carcinogenic. Recently, it is shown that autocrine TGF-beta signaling plays an essential role in retention of stemness of glioma-initiating cells (GICs) through an induction of transcription factors including Sox2 (Ikushima et al. 2009).

A number of other cytokines are also indicated to play important roles in stemness regulation of cells. For example, normal intestinal epithelial cell line IEC-18 cells grow as a monolayer in serum free medium in the absence of IL-1beta, but spheres were formed when IL-1beta was added into the medium. The IL-1beta treated cells demonstrated higher colony formation ability with higher levels of stemness gene expressions including Nanog (Wang et al. 2012). In the same time, it seems there is an interaction between cytokines and stemness factors. Oct3/4 overexpression enhances CS cell-like properties of colorectal cancer cells, including sphere formation, cell colony formation, cell migration, invasiveness and drug resistance. In addition, IL-8 and IL-32 were upregulated in the Oct3/4 overexpressing cells. Neutralization of either IL-8 or IL-32 with specific antibodies partially blocked the tumorigenic effects induced by either Oct3/4 overexpression or by the addition of conditioned media from Oct3/4-overexpressing colorectal cancer cells (Chang et al. 2011).

In deed, there is a long list of cytokines positively or negatively influencing stemness of cancer cells, and their molecular effect may also be cell type dependent. Therefore characterization of individual cytokines in different type of cancer cells and optimization of different combinations of different cytokines for a given cancer type will be necessary before it is possible to maintain and propagate CS cells in vitro in consideration of cytokines application.

Expression of the Stemness Factors in Solid Tumor Cells

Numerous reports focus on RNA and or protein expression of the stemness factors Oct3/4, Sox2 and Nanog in solid tumors. In a bladder cancer study, Chang et al. (2008) demonstrated both RNA and protein expression Oct3/4 in human bladder transitional cell carcinoma samples and related cell lines. Bae et al. (2010) verified that mRNA expression of transcription factors Oct3/4 and Sox2 highly correlated in primary prostate tumor tissue samples. When isolated from the DU145 and PC3 prostate cancer cell lines by flow cytometry, stem cell-like tumor cells expressing high levels of Oct3/4 and Sox2 showed high tumorigenecity in immunodeficient mice. In a study of tissue mRNAs of Oct-3/4, Sox-2 and Nanog in esophageal cancer patients using reverse-transcription polymerase chain reaction, Bahl et al. (2012) observed increased transcript levels of these factors in the esophageal cancer tissues compared with the matched distant normals, and most of the preneoplastic tissues exhibited increased transcript levels of these stemness markers as well. Similarly, in an immunohistochemical study of 162 consecutive esophageal squamous cancer patients in a high-risk population in China we demonstrated 17.90% and 22.84% of the tumors highly expressed Oct3/4 and Sox2 proteins, respectively (Wang et al. 2009). In a tissue microarray study of a series of 226 sporadic node-negative invasive breast

carcinomas by immunohistochemitry for Sox2 protein expression, it was found that Sox2 was positive in 16.7% of the tumors and its expression was significantly more frequently observed in basal-like breast carcinomas. Furthermore, Sox2 expression showed a statistically significant inverse association with ER and PR, and direct association with CK5/6, EGFR and vimentin, respectively, highly suggesting that Sox2 is preferentially expressed in tumors with basal-like phenotype with less differentiated/'stem cell' phenotypic characteristics (Rodriguez-Pinilla et al. 2007).

It should be noted that some variants of these factors may be closely associated with stemness of tumor cells. While an increasing number of reports deal with the expression of Oct3/4 RNA or/and protein by studying the Oct3/4A, Wang and Dai (2010) has been focusing on alternative splicing and translation of Oct3/4. This group has shown that the human Oct3/4 gene can generate at least three transcripts (Oct3/4A, Oct3/4B, and Oct3/4B1) and four protein isoforms (OctT3/4A, OctT3/4B-190, Oct3/4B-265, and Oct3/4B-164) by alternative splicing and alternative translation initiation. Thus, in embryonic stem (ES) cell the Oct3/4A is a transcription factor responsible for the pluripotency properties, but Oct3/4B cannot sustain ES cell self-renewal. However, it was discovered by Atlasi et al. (2008) that Oct3/4B1 was sharply down-regulated during the course of all-trans retinoic acid-induced differentiation of human ES cells, strongly suggesting a potential involvement of Oct3/4B1 in maintaining the pluripotential of ES cells. In a study of gastric cancer tissues Asadi et al. (2010) have shown that Oct3/4B1 is expressed in both tumor and non tumor samples of stomach, with 86.1% (31/36) positivity in the tumor samples and 55.5% (20/36) positivity in the marginal counterparts of the same tumors. But, the level of expression was much higher in the tumor samples compared to the apparent normal tissues obtained from the margin of same tumors ($p < 0.002$). Furthermore, Oct3/4B1expression was much higher in the high-grade tumors compared to the low-grade ones ($p < 0.05$). Gastric cancer cell line Oct3/4B1 siRNA functional study indicated its antiapoptotic role. It will be interesting to know whether Oct3/4B1 plays similar role in stemness maintenance in cancer cells.

Nanog expression has been documented in numerous somatic cancers. Recent studies reveal that Nanog1 and Nanogp8 are differently expressed in different colon cancer cell lines (Ishiguro et al. 2011). In prostate cancer cell lines study, Jeter et al. (2011) confirmed that Nanog mRNA expression in prostate cancer cells was primarily originated from the Nanogp8 locus on chromosome 15q14. In additional functional study of Nanogp8 using green fluorescence protein (GFP) reporter, it was discovered that Nanogp8-GFP(+) prostate cancer cells showed cancer stem cell (CSC) characteristics such as enhanced clonal growth and tumor regenerative capacity. Nanog induction promoted drug resistance in MCF-7 breast cancer cells, tumor regeneration in Du145 prostate cancer cells and castration-resistant tumor development in LNCaP prostate cancer cells. In line with this finding, upregulatetd stemness of prostate cancer cells in hypoxia was positively correlated to the induction of Nanogp8 (Ma et al. 2011).

Most importantly, the expression of these stemness factors Oct3/4, Sox2 and Nanog has been detected mainly in the CS cells or cancer initiating cells. If prostate cancer cell lines were comparatively cultivated in normoxia or hypoxia (1% O_2) for a period of 48 h, there was no obvious growth difference for these cells. However, if the prostate cancer cell lines were pretreated in hypoxia for 48 h and then brought back to normoxia, the cells showed significantly higher colony and sphere formation efficiencies than the control cells did. In response to the hypoxia pretreatment, G_0/G_1 stage was extended, side population cells were increased and CD44 and ABCG2 expressions were also upregulated. Furthermore, the induced ABCG2 positive cells were predominantly $CD44^{bright}$ cells, and the sorted $CD44^{bright}$ cells showed even greater colony and sphere formation capabilities than the sorted $CD44^{dim}$ cells, suggesting that $CD44^{bright}$ cells are cancer stem-like cells and their stemness is associated with the induction of Oct3/4 and Nanog (Ma et al. 2011).

Upregulation of Cancer Cell Stemness and Clinicopathological Impact

Increasing in vitro and in vivo evidence strongly indicates that the levels of the master transcription factors Oct3/4, Sox2 and Nanog in cancer cells are correlated to their cell stemness. It has been shown that lung adenocarcinomas express elevated levels of Oct3/4 and Nanog, especially in the high grade tumors, and the expression of Oct3/4 and Nanog is associated with a poorer prognosis. In addition, ectopic expressions of Oct3/4 and Nanog in lung adenocarcinoma cell line showed increased stemness feature including increasing CD133-expression subpopulation, greater sphere formation, enhanced drug resistance and promoted EMT process (Chiou et al. 2010). In a p53-deficient mouse astrocyte model, the mouse $p53^{-/-}$ astrocytes were transduced with virus encoding Nanog, and the enforced Nanog expression in these cells not only increased the growth rate and but also transformed their phenotypes in vitro and in vivo, i.e. the enforced Nanog expression drove the cells towards a dedifferentiated cancer stem cell phenotype (Moon et al. 2011). In an experiment of human squamous esophageal cancer cell lines, enforced expression of Oct3/4 with the eukaryotic expression vector pIRESpuro2-Oct3/4, in addition to upregulation of cell stemness, could greatly increase the expression of Sox2, indicating the collaboration these factor in cancer cell stemness regulation, similar to the findings in ES cells. Oct3/4 and Sox2 expressed variably in Kyse70, Kyse140 and Kyse450 human squamous esophageal cancer cell lines, but weakly or negatively expressed in the virus transformed human squamous esophageal epithelial cell line Het-1A. Further examination of their expressions in a series of 162 consecutive esophageal squamous cell carcinomas in a high-risk population in China showed that 17.90% and 22.84% of the tumors highly expressed Oct3/4 and Sox2, respectively, and the expressions of these factors were significantly associated with poor survival (Wang et al. 2009). Similarly, the enriched oral cancer stem like cells originated from either oral squamous cell carcinoma cell lines or from primary cultures of oral squamous cell carcinoma expressed stem cell markers including Oct3/4 and Nanog, and Oct3/4/Nanog/CD133 triple positivity was significantly associated with poorer clinical survival (Chiou et al. 2008).

Debating Reports

Although there are numerous reports describing positive Oct3/4, Sox2 and Nanog expression in either tumors in general, or in isolated CS cells or CS-like cells in special, it is worthy to note that some reports claim no expression of these factors in cell lines and clinical samples. To determine Oct3/4 expression in the cervical carcinoma cell line HeLa and the breast cancer cell line MCF7 in comparison with the human teratoma cell line nTera, Cantz et al. (2008) were unable to detect Oct3/4 transcription factor in the nucleus of HeLa and MCF7 cells by immunofluorescency using two different monoclonal antibodies. Faint cytoplasmic staining in HeLa and MCF7 cells was observed; however, no Oct3/4 signal could be detected by Western blot analysis. In addition, the levels of Oct3/4 mRNA in HeLa and in MCF7 cells detected by RT-PCR were low. Furthermore, the authors discovered that the Oct3/4 promoter region was highly methylated in these cells. Therefore the authors concluded that there was no Oct3/4 expression in somatic cancer cell lines. Later on another similar finding was reported. In this study, the authors analyzed 42 human tumor cell lines from 13 entities and human bone marrow-derived mesenchymal stem cells (MSC) in addition to some solid tumors with the methods of RT-PCR, Western blotting, immunocytochemistry and immunohistochemistry. With the exception of typical embryonal carcinoma cells, the authors did not observe reliable Oct3/4 expression in these cells (Mueller et al. 2009).

To our knowledge, these negative findings, in contrast to the positive Oct3/4, Nanog and Sox2 in tumor cell lines and solid tumors reported in literature, may be due to the controls used. As shown in Fig. 12.1, tumor cell lines in vitro could

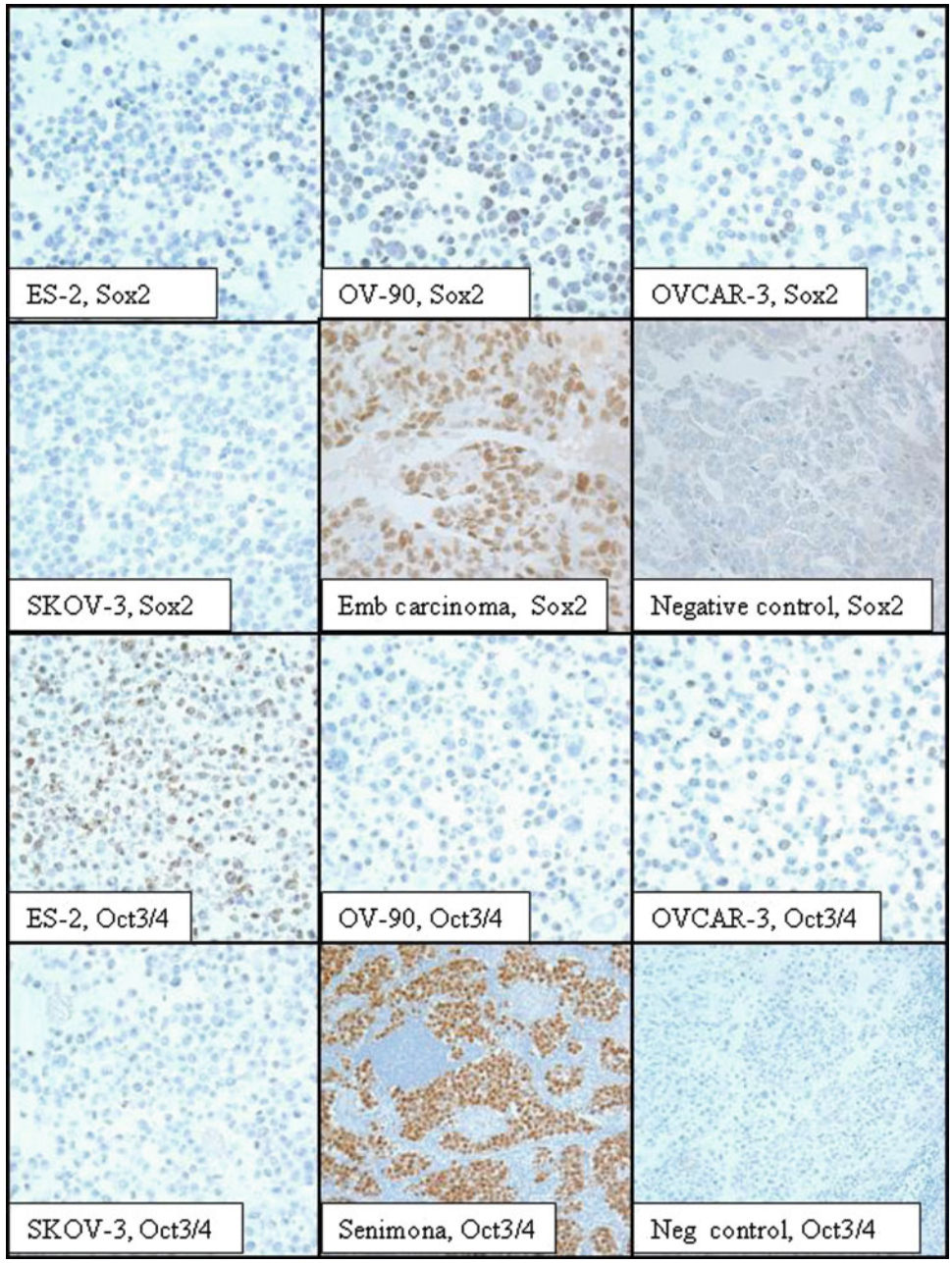

Fig. 12.1 Immunocytochemical staining for Sox2 and Oct3/4 in human ovarian cancer cell lines using a monoclonal rabbit anti-human Sox2 (clone D6D9, Cell Signalling Technology) and a goat anti-human Oct3/4 antibody (AF1759, R&D Systems) showing variable reactions in these cell *lines*. All photos, except the Oct3/4 *negative* control at 200×, were taken at 400× magnification

be detected with variable positive nuclear staining of Oct3/4 and Sox2. However, compared to the seminoma (positive control for Oct3/4) and embryonic carcinoma (positive control for Sox2) where strong immunointensities are shown, the ovarian cancer cell lines are often weakly or moderately positive. But, if the immunocytochemical procedure is optimized in such a condition

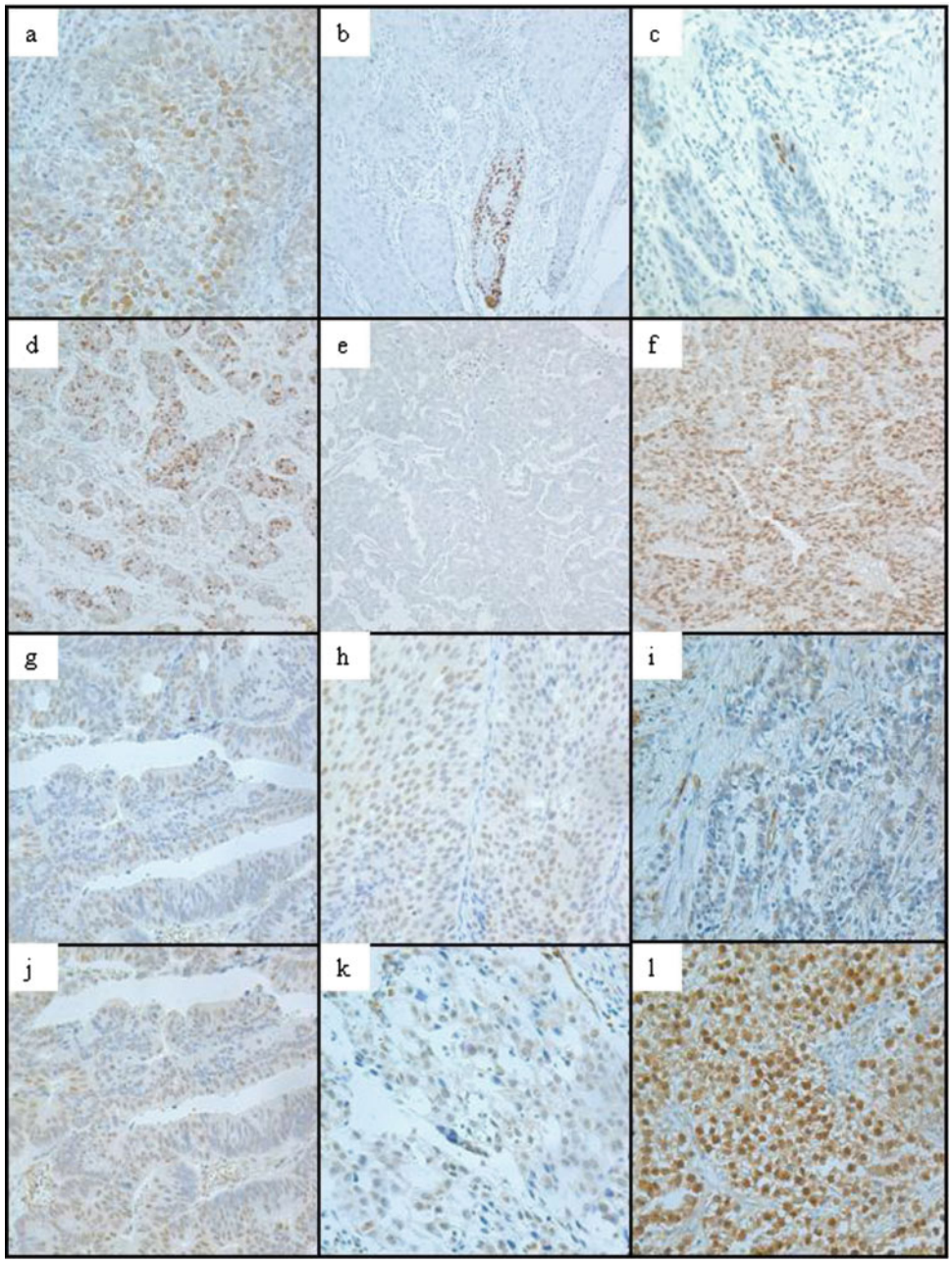

Fig. 12.2 Immunohistochemical staining for Sox2 and Oct3/4 in human solid tumors using a monoclonal rabbit anti-human Sox2 (clone D6D9, Cell Signalling Technology) and a goat anti-human Oct3/4 antibody (AF1759, R&D Systems). Variable Sox2 immunoreactivities of urine bladder (**a**), vulvar (**b** and **c**) and gastric carcinomas (**d**) are shown. Other cell populations of the same gastric tumor as shown in d are always negative (**e**). Comparatively, the Sox2 immunostaining in embryonal carcinoma (**f**) is much stronger. Moderate Oct3/4 immunoreactivities in urine bladder carcinoma cells are shown in **g** and **h**. There are weak (**i** and **j**) and moderate (**k**) Oct3/4 immunoreactivities in gastric carcinomas. Comparatively stronger Oct3/4 immunostaining in a seminoma is shown in (**l**). All photos, except **b** in 200×, were taken at 400× magnification

that soon after the positive controls show positivity and the whole procedure will be terminated, it may be not enough for the detection of the weak signals. Our experience is that as long as no background staining appears, increasing first antibody concentration/incubation and longer colorization development are necessary. For solid tumors, proper immunohistochemical procedure permits positive Oct3/4 and Sox2 detection as well, although their expression is, in general, in lower levels, compared to the Oct3/4 expression in seminoma or the Sox2 expression in embryonal carcinoma (Fig. 12.2). However, it is still useful to explore the function or clinical correlation of the low levels stemness factors expression in solid tumors, even though some times there are only a few tumor cells positive for the stemness factors revealed by immunohistochemical method in some tumors.

CS Cell Line Development

Mouse ES cell line development took several decades of efforts. Naturally, the ICM cells are maintained in vivo for several days. In the human embryo, ICM cell formation starts at day 3 after fertilization, and differentiation is beginning after day 7. Although much about human ES cells is still to be explored, successful establishment of mouse ES cells has shown that cultivation of these isolated ICM cells with typical feeder cells, now can be replaced with defined factors including leukaemia inbitory fator, LIF, in addition to special medium, and the cell stemness of the isolated ICM can be maintained and propagated in vitro without stemness change.

Although great progress has been achieved during the past years in the CS field, CS cell lines are still missing. It is mandatory to know how cancer cell stemness can be regulated and how CS cells can be maintained and propagated in vitro. It is attempting to believe that the best way to decode CS cells is by building models of CS cell lines in vitro. As shown in Fig. 12.3, ES cell line establishment (mainly from the work of mouse ES cell lines) has shown that some

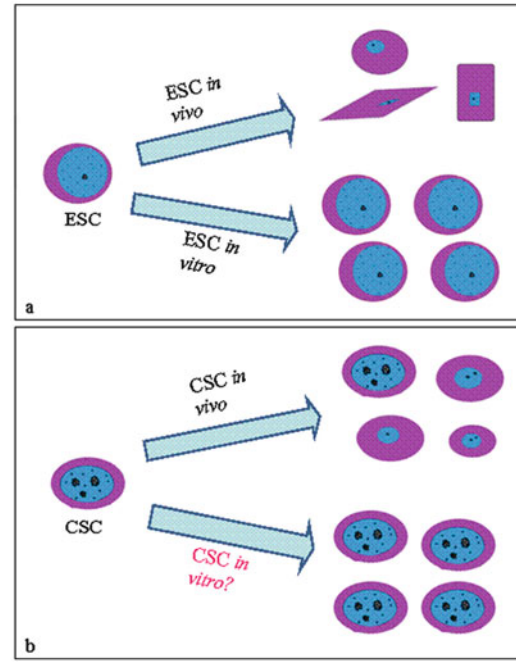

Fig. 12.3 The fate of ES and CS cells. (**a**) Naturally (in vivo), the undifferentiated cells from the inner cell mass where the ES cells are derived in culture will differentiate into more than 200 different cell types after embryo implantation into the uterine epithelium; however, with proper technologies these ES cells can be maintained and propagated in vitro without stemness alteration now. (**b**) Tumors in vivo (in tumor tissues) produce variable tumor cells where CS cell feature is kept in only a very small population cells, and all other tumor cells in the tumor mass are non-tumorigenic. The development of new technologies to isolate, maintain, and propagate CS cells in vitro without stemness changes like we see in ES cells, will greatly improve our understanding of stemness regulation of cancer cells in order to establish CS cell lines

transcription factors play more important roles than others in stemness maintenance and ES cell propagation. Various factors like oxygen concentration, cytokines, chemokines, chemotherapeutic reagents and all biological active molecules which may positively or negatively influence the stemness of cancer cells are all of great significance in the field of CS research. It is envisaged that it is possible to establish CS cell lines in vitro through extensively exploring these key factors. Furthermore, the models CS cell lines will be applicable to additional basic and clinical CS targeting studies.

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Induced Differentiation of Brain Tumour Stem Cells

13

Verena Kuchler, Euan S. Polson, Anjana Patel, and Heiko Wurdak

Contents

Abstract

Glioblastoma multiforme (GBM) is considered the most common and deadliest form of tumour arising in the human central nervous system. GBMs are inherently resistant to therapy and complete surgical resection is rarely achieved, and consequently, patient prognosis is poor. The discovery of stem-like cells in GBM channelled research towards the analysis of this 'brain tumour stem cell' (BTSC) population. Altered growth factor signalling combined with genetic, epigenetic and microRNA (miRNA) networks act to maintain the BTSC phenotype and aggressive nature of GBM tumours. Differentiation therapy presents as an intriguing option to specifically target BTSCs through 'forced' differentiation towards neuronal and glial lineages, thus eliminating BTSCs and sensitising the remaining tumour mass to current therapeutic strategies. Here we review current research and opinions surrounding this relatively recent concept in brain cancer biology that may prove to be a tool to treat high-grade brain tumours and improve patient survival.

Introduction

Despite its moderate incidence rate (approximately 7 in 100,000 people), brain cancer is a devastating disease with a very high mortality rate. The majority of brain tumour-associated

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deaths are attributed to the most frequent and aggressive form, glioblastoma multiforme (GBM). This common high-grade brain tumour (also known as WHO grade IV glioma/astrocytoma) is associated with extremely poor median patient survival rates (approximately 12–17 months) and high-grade brain tumours are often difficult to treat in the clinic due to the brain-infiltrative growth of the cancer. Even surgical resection combined with radio- and chemotherapy does not always provide a cure, and hence, brain tumour recurrence is common and ultimately fatal. Thus, for improving prognosis for GBM, novel more efficient therapies combined with a better understanding of brain tumour biology are urgently needed.

A potential break-through in brain tumour biology occurred within the last decade with the identification of cells with stem-like characteristics, herein termed brain tumour stem cells (BTSCs). Indeed, initial studies, combined with a vast amount of more recent data, provide compelling evidence that BTSCs represent the most dangerous cellular subpopulation within high-grade brain tumours (reviewed in Dirks 2010). BTSCs are thought to be responsible for brain tumour initiation, progression, and recurrence. However, BTSC behaviour and their molecular characteristics, including the correlation between distinct surface markers and BTSC phenotypes, may change dynamically during tumour development and progression. Therefore, while BTSCs share features such as cell fate marker protein expression, self-renewal and differentiation capacity with normal neural stem/progenitor cells (NSCs), BTSCs remain 'operationally defined'. BTSC enrichment from patient tissue specimens revolves around two major methods: (1) the use of specific cell surface markers and flow cytometry (e.g., Dirks 2010), or (2) propagation of unsorted primary cells in serum-free culture conditions that select for a homogeneous BTSC phenotype (e.g., Lee et al. 2006). Prominin-1 (PROM1; also known as CD133) is so far the most widely utilised antigen for BTSC isolation, although, in line with the emerging notion of BTSC complexity, other markers including CD15/SSEA-1 have been discussed (Dirks 2010). However, the BTSC numbers that can be yielded by FACS-based enrichment are limited and the surface marker phenotype of the pre-sorted BTSC population is likely unstable during culturing and prolonged cell propagation.

Irrespective of the isolation method, the authentication of BTSCs occurs through functional assessment of two key 'stem-cell' characteristics: self-renewal and differentiation capacity. Utilisation of the neurosphere assay and/or adherent clonal growth assays allows for the assessment of self-renewal and the expression of markers that are indicative of an undifferentiated NSC-like cell state (e.g., NESTIN, SOX2), or a more differentiated cell state (e.g., GFAP and TuJ1). For example, differentiation can be robustly induced in BTSC cultures by the addition of ~10% Serum. Importantly, Lee et al. (2006) showed that continuous culture in serum-containing conditions leads to loss of molecular and phenotypic BTSC features and propagation of cells that are biologically and genetically different to the parental tumour. The gold standard for assessing BTSC characteristics, however, is the orthotopic xenograft model. Here, BTSCs are typically intracranially injected into the brains of immunodeficient mice because the resulting primary tumours have been shown to resemble high-grade brain tumours in patients in terms of histological and molecular features (e.g., Lee et al. 2006; Wurdak et al. 2010). As a result of their therapy resistance (e.g., upon induced DNA damage) and their undifferentiated cell state, BTSCs conceivably repopulate the tumour mass after surgical removal/DNA-damaging therapy, and thus, their elimination is expected to improve patient outcomes (Dirks 2010).

Although a relatively recent concept in GBM, differentiation therapy targeting undifferentiated (stem cell-like) cancer cells was originally proposed in the 1970s in myeloid leukaemia and later applied clinically for acute promyelocytic leukaemia (APL) with the use of *all-trans* retinoic acid (RA). This compound targets the PML-RARα fusion protein triggering its degradation and activating a normal differentiation program in APL cells, ultimately leading to APL cell death (Zhou et al. 2005). Interestingly, recent

studies suggest that several growth factors (e.g., bone morphogenetic proteins (BMPs)) as well as small molecules (including RA) can critically regulate the balance between BTSC maintenance and differentiation. Importantly, depleting BTSCs by their 'forced' differentiation is expected to render high-grade brain tumours more susceptible to conventional therapies. Here, we give an overview on the current status of BTSC differentiation paradigms and discuss the targeted differentiation of BTSCs in the light of tumour complexity.

Growth Factor Signalling

Growth factor and cytokine signalling, such as the transforming growth factor beta (TGF-β) and Notch pathways, have been widely implicated in the regulation of NSCs and aberrant activation of these pathways can promote tumourigenesis (e.g., Massague 2008). Proteins of the TGF-β family signal through their respective receptors leading to phosphorylation of Smad proteins (Smad1, 5 and 8 in the case of BMP signalling). Once activated, Smads form heteromers with Smad4, a signalling protein shared between TGF-β, Activin and BMP signalling pathways. Subsequently, Smad heteromers translocate into the nucleus where they interact with other transcriptional co-factors, resulting in transcriptional activation or repression of target genes depending on the cellular context (Massague 2008). Notably, BTSCs have been shown to express BMP receptors and they consequently exhibit a response to BMP. In their hallmark study, Piccirillo et al. (2006) showed that BMP4, and also BMPs 2, 5, 6 and 7, reduce the proliferation of short term-cultured BTSC populations, both unsorted and enriched for the cell surface marker CD133. Importantly, the authors linked growth retardation of BMP-treated BTSCs to a strong pro-differentiation phenotype that manifests itself in overt cell morphology changes and markedly increased expression of the astroglial marker GFAP. Consistent with the in vitro results, in vivo delivery of BMP4 had an inhibitory effect on the development of brain tumours in mice injected with BTSCs (Piccirillo et al. 2006). In a more recent study by Tate et al. (2012), BMP7 was used to investigate the role of BMP signalling as a key regulator of the BTSC phenotype. The authors demonstrated that BMP7 reduces cell growth and decreases the expression of stem cell markers SOX2, NESTIN, OLIG2 and NANOG, concomitantly increasing the expression of differentiation markers GFAP and TuJ1 in at least three different BTSC lines. Moreover, they observed that BMP7 treatment reduced the invasiveness of xenograft tumours and decreased their angiogenic potential (Tate et al. 2012). Along this line, Chirasani et al. (2010) investigated a potential anti-tumourigenic effect of BMP7-expressing endogenous NSCs. Using a mouse model the authors analyzed the migration of GFP-expressing NSCs towards intracranially injected astrocytoma cells. Indeed, endogenous NSCs were recruited from the subventricular zone to the developing tumour site and these cells abundantly expressed and released BMP7 resulting in an anti-tumourigenic effect and downregulation of the transcription factor OLIG2, a known marker for diffuse brain tumours (Chirasani et al. 2010).

Taken together, strong evidence indicates that various BMP isoforms (including BMP2, 4, 5, 6, and 7) elicit a pro-differentiation effect in BTSCs; however, signal transduction downstream of BMPs is not well understood, for example at the level of BMP receptor utilisation. BMP receptors are serine/threonine kinases that include the type I receptors BMPR1A, BMPR1B, and the type II receptor BMPR2. Different BMPs may trigger different BMPR homodimer or heterodimer formations in different cellular contexts and BMP receptor activity appears to play an important role in BTSC behaviour. Indeed, significant downregulation of BMPR1B mRNA has been reported in approximately 20% of GBMs, likely due to epigenetic silencing (Lee et al. 2008). Significantly reduced BMPR1B expression levels can also be found in certain BTSC populations and these BTSCs become irresponsive to BMP2/4-induced differentiation, while responding to BMP signals with increased proliferation and self-renewal capacity; notably, BMPR1 overexpression can reverse this effect

(Lee et al. 2008). In addition, the pro-differentiation effect of BMPs will likely depend on the tumour microenvironment: for example, low oxygen tension (2% oxygen) has been shown to block BMP-triggered differentiation of BTSCs in paediatric brain tumour-derived precursor cells (Pistollato et al. 2009). Along this line, different BTSC niches within a tumour have been recently discussed (Lathia et al. 2011), and conceivably, BTSCs localized in certain hypoxic tumour regions may not as efficiently differentiate upon exposure to BMPs as BTSCs that grow under normoxic conditions.

For the regulatory cytokine TGF-β, a dual role has been reported depending on the cell/tumour type and their developmental stage. In normal epithelial cells, TGF-β signalling has an inhibitory effect on proliferation; however, it acts as an oncogene in glioma inducing abnormal cell proliferation (Massague 2008). The cytokine leukaemia inhibitory factor (LIF) has been shown to connect TGF-β signalling to the JAK-STAT pathway resulting in the activation of the self-renewal capacity of BTSCs (Penuelas et al. 2009). In a study conducted by Ikushima et al. (2009), SOX4 was found to be a direct target of TGF-β acting as a mediator of TGF-β-induced expression of SOX2. The authors therefore suggest that this TGF-β-SOX4-SOX2 pathway plays a critical role in the maintenance of the BTSC phenotype (Ikushima et al. 2009). TGF-β signalling can also induce the expression of the transcription factor inhibitors of DNA-binding (ID) proteins that are known regulators of cell differentiation and stem cell self-renewal. The effect of TGF-β on the expression of ID1 was shown in a BTSC population enriched for expression of the surface marker CD44. Notably, high CD44 levels correlated with elevated expression of ID1, hence defining a tumour stem cell population susceptible to treatment with TGF-β inhibitors (Anido et al. 2010). Additionally, these three independent studies reported that TGF-β treatment impedes BTSC differentiation by inducing expression of SOX2, resulting in the maintenance of a cellular 'stemness' program. Consistently, inhibition of TGF-β signalling by several different TGF-β inhibitors results in drastically reduced tumourigenicity of BTSCs in vitro and in vivo (Ikushima et al. 2009; Penuelas et al. 2009; Anido et al. 2010).

There is a plethora of studies illuminating the role of Notch signalling in stem cell biology and, in glioma, expression of the receptor NOTCH1 has been reported to directly correlate with the degree of tumour progression, with NOTCH1 expression levels increasing from glioma grades I to IV (Jiang et al. 2011). Consistently, a recent study showed that inhibition of Notch signalling by γ-secretase inhibitors (GSI), which block downstream signal transduction by proteolytic cleavage of the intracellular Notch receptor domain, reduces proliferation and self-renewal of mouse NSCs and glioma-derived BTSCs in vitro (Hu et al. 2011). However, in the case of BTSCs, this was only observed in long-term GSI-treated cultures (7–14 days), whereas at early stages of treatment, the three tested BTSC lines showed resistance to the inhibitor. The same study demonstrated enhanced differentiation of BTSCs into intermediate NSCs as shown by increased neurite formation and neuronal marker expression from tumour spheres treated with GSI compared to untreated spheres (Hu et al. 2011). The Notch signalling pathway has been identified as being targeted by microRNAs such as miR-34a or miR-146a, which directly downregulate NOTCH1, inducing differentiation of BTSC lines (Guessous et al. 2010; Mei et al. 2011). In addition, Notch signalling can be negatively affected by RA (Ying et al. 2011).

To date, the anti-tumourigenic effect of RA has been demonstrated in several cancers including brain tumours. The RA differentiation pathway appears to be deregulated in malignant glioma as several RA signalling molecules (e.g. CRBP1, ALDH1A1, CYP26B1, FABP5) show an aberrant expression profile (Campos et al. 2011). In GBM, RA induces the expression of TuJ1 and GFAP, and reduces the expression of the progenitor cell marker NESTIN in tumour sphere cultures. Moreover, RA treatment leads to reduced growth of BTSCs in vivo (Ying et al. 2011). Interestingly, another study showed a role for nuclear receptor corepressor (N-CoR) in the differentiation

response of glioma cells triggered by RA and a synergistic effect of RA and the protein phosphatase-1 inhibitor okadaic acid that promotes N-CoR phosphorylation and subsequent translocation to the cytoplasm (Park et al. 2007).

In summary, several growth factors and/or cytokines and their downstream molecular pathways have been critically implicated in the maintenance of the BTSC phenotype. It is expected that the rapidly growing number of studies investigating the role of signalling molecules involved in BTSC proliferation and differentiation will provide both novel insight into the regulation of the BTSC phenotype and a wide range of potential therapeutic brain tumour targets.

MicroRNAs

MicroRNAs (miRNAs) are 20–23 nucleotides-long non-coding RNAs that act as post-transcriptional regulators of gene expression in a cellular context-dependent manner. Consequently, it has been suggested that the deregulation of miRNAs that are critical for neural development and normal stem cell function, is also integral to the development of GBM. Recent studies have revealed characteristic miRNA expression patterns common to GBM when compared to the surrounding normal brain. They show consistently high expression of miR-21 and miR-9, and reduced expression of miR-7, miR-34a, miR-124, miR-137 and miR-128 (Godlewski et al. 2010; Guessous et al. 2010). The miRNAs showing high expression in GBM are now being linked to the maintenance of an undifferentiated BTSC phenotype. In particular, miR-9/9* are highly abundant in CD133-positive BTSCs with their inhibition resulting in reduction of primary, secondary and tertiary neurosphere formation and stimulation of a differentiation phenotype (Schraivogel et al. 2011).

While some miRNAs can promote the BTSC phenotype, miRNAs inducing BTSC differentiation have also been identified. For example, miR-124 and miR-137 were found to significantly increase the percentage of TuJ1-positive cells whilst reducing the percentage of GFAP-positive cells in BTSCs, consistent with a neuronal-like differentiation phenotype (Silber et al. 2008). In addition, non-specific BTSC differentiation, potentially into cells showing certain phenotypic similarities with astrocytes, neurons and oligodendrocytes, can occur as a result of miR-34a expression in BTSCs and its interactions with the Notch pathway (Guessous et al. 2010). Recently, miR-146a has been linked to a negative feedback loop that may act as a secondary tumour suppressor mechanism of glioma tumourigenesis through the downregulation of NOTCH1. In this context, another study showed cumulative upregulation of miR-146a in the presence of GBM-driving factors such as constitutive EGFR activation and PTEN loss in *Ink4a/Arf*$^{-/-}$ astrocytes. However, miR-146a overexpression attenuated proliferation, migration and the tumorigenic potential of the same astrocytes, suggesting that instead of functioning as an onco-miR as predicted, miR-146a may act as a tumour suppressor (Mei et al. 2011). The final miRNA highlighted above, miR-128, has been shown to be involved in key pathways regulating BTSC growth and self-renewal. It has been implicated in the repression of mitogenic signalling mediated by EGFR and PDGFRα, both of which act to maintain BTSCs (Papagiannakopoulos et al. 2012). Moreover, miR-128 is linked to downregulation of the master regulatory polycomb group (PcG) gene BMI1, hence specifically blocking BTSC self-renewal (Guessous et al. 2010). Clearly, miRNAs are emerging as a common feature of not only neural development but also in GBM biology where they are functioning either to promote ‘stemness’ or inhibit differentiation and thus maintain tumourigenesis. Therefore, miRNAs and their direct targets may provide new options for therapeutic strategies against high-grade brain tumours, particularly GBM.

Small Chemical Compounds

In addition to the previously discussed miRNAs, several natural as well as synthetic chemical compounds have been found to impact the fate of BTSCs. One example is the naturally occurring

metabolite of Vitamin A, retinoic acid, whose pro-differentiation effect has been described above. Other natural compounds that have been tested for their effect on BTSCs are cannabinoids. Aguado et al. (2007) used primary GBM cell lines as well as widely used glioma cell lines for treatment with various cannabinoid receptor agonists and antagonists. Indeed, the agonist-treated cell lines showed an increased expression of glial and neuronal markers and decreased tumour growth in xenograft assays, suggesting a receptor-dependent initiation of tumour cell differentiation (Aguado et al. 2007). Zhuang et al. (2012) investigated the effect of curcumin and showed that this natural phenol can promote differentiation of BTSCs. Curcumin-treated BTSC tumour spheres showed a reduced expression of the BTSC markers NESTIN and CD133 as well as raised levels of differentiation and autophagy markers (Zhuang et al. 2012). The induction of autophagy was also observed in glioma cells upon treatment with 2-Hydroxyoleate (2OHOA), a synthetic fatty acid that activates sphingomyelin synthase and inhibits several pathways involved in cell proliferation and differentiation, such as the RAS-MAPK, Cyclin-CDK-DHFR, and PI3K-AKT pathways. In glioma cell lines, 2OHOA can initiate glioma-to-glial cell differentiation concomitantly leading to autophagy. Interestingly, the effect of 2OHOA seems to be more specific for cancer cells compared with normal somatic cells, hence suggesting a potential therapeutic window in cancer treatment for 2OHOA (Teres et al. 2012), which is used in the clinic as a blood pressure lowering drug.

Genetic Factors

A fundamental question in brain tumour biology regards the genetic pathways utilised by BTSCs to preserve their undifferentiated phenotype and enhanced DNA-repair mechanisms. To this end, NSC regulatory networks have been investigated in an attempt to identify the main factors involved in BTSC self-renewal and maintenance. Despite the overall complexity of the pathways involved, key players regulating the BTSC phenotype are emerging. Recent examples are proteins from the PcG and numerous oncogenes/tumour suppressors. PcG proteins form large multimeric protein complexes allowing for stable silencing of gene expression through histone modifications. Two key components of these complexes, histone-lysine N-methyltransferase (EZH2) and BMI1, have been shown to be associated with cancer stem cell self-renewal and maintenance in a number of human cancers including GBM (Valk-Lingbeek et al. 2004). Work by Abdouh et al. (2009) showed overexpression of both EZH2 and BMI1 in human GBM tumours, specifically in the CD133-positive BTSC population. Further, the authors focused on BMI1 function and showed that BMI1 knockdown promotes the induction of cell death and differentiation pathways, and abolishes tumour growth in xenograft tumour assays (Abdouh et al. 2009). Another study shed light onto an *Ink4a/Arf*-independent role for Bmi1 in a mouse model of high-grade glioma. Interestingly, the authors report that *Ink4a/Arf and Bmi1*-deficient tumours show a reduction in their differentiation capacity (Bruggeman et al. 2007).

The prominent proto-oncogene MYC has also been implicated in the maintenance of GBM-derived BTSCs as its knockdown blocked cell cycle progression and induced apoptosis in a CD133-positive cell population, potentially through the regulation of cyclin D1 and $p21^{WAF1/CIP1}$ (Wang et al. 2008). Interestingly, a key dual regulator of MYC, EP300 has also been linked to BTSC differentiation as EP300 overexpression biased BTSCs specifically towards an astrocyte-like phenotype. However, in the presence of MYC this effect was reversed, with BTSCs remaining in an undifferentiated state (Panicker et al. 2010). Zheng et al. (2008) recently proposed that the upregulation of MYC in GBM is a result of dual inactivation of *Trp53* and *Pten* based on studies using a murine CNS tumour model. Their data showed that dual, but not single, inactivation of these tumour suppressors blocked the differentiation potential of NSCs (in response to 1% FBS), concomitantly leading to a malignant transformation that in turn led to the development of glioma (grade III and IV in 73% of mice; Zheng et al.

2008). Other recent studies suggest the involvement of additional distinct genetic factors in the maintenance of the BTSC phenotype. Factors whose downregulation consistently inhibit neurosphere formation and the tumour-driving capabilities of BTSC populations include the actin-binding protein Girdin that is encoded by the human *CCDC88A* gene (Natsume et al. 2012), transcription factors SOX2 (Gangemi et al. 2009) and YB-1 (Fotovati et al. 2011) that are both critically linked to NSC biology, and the known glioma driver signal STAT3 (e.g., Sherry et al. 2009). Using a kinome-wide RNAi screen, genes responsible for directly regulating differentiation in several primary BTSC lines were recently identified (Wurdak et al. 2010). This screen revealed a number of candidate factors previously linked to GBM (e.g., LYN kinase), and importantly, also uncovered novel factors whose knockdown induced a strong differentiation phenotype in BTSCs. Among these factors, it was the adapter protein transformation/transcription domain-associated protein (TRRAP) that induced the strongest and most consistent differentiation phenotype across five molecularly distinct BTSC lines. Further analysis of the downstream effects of TRRAP knockdown highlighted its function in preventing BTSC cell cycle progression through cyclin A2 downregulation. In addition, TRRAP knockdown significantly suppressed BTSC tumourigenicity in a CB17/SCID xenograft model (Wurdak et al. 2010).

Current Challenges and Future Directions

The invasive and treatment-resistant nature of GBM tumours continues to hamper a good patient prognosis. The discovery of BTSCs has proven to be a significant advance for the identification of alternative research targets and the development of novel therapeutic strategies. As discussed within this chapter, the induction of differentiation pathways that result in a shift from the self-renewing BTSC phenotype to a less-tumourigenic cell status are paramount in current BTSC and GBM research strategies. Genetic studies identified three critical signalling pathways that are frequently altered in GBM: p53, RB and RTK-RAS-PI3K pathways. Based on the identified genomic alterations and gene expression patterns, GBMs can be classified into different subtypes – classical, mesenchymal, neural, and proneural (e.g., Verhaak et al. 2010). Clearly, future profiling efforts are expected to highlight the intricate nature of GBM subtyping, where the number of features analysed will influence the final segregation of each subtype. Interestingly, the subtype classifications of GBM, although originally encompassing primary human GBM tissues, also seem to be applicable to their BTSC component, with a very recent study showing that BTSC neurospheres can be categorised into mesenchymal or proneural subtypes (De Bacco et al. 2012). However, the important question arises as to whether BTSCs are themselves a heterogeneous cell population with a high degree of plasticity. This potential plasticity may be the consequence of changes within intrinsic cellular features (e.g., random accumulation of mutations) and/or driven by the tumour microenvironment. Unfortunately, it is still unclear as to how BTSCs interact with other cancer or non-cancer cells during tumour progression, how molecularly distinct BTSC-like populations emerge within the tumour environment, and how this affects differentiation therapies directed at BTSCs. One plausible theory is that the highly infiltrative phenotype of GBM may contribute to the diversity observed within a single tumour, as spatially distinct BTSC populations may respond to unique signals present within each individual 'niche'. In a recent review Lathia et al. (2011) propose the existence of three distinct BTSC niches within a high-grade brain tumour: the hypoxic niche, the perivascular niche and the invasive niche, where each microenvironment is supportive of BTSC maintenance. Moreover, BTSCs themselves are likely actively involved in generating these niches through complex cross-talk with the immediate and also distant microenvironments of the tumour (Lathia et al. 2011). Notably, similar to NSCs, BTSCs are able to migrate to other regions of the brain, and thus, it is conceivable that their gene expression patterns

evolve along these migration routes in response to the changing microenvironment. This presents a further challenge to BTSC research, whereby efficient targeting of these cells will have to consider their highly motile properties and the consequences this may have on any single target gene. In this context, future studies will likely benefit from sophisticated in vivo model systems that allow for BTSC lineage tracing and analysis of the interplay between single BTSCs and cells of their microenvironment.

Overall, the wealth of new data emerging from GBM research is encouraging in the search for an effective therapy including 'forced' BTSC differentiation. Current challenges relate to specific gene functions as well as key regulatory networks in diverse BTSC populations and GBM subtypes. A better understanding of BTSC populations in multiple contexts, for example in the context of a complex brain tumour microenvironment, will ultimately push current and future research towards the development of more targeted treatments in diverse brain tumour model systems, and ultimately, in patients. The elimination of BTSCs, which are resistant to chemotherapy and responsible for tumour recurrence after surgical removal/DNA-damaging therapy, constitutes an important goal in current research. Overall, differentiation therapy targeting BTSCs represents a potential tool as part of a combinatorial therapy to improve the overall treatment outcome of high-grade brain tumours.

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Role of Microenvironment in Regulating Stem Cell and Tumor Initiating Cancer Cell Behavior and Its Potential Therapeutic Implications

14

Ana Krtolica

Contents

Abstract

Microenvironment plays a key role in controlling stem cell fate and thereby regulating tissue homeostasis and repair. It consists of acellular and cellular components that interact with stem cells and their progenitors and through signaling cascades influence balance between self-renewal, differentiation and dormancy. Under pathological conditions, disruptions in microenvironment can generate signals that stimulate untimely or aberrant stem cell differentiation or self-renewal, or activate de-differentiation of progenitor cells, leading to diseased states such as cancer. However, while unaltered microenvironment can restrain transformed cell behavior inhibiting malignant phenotypes, transformed cancer cells that exhibit resistance to conventional therapies and tumor initiating capacity are capable of inducing more permissive and immunotolerant microenvironment that promotes tumor growth and metastasis. Better understanding of their behavior and interactions with microenvironment opens up novel avenues for devising more efficacious cancer therapies.

Introduction

Stem cells represent populations of cells within organism that are capable of self-renewal and differentiation into one or more cell types that form a specific tissue (uni- or multi- potent stem cells, respectively) or the whole body

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(pluripotent or omnipotent stem cells). Orderly development and tissue repair require tight balance between arrested stem cell state (dormancy), stem cell self-renewal and differentiation, and in case of multipotent and pluripotent stem cells, between multiple differentiation pathways. This is achieved by stringent control of stem cell behavior exerted through signals from their microenvironment (niche). For instance, signals stemming from cell-cell and cell-matrix interactions, changes in oxygen concentration (i.e. hypoxia) and nutrient availability, all affect stem cell state and phenotype. Escape from and/or deregulation of the signaling cues present in microenvironment loosens control over stem cell function and results in disruption of tissue homeostasis leading to pathological states. Understanding the mechanisms involved in the cross talk between acellular and cellular elements of the microenvironment and their effects on normal and cancer stem cell behavior is crucial for the development of more efficacious treatments for pathological states associated with aberrant stem and/or progenitor cell phenotypes such as cancer.

Stem Cells' Behavior Is Controlled by Their Niches: Examples of Epidermal Stem Cell Niche and of Hypoxia

Embryonic development and multiple physiologic processes depend on timely and regulated activation and differentiation of stem cells, processes controlled by signals from the niche. Typically, the stem cell niche is a defined anatomical compartment that includes cellular and acellular components that integrate both systemic and local cues to regulate the biology of stem cells. Cells, blood vessels, matrix glycoproteins, and the three-dimensional space that is formed from this architecture provide a highly specialized microenvironment for a stem cell. Stem cell niches facilitate the interaction between the stem cell and surrounding cells in a spatially and temporally defined manner that maintains tissue homeostasis. Niche includes extracellular components as well as diffusible factors to provide the proper regulation of the stem cell.

Epidermal stem cell niche. We will illustrate the role of the niche with an example of interfollicular epidermal stem cells in human skin. In humans large portions of the skin lack hair folicules and stem cells appear to be dispersed along the basal compartment of the interfollicular epidermis. While here, specific niche is difficult to define in morphological terms, patterns or gradients in structural elements and/or positive and negative signals generate niches that enable maintenance and functionality of the stem cell population. Even in the absence of morphological separation present in more commonly described epidermal stem cell hair buldge niche of the mouse, it still incorporates all the key interactions present in other stem cell niches. In both cases, epidermal stem cells reside in fine-tuned microenvironments that are controlled by constant cell–cell and cell–matrix interactions. They sit on and, through integrin and other receptors interact with, a specialized extracellular matrix layer called basement membrane that provides an interface between the epidermis and the underlying dermis. It is made from a complex network of extracellular matrix molecules, including several laminins, type IV collagen, nidogen and perlecan, all of which are necessary for its native structure and epidermal tissue formation. Cellular constituents of the niche include fibroblasts, endothelial cells and inflammatory cells and, presumably, also neighboring keratinocytes and melanocytes, Merkel cells and Langerhans cells. When three-dimensional in vitro model of human skin (dermal equivalent), was modified in a way that allowed for the formation of an authentic dermis-like matrix, it enabled long-term regeneration of the epidermis (Boehnke et al. 2007). These studies also confirmed the major impact of keratinocytes on ECM assembly and maturation in the dermis and clearly underlined the relevance of mutual epithelial–mesenchymal interaction for establishing a proper stem cell niche.

Epidermal stem cell maintenance is likely regulated by Notch ligand Delta1 whose expression is confined to the basal layer of human epidermis, with highest levels in regions presumably

harboring stem cells. Furthermore, epidermal stem cell differentiation and maintenance of interfollicular epidermis are determined by a fine-tuned balance between intracellular levels of Notch and p63. Another crucial regulator of homeostasis in the interfollicular epidermis is the EGFR with its multiple mediators such as amphiregulin, epiregulin, heparin binding-epidermal growth factor and transforming growth factor (TGF)-α, all acting in an autocrine and paracrine manner. The role for several major EGFR-activated pathways is described including MAPK, PI3K/AKT, JAK/STAT and PKC cascades. Antagonizing interaction of EGFR and Notch family members in the differentiating epidermis resulted in their mutual downregulation strongly arguing for their essential functional interdependence.

Role of hypoxia. Stem cell fate is further regulated by conditions in the microenvironment. For example, low oxygen tension (hypoxia) maintains undifferentiated states of embryonic, hematopoietic, mesenchymal, and neural stem cell phenotypes and also influences their proliferation and cell-fate commitment. It has been hypothesized that the presence of low oxygen tension in stem cell niches offers a selective advantage that is well suited to their particular biological role. That is, essentially all cells that undergo aerobic metabolism are subject to some degree of oxidative stress through the generation of reactive oxygen species that can damage DNA. This effect is demonstrated by the fact that mouse embryonic fibroblasts accumulate more mutations and senesce faster when cultured under 20% oxygen than cells cultured under 3% oxygen (Parrinello et al. 2003). By residing in anatomical compartments that experience relatively low oxygen tensions (in the range of 1–9%), stem cells may escape this damage and maintain low proliferation rate. In addition, hypoxia has been shown in multiple stem cell systems to activate molecular pathways that control Oct4 and Notch signaling, two important regulators of stemness. Indeed, human ESC derivation from single embryonic cells (blastomeres) has been enhanced under mildly hypoxic conditions (8%) and eliminated the need for serum, essential ingredient for blastomere derivation under 20% oxygen. This gives further support to the notion that lower oxygen tension promotes better survival and self-renewal of pluripotent ESC (Ilic et al. 2009). Finally, oxygen tensions as low as 1% appear to decrease proliferation and maintain ESC pluripotency, while higher oxygen tensions (3–5%) appear to maintain pluripotency with no effect on proliferation. These results suggest that proliferation and perhaps even stem cell dormancy may be regulated by gradients of oxygen tension supplied by stem cells' local niche.

Hypoxia appears to maintain an immature blast-like quality in mouse hematopoietic stem cells (HSC) with a primitive phenotype and enhanced engraftment capabilities (Eliasson and Jonsson 2010). Several investigators have demonstrated that slow-cycling HSC are more likely to localize in the low oxygen areas of the marrow, away from blood vessels, whereas fast cycling early hematopoietic progenitors with limited capacity for self-renewal reside in areas much closer to vasculature. Although hypoxic cultivation of bone marrow cells has been shown to increase their ability to repopulate and engraft, it is still unclear whether these effects are due to direct action on HSCs or other stromal elements, as many of these experiments were performed with whole marrow or partially purified cell populations (Eliasson and Jonsson 2010). HSCs present in the hypoxic niche express higher levels of Notch-1, telomerase, and the cell-cycle inhibitor p21 than cells closer to the vasculature (Jang and Sharkis 2007). Remarkably, extremely low oxygen tensions (0.1%) push CD34+ cells into an essentially quiescent state (Hermitte et al. 2006). HIF-1 has emerged as a likely candidate for this regulatory mechanism, as several groups have demonstrated that HIF can mediate cell-cycle arrest in multiple cell lines. In addition, mice with defective HIF signaling exhibit numerous hematopoietic pathologies with prominent defects in hematopoiesis that are embryonically lethal (Eliasson and Jonsson 2010). Collectively, this evidence suggests that hypoxia is a critical component of the HSC niche, and exposure of HSC to elevated oxygen tensions negatively affects their self-renewal while promoting cell-cycle entry and differentiation.

Hypoxia also likely plays a role in a neural stem cell (NSC) maintenance. In the human brain, partial oxygen pressure (pO_2) varies from approximately 3% (23 mmHg) to 4% (33 mmHg), demonstrating a physiological oxygen gradient that is the highest in the alveolar space and the lowest in tissues where NSC likely reside. It is thus, likely that NSCs in the brain are located in a relatively hypoxic environment. Thus, in addition to the intercellular signals, soluble factors, blood vessels, and the extracellular matrix proteins found in neurogenic niches, oxygen tension may be an additional important component of the neural stem cell niche. In vitro, hypoxia is able to promote an undifferentiated state in neural crest stem cells and NSC. Observations regarding an enhancement in survival and proliferation of NSCs in mild hypoxic conditions have also been made (Pistollato et al. 2007). It has been shown that p53 phosphorylation increases in cultures maintained at 20% oxygen resulting in cell-cycle arrest, decreased proliferation, and differentiation of NSCs toward the glial lineage (Pistollato et al. 2007). This finding suggests that oxygen tensions in the environment influence both NSC stemness (or the maintenance of an undifferentiated state) by inhibiting their differentiation and their specific fate by modulating important intracellular pathways such as p53 and Notch signaling (Pistollato et al. 2007). Therefore, oxygen tension in the neural niche functions to maintain stem cell self-renewal and an undifferentiated state. Although direct measurements of oxygen tension of the subventricular zone (SVZ) have not been made, our current understanding of the cytoarchitecture and its relation to adjacent blood cells suggests that oxygen can be limiting near the ependymal surface where the neural stem cells reside. In conclusion, a hypoxic microenvironment facilitates stemness and prevents NSC from differentiating. Changes in redox status or other local cues mobilize the NSC population to proliferate, differentiate, or migrate.

Other factors such as nutrient availability also affect stem cell behavior. The mammalian target of rapamycin (mTOR) seems to play a key role within a cell in integrating a myriad of external and internal signals including niche oxygen levels and nutrient availability, cell energy status, presence of cellular stressors, and growth factors. The finely tuned response of mTOR to these stimuli results in alterations in stem cell metabolism, differentiation and cell growth, playing a major role in stem cell homeostasis and lifespan determination (reviewed in (Russell et al. 2011)).

Disruption of Niche-Stem Cell Interactions Induces Pathological States: Premature Aging and Tumorigenesis

Disruptions/failures in stem cell regulation lead to pathological states such as premature aging and cancer. For example, untimely and altered differentiation of mesenchimal stem cells (MSC) by upregulated downstream Notch signaling causes premature-aging disease Hutchinson–Gilford Progeria Syndrome (HGPS). This is due to a mutant version of lamin A protein called progerin that increases availability of the SKIP, nuclear matrix-associated co-activator of the Notch targets' transcription. Significantly, activation of Notch pathway not only induced premature differentiation of MSC, but also enhanced osteogenesis while inhibiting differentiation of hMSCs into adipose tissue altering a balance between multiple differentiation pathways (Scaffidi and Misteli 2008). Alterations in stem cell regulation can also be caused by external signals and insults from the microenvironment such as ionizing radiation. For example, ionizing radiation-induced premature differentiation of melanocyte stem cells and resulting melanocyte stem cells depletion lead to irreversible hair graying (Inomata et al. 2009).

The altered niche environment can also induce aberrant activation of stem cell phenotype and self-renewal, the phenomenon that may lead to tumorigenesis. There is strong evidence to support the role of permissive microenvironment in promoting tumorigenesis both at premalignant and malignant stages. Multiple studies have shown that tumor-associated stroma can promote tumorigenesis by creating pro-inflammatory microenvironment. For example, tumor associated-fibroblasts

isolated from the tumor stroma produce plethora of pro-inflammatory cytokines and growth factors and stimulate malignant transformation of multiple epithelial cell types including breast and prostate (Aboussekhra 2011). Pro-inflammatory secretome including IL-1, IL-6, IL-8 and GROα, has also been shown to contribute to pro-carcinogenic microenvironment associated with aging and stress-induced cell senescence (Coppe et al. 2010). Moreover, these cytokines have been shown to play key role in supporting cancer stem cell phenotypes and stem cell self-renewal (Krtolica et al. 2011; Korkaya et al. 2012). Indeed, recent evidence suggests that secretion of IL-1 by carcinoma cells attracts MSC to tumor-associated stroma and via prostaglandin E2 signaling induces MSC to generate pro-inflammatory cytokines that promote β catenin activation and cancer stem cell phenotype (Li et al. 2012).

However, microenvironment can also restrict cell behavior and, in some instances, restrain frankly malignant state through direct control of growth and invasiveness. For example, Weaver and colleagues have shown that malignant phenotype of breast tumor cells can be reversed in three-dimensional culture and in vivo by integrin β1 blocking antibodies which induced them to form polarized acini and cease growth (Weaver et al. 1997). Additionally, microenvironment can exert control through immunosurveillance – immune system-mediated tumor cell recognition and consequent destruction (see discussion below).

Tumor-Initiating and Cancer Stem Cell-Like State Is Affected by Niche/Microenvironment

There is currently growing evidence for the presence of cancer stem-like or tumor initiating cells in multiple tumor types – both hematological malignancies and solid cancers (Bonnet and Dick 1997; Reya et al. 2001). Cancer stem cells (CSC) or tumor initiating cells (TIC) are functionally defined by their potential to recapitulate tumor from which they were isolated at the single cell level. To this end, they are usually identified using serial transplantation experiments where limited number of cells isolated from the original tumor are transplanted into recipient animals and, once tumor is formed, this procedure is repeated multiple times with additional animals demonstrating CSC/TIC ability to initiate new tumors. New evidence demonstrates CSC/TIC clonal capacity even within natural tumor niches (Chen et al. 2012; Driessens et al. 2012; Schepers et al. 2012). These characteristics imply an unlimited proliferative capacity and also an ability to differentiate into all cell types present in the given tumor. What makes CSC/TIC-like populations within tumor even more therapeutically relevant is that their phenotype is often associated with high resistance to common therapeutic modalities of cancer treatment: chemotherapy and ionizing radiation. It is therefore, hypothesized, that they may be the major source of tumor re-growth and patient relapse after therapy. Indeed, there is growing clinical evidence that this may be the case (Li et al. 2008).

The ability of cancer cells to establish themselves in a foreign cellular environment is an essential characteristic of successful metastasis and a defining characteristic of CSC/TICs. Furthermore, there is some evidence that CSC/TIC phenotype may significantly overlap with phenotype of cells undergoing epithelial to mesenchymal transition (EMT), a phenomenon associated with increased tumor aggressiveness and metastasis. EMT is driven by transcription factors, including SNAIL1/2, ZEB1/2, or TWIST1/2, which increase the invasiveness of epithelial cells. In several studies, the induction of EMT has been shown to enhance self-renewal and the acquisition of CSC characteristics (Ansieau et al. 2008). In contrast, some studies demonstrate that tumor cells with an epithelial, not mesenchymal, phenotype survive in the circulation and form distant metastasis (Celia-Terrassa et al. 2012). For example, in prostate cancer cell lines, subpopulations with a strong epithelial gene program were enriched in highly metastatic CSC/TIC, whereas mesenchymal subpopulations showed reduced numbers of CSC/TIC. Collectively, these studies illustrate cancer stem cell plasticity and the fact that cell-type specific characteristics govern their self-renewal and

mesenchymal gene interactions (Celia-Terrassa et al. 2012). Nevertheless, these data taken together with CSC/TIC chemo- and radiation-therapy resistance and capacity to form new tumors, suggest that it is quite likely that the cells with CSC/TIC characteristics may be the main sources of metastasis.

Importantly, every aspect of CSC/TIC behavior is under influence of microenvironment. For example, presence of drugs in tumor microenvironment and circulation may support survival and provide growth advantage to drug resistant tumor cells such as those expressing CSC/TIC phenotypes. Another example are hypoxic tumor microenvironments that often harbor quiescent (non-dividing) and tumor initiating cell populations. Hypoxia maintains the stem-like phenotype and prevents differentiation of CSC/TIC. It has been shown to promote self-renewal of glioblastoma and colorectal cancer stem cell-like CSC/TIC populations by inducing PI(3)K and ERK 1/2 pathways and regulating CDX1 and Notch1, respectively. While the degree to which quiescent and CSC/TIC populations overlap in hypoxic regions remains to be elucidated and may vary between different stages and types of tumors, it is clear that hypoxic tumor cells exhibit high resistance to common chemotherapeutic agents and are thus, likely responsible for tumor reoccurrence and, potentially, metastasis. Consequently, tumor hypoxia has been shown to correlate with poorer patient outcomes in multiple cancer types including colon, breast, prostate, and brain cancer.

Yet another effect of microenvironment is exerted through provisional extracellular matrix laid down by tumor-associated fibroblasts. It may promote migration and invasiveness thru integrin-fibronectin interactions and support survival of cells that have undergone EMT and are capable of metastasizing from the primary tumor. Tumor-associated fibroblasts and MSC present in tumor-activated stroma can also secrete a number of cytokines and growth factors creating a pro-inflammatory environment. This, in turn, can both directly affect CSC/TIC proliferation and motility (see discussion above) and also, modulate immune response (see next section).

Modulating the Effects of Microenvironment: Examples of Escaping Immunosurveillance and of Promoting Epithelial-to-Mesenchymal Transition

Tumor cell microenvironment contains, and is altered by, various components of both innate and adaptive immune system and their products. Depending on tumor immunogenicity, both initial tumor formation and progression of the disease can be significantly affected by host anti-tumor responses. By targeting premalignant and malignant cells in a process called immunosurveillance, immune responses can eliminate cancer cells prior to tumors becoming clinically apparent or can attenuate tumor growth and progression. However, mounting evidence shows that CSC/TIC effectively evade host immunosurveillance through multiple mechanisms including altered immunogenicity, production of immunomodulatory factors and direct interactions with tumor-infiltrating immune cells. We will discuss these different mechanisms in the following paragraphs.

MHC class I molecules are one of two primary classes of major histocompatibility complex (MHC) molecules and are found on every nucleated cell of the body. Their function is to present fragments of proteasome-degraded cellular and external proteins to cytotoxic T cells (CTL, CD8+). T cell receptors (TCR) and CD8 co-receptors on CTL plasma membrane interact with MHC I molecules that present the foreign protein fragments (antigens) on the surface of the affected cell. This interaction activates CTL to lyse the foreign antigen presenting cell.

MHC class I molecules play a key role in the immune recognition of transformed cells. It was recently reported that CSC/TIC may predominantly lose MHC class I molecules, and selectively silence the expression of tumor-associated antigens (TAAs), whose presence is associated with differentiated state, leading to resistance to immune rejection. Indeed, selective or general downregulation of MHC class I molecules may suppress the ability of class I MHC-restricted CTL to lyse CSC/TIC.

Consequently, flow cytometry analysis of glioblastoma multiforme (GBM) and astrocytoma CSC/TIC-enriched CD133+ cell fractions revealed that the majority of CD133+ cells did not express detectable MHC class I molecules or natural killer (NK) cell activating ligands on their cell surface (Wu et al. 2007). This may render them resistant to adaptive and innate immune surveillance. In addition, defects were found in the expression of antigen-processing machinery (APM) components in the cultured population of GBM-derived CSC/TIC (GSC; (Di Tomaso et al. 2010)). APM molecules included MHC class I molecules and their heavy chains (i.e., A-HC), β2-microglobulin, constitutive proteasome subunits (δ, MB1, and Z), immunoproteasome (LMP2, LMP7, and LMP10), transporter molecules (TAP), and chaperon molecules (tapasin, calnexin, calreticulin, and ERp57). While low levels of expression were also detected in corresponding fetal bovine serum-derived tumor cell lines in most cases expression was higher in tumor cell lines than in CSC/TIC (CSC/TIC are typically isolated under serum-free conditions, since serum constituents induce their differentiation into progenitor tumor cell types that have lower tumorigenic capacity). These results are in line with the previously reported decreased expression of MHC and APM molecules in a variety of human tumors and derived cell lines and suggest that in CSC/TIC isolated from these tumor types there is low efficiency in antigen processing and presentation. Therefore, CSC/TIC may display unique immunophenotypes, such as downregulation of MHC class I molecules, differentiation-associated TAAs and APM components, that enable them to effectively evade host immunosurveillance.

Another mechanism by which CSC/TIC may avoid the attack of immune system, is by inducing immunogenic tolerance through functional inactivation of antigen-reactive T lymphocytes or activation of regulatory T cells (Treg). Lymphocyte tolerance or anergy is likely induced by direct T cell inhibition via secretion of immunosuppressive cytokines, including IL-4, IL-10 and TGF-β. For example, researchers detected high levels of IL-4 and IL-4R in CD133+ stem-like cells in colon cancer. IL-4 has previously been reported to suppress apoptosis by enhancing the expression of anti-apoptotic proteins cFLIP, Bcl-xL, and PED in many tumor cell lines, including chronic lymphocytic leukemia B cells, as well as prostate, breast, and bladder tumor cell lines. Additionally, IL-4 has the capability of inhibiting the proliferation and immune responses of helper T (Th, CD4+) cells, and also exhibits immunoregulatory effect on B cells, mastocytes, and macrophages. Recent evidence shows that GSC can prevent CTL mediated specific immune cytotoxicity, and GSC can strongly inhibit the proliferation of Th cells. Effects of CSC/TIC IL-4 signaling may include autocrine inhibition of the apoptosis and induction of immune tolerance. In addition, TGF-β signaling pathway is specifically activated in the CSC/TIC fraction of breast cancers (Shipitsin et al. 2007), and secreted morphogens in the TGF-β super family as well as their receptors are preferentially expressed by CD133+ brain tumor CSC/TIC (Piccirillo et al. 2006) and by ABCB5+ malignant melanoma CSC/TIC (Schatton et al. 2008). It was shown that TGF-β negatively influences antitumor capabilities of host CTL. This activity is multi-directional and is based on the impairment of Fas-mediated apoptosis of tumor cells, downregulation of IFN-γ secretion and disturbed expression of perforin and granzymes by CTL. Indeed, T cell-specific blockade of TGF-β signaling was found to enhance the antitumor immune response in mice challenged with live tumor cells. Moreover, CSC/TIC may induce high levels of Treg cells to suppress the antitumor immune response and ultimately promote tumorigenic growth.

Immunogenic tolerance may also be achieved through clonal anergy of macrophages and dendritic cells (DC). Tumor associated macrophages (TAM) constitute one of the major immune cell populations responsible for both tumor rejection and promotion. The high expression of CD47 on leukemia stem cells (LSC) of AML patients can reduce the macrophage-induced phagocytosis of LSC and thus, decrease LSC clearance by innate immune system. CD47, also known as integrin-associated protein (IAP), can inhibit macrophage-mediated

phagocytosis by binding signal-regulatory protein-α (SIRPα) on their surface (Barclay and Brown 2006). Disruption of the CD47–SIRPα interaction with a monoclonal antibody against CD47 preferentially enabled the phagocytosis of AML LSC by human macrophages. In addition, CD47–SIRPα interaction between CD47 expressed by LSC and SIRPα present on DC surface can also inhibit DC activation (Barclay and Brown 2006). Moreover, functional inactivation of DC, a major type of antigen-presenting cells, can affect the activation of initial T cells and inhibit the adaptive immune response.

Expression of another immunosuppressive protein, CD200, was significantly higher in the CSC/TIC relative to differentiated tumor cell fractions isolated from prostate, breast, colon and brain tumors. Additionally, CD200 was co-expressed with CSC/TIC markers. CD200 is a type Ia membrane protein which exerts suppressive effects through binding to its receptor, CD200R. CD200R is present on the surface of myeloid DCs, monocyte/macrophage lineage and on T lymphocytes. It was shown that the stimulation of CD200R on DCs triggered tumor supporting reactions mediated by Th2 cytokines and increased Treg activity, thought to hamper tumor-specific effector T cell immunity (Curiel et al. 2004). Conversely, blockade of CD200/CD200R interactions with monoclonal anti-CD200 antibodies resulted in a shift towards Th1 activity and attenuated immune tolerance (Rygiel et al. 2012).

While attenuation of the immune response may promote carcinogenesis, the activity of immune system itself can also promote tumor development. For example, chronic inflammatory responses mediated by activated B cells and associated antibodies have been directly shown to be critical in the initiation of skin cancer in K14-HPV16 mice (de Visser et al. 2005). Furthermore, tumor growth could be promoted by TAM. TAM can contribute to either a pro-tumorigenic or anti-tumorigenic environment depending on their capacity to present antigens, produce inflammatory cytokines, stimulate angiogenesis, and enable cytotoxic activity. While tumors evade macrophage phagocytosis through the expression of anti-phagocytic signals, including CD200 and CD47 as discussed above, cytokine production and antigen presentation by macrophages have also been shown to directly impact tumor growth.

Moreover, the evidence suggests that immune effectors can induce EMT following an acute or chronic inflammatory response. Likely, CTL cells trafficking into the tumor microenvironment can produce direct mediators of EMT, or alternatively, can produce other cytokines or chemokines (e.g., MCP-1), which can attract additional immune effectors (e.g., macrophages) that provide the stimuli. When epithelial tumors from neu-transgenic mice, that express the cell surface neu oncogene under control of the mammary epithelial cell-specific mouse mammary tumor virus (MMTV) promoter, were transplanted into nontransgenic syngeneic mice, a T-cell-dependent rejection occurred. However, the mice subsequently relapsed with tumors enriched in neu-negative variant tumor cells that had a mesenchymal phenotype. CTL cells were required for outgrowth of the neu-negative mesenchymal variants suggesting local induction of EMT (Santisteban et al. 2009). Furthermore, CTL cells isolated from mice primed with neu positive tumor cells were able to induce antigen loss when co-cultured with neu-positive tumor cells. Tumor cells isolated from relapsed mice showed that these tumors had characteristics of breast cancer stem cells (BCSC), as indicated by the cell surface CD24−/loCD44+ marker profile, enhanced mammosphere formation and tumorigenicity, elevated expression of drug transporters, DNA repair enzymes, and enhanced resistance to chemotherapy and radiation. In accordance with characteristics of true CSC/TIC, BCSC gave rise in vivo to tumors with a heterogeneous tumor population consisting of both CD24− and CD24+ tumor cells with predominant neu-positive epithelial phenotype, suggesting that the immune induced EMT was fully reversible. Thus, in contrast to their typically ascribed protective role, CTL cells were capable of inducing tumors to undergo EMT and to acquire BCSC properties and a more aggressive tumor phenotype.

Therapeutic Implications

In devising anti-cancer therapy, it is important to take into account the effects of microenvironment. Probably best known example and most used cancer treatment that relies on altering microenvironment, is inhibition of angiogenesis by anti-VEGF antibody (bevacizumab/avastin) and thus, deprivation of tumors of their oxygen and nutrition supply. Another example is inhibition of hedgehog pathway through inactivation of smoothened (Saridegib/IPI-926, GDC-0449/vismodegib, LDE-225/erismodegib) that may act to eliminate fibrous tissue that hinders drugs from reaching the cancer, while also directly affecting TIC/CSC growth. Tumor site allografts of healthy endothelial cells embedded in polymer matrix (PVS-30200) delivered at the time of surgical tumor removal to block tumor growth have also shown promise in preclinical studies.

There are multiple other novel approaches that may be tackled and are at different stages of preclinical/clinical development. For example, eliminating CSC/TIC through stimulating external signals that activate their differentiation may serve to sensitize tumor cells to standard therapy. Oxygenating hypoxic regions of tumors has the potential to promote cell cycle entry of quiescent tumor cells and to induce differentiation of hypoxic niche-dependent CSC/TIC, reducing resistance to antineoplastic therapies. Inhibiting promotion of EMT and lowering chronic inflammation while activating anti-tumor immune responses could provide additional approaches. Chronic inflammation is often associated with increased cancer risk in humans: patients with inflammatory bowel disease have an increased risk of colorectal cancer; Helicobacter pylori infection is associated with gastric adenocarcinomas and mucosa associated lymphomas; and chronic hepatitis is associated with hepatocellular carcinoma. For patients with chronic inflammatory conditions, therefore, suppressing the immune response can reduce subsequent cancer development. Considering the complexity of the disease, most likely the best ways for treating cancer patients are going to be individualized combination therapies based on well stratified patient populations and may include one or multiple of aforementioned modalities in conjunction with more traditional therapies.

A series of therapeutic strategies targeting CSC/TIC have been developed, such as inhibiting proliferation, promoting differentiation, inducing apoptosis, and enhancing the sensitivity of chemoradiation. Preliminary experimental results indicate that these strategies can target CSC/TIC and inhibit their functions albeit so far with limited success. Therefore, there is an urgent need for further in-depth investigations of the mechanisms that may lead to rational basis for treatment development.

Although identification of therapies that selectively target CSC/TIC is an important goal, a parallel and perhaps equally efficacious approach would be to target the mechanisms of plasticity that generate and maintain the CSC/TIC population in tumors, namely elements of their microenvironment. These include both extracellular factors controlled on systemic and local levels such as hypoxia, cytokines, growth factors and extracellular matrix, as well as different cellular components, including niche and stromal cells and various constituents of the immune system that contribute to tumor milieu. For example, it has been suggested by Reiman and colleagues (Reiman et al. 2010) that because immunity is able to induce BCSCs, one approach would be to define and to target the specific immune effectors of this process. Although activated CTL cells are the critical effectors of EMT in mouse mammary tumor cells, it is not possible to generally target CTL cells given their important role in protection against infection. Skewing of the macrophage response within the tumor microenvironment from a M2 (tumor-promoting) to M1 (tumor-eradicating) phenotype may have the potential to reduce tumor invasion and metastases. Having a Th2 or Treg response may promote breast cancer metastases, suggesting that agents (e.g., vaccines) that shift from Th2/Treg to an antitumor Th1 response may be useful.

Another approach to inhibit EMT-associated tumor progression is to target the pathways involved in the induction of EMT that specifically lead to the acquisition of CSC/TIC characteristics,

as recently shown for inhibitors of EMT mediated by TGFβ or Hedgehog pathways. The immune system has long been viewed as a co-conspirator with developing tumors; more recent data have shown that it can also selectively target tumor cells at early stages of cancer progression. An important goal now is to identify how to reduce the tumor promotion abilities of the immune system while preserving or increasing its ability to eliminate tumor cells.

In conclusion, while tumor cell populations undergoing malignant transformation may not in some cases represent CSC or TIC (and in many cases may not arise from normal stem cells), they inevitably harbor within their cells that share with CSC/TIC some of their key properties: the ability to self-renew and to give rise to tumors and, often, also the capacity to differentiate into multiple tumor-associated cell types. It is these characteristics that may be selected for and/or supported by permissive microenvironment and are thus, important to be studied in that context. The underlying mechanisms promise to open up novel approaches to developing drug targets and therapies that may lead to increased disease-free survival and reduction in metastatic disease.

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Identification of Ovarian Cancer Stem Cells: Molecular Pathways

15

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Contents

Abstract

Ovarian cancer is one of the major causes of cancer-related deaths among the female reproductive tract cancers worldwide. The foremost problem with ovarian cancer is in its heterogeneity, different subtypes and drug resistance. Moreover, the different types of ovarian cancer, such as endometrioid, serous, clear cell and mucinous appends to the complexity of this cancer. These tumors contain a small population of cells known as cancer stem cells (CSCs), which have the capacity to self-renew and are highly drug resistant, leading to tumor relapse. CSCs have attained tremendous attention in the last decade and have been shown to be a major target in various cancers including ovarian cancer. Understanding the molecular pathways implicated by ovarian CSCs is crucial to combat the residual disease following chemotherapy and combinational therapies. Several markers, such as CD24, CD44, CD117, CD133 and ALDH1, are found to be highly expressed on the surface of ovarian CSCs and can be used as a target as well as to battle ovarian cancer. This book chapter focuses on CSCs and their associate molecules and molecular pathways involved in the maintenance of ovarian CSCs, specific targets and the novel therapeutic implications of ovarian CSCs.

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Introduction

Ovarian cancer is one of the deadliest gynecologic diseases worldwide. It accounts for 3% of cancer-related deaths in women with a relative survival rate of 75%, 44%, and 35% for 1, 5 and 10 years, respectively. The increased mortality rate of ovarian cancer patients is attributed to late stage diagnosis and the lack of early symptoms. Identifying the tumor initiating cell in cancer has remained elusive, as the tumor initiating cell is usually indistinct. The cells responsible for origin of tumor are typically referred to as CSCs

CSCs allude to cancer cells propagating to subsequent progeny. Stem cells are unspecialized cells capable of self-renewal which can generate transit amplifying cells through asymmetric division giving rise to differentiated progenitor cells. More than 70% of ovarian cancer patients are developing drug resistance against chemotherapeutic drugs which could possibly be due to the cancer stem cell population.

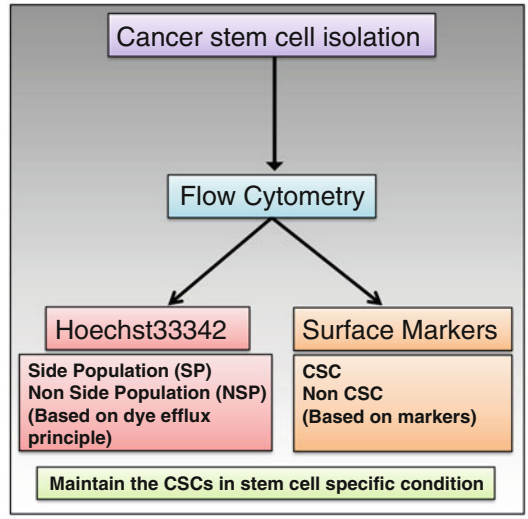

Fig. 15.1 Cancer Stem Cell isolation. Two different isolation methods were followed to isolate the cancer stem cells, such as Hoechst33342 dye based isolation and surface markers based isolation by FACS method. After isolating these, cancer stem cells were maintained in a stem cell specific culture condition

Cancer Stem Cells in Ovarian Cancer

CSCs constitute a very minor sub-population of cells from the entire main population which have some special characteristics due to which they are very distinct from the rest of the population. The main features that make the CSC population unique are as follows: (i) The ability to renew itself indefinitely, i.e. to produce a daughter cell and a differentiated cell as well; (ii) Resistance to chemotherapeutic drugs; (iii) Extensive proliferative capacity; (iv) Arrangement of a set of markers specific to each type of cancer; and (v) Ability to grow in a unique CSC specific niche. Another interesting characteristic feature of CSCs is that, when injected into mice, only a very minimal number (100–1,000 cells) of CSCs results in seeding new tumors rapidly compared to that of the non-cancer stem cell counterpart. These features were identified in CSCs isolated by different methods such as Hoechst 33342 dye exclusion assay and surface marker based flow sorting (Figs. 15.1 and 15.2). Over the last decade there were a lot of controversies over the CSC theory. Very recently, three independent groups showed outstanding work to prove that CSCs exist in different cancers. Schepers et al. (2012) showed that stem cells prevailing in the primary intestinal adenomas served as a precursor to form intestinal cancer and they also found that these cells, which have stem like properties have a cell division rate of twice a day.

Broadly, ovarian cancer is histologically classified into three different types: stromal tumors, germ cell tumors and epithelial ovarian carcinoma (EOC). Out of the three types, around 90% of ovarian cancer cases arise from EOC and the other two types of OC account for only 5% of ovarian tumors. EOC is a frequently occurring disease and is associated with a high mortality rate. Ovarian surface epithelium (OSE) forms the outer covering of the ovary and is made of a layer of uncommitted mesothelial cells. In the course of a woman's reproductive life, the ovarian follicles are susceptible to a lot of physical strain (wear and tear) during the process of follicular rupture and release of oocyte, thereby breaching the ovarian surface epithelium. OSE is also exposed to reactive

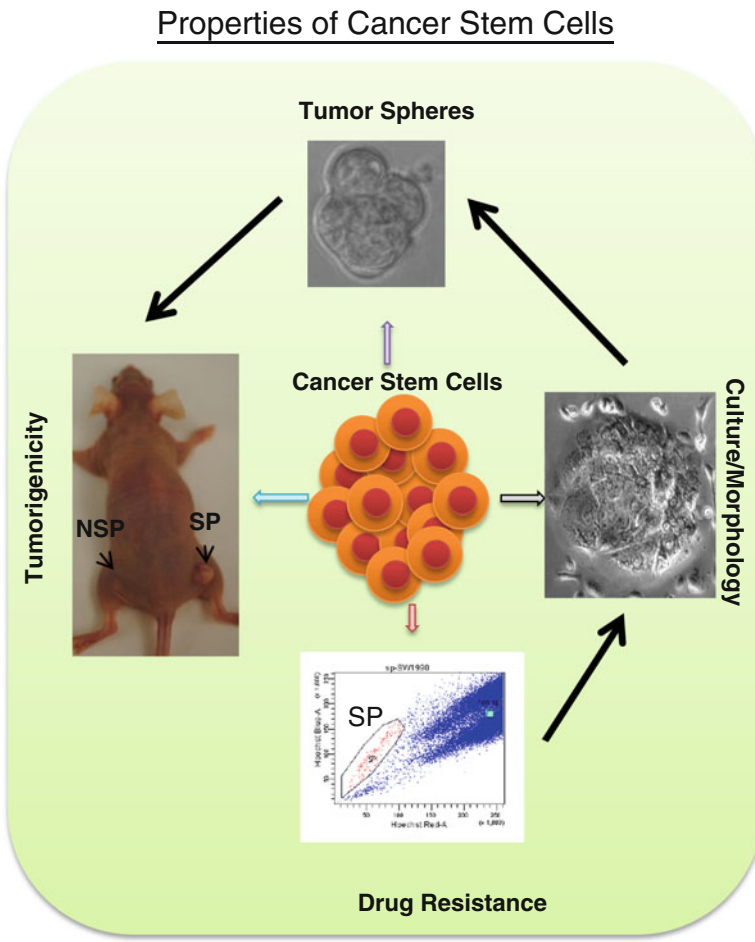

Fig. 15.2 Properties of cancer stem cells. Cancer stem cells carry some specific features such as cobblestone phenotype (culture/morphology), tumorsphere formation, drug resistance and aggressive tumorigenicity

oxygen species (ROS) as well as inflammatory cytokines that lead to damage of DNA. Due to the different types of stress undergone by the OSE there may be an increased chance for its susceptibility to transformation. Apart from the stress imparted to the cells, age is another parameter to be considered which contributes to OC formation. As they age, several invaginations emerge from the ovarian surface and project into the cortical stroma which slowly pinches off and gets entrapped in the stroma, thereby forming cortical inclusion cysts (CICs). Hormonal stimulation on the ovary leads to growth inducing properties which may result in epithelial cell proliferation within the CICs. If these epithelial cells carry damaged DNA, they may become the seeds causing neoplastic transformation which eventually emerges into ovarian cancer. In addition to the aforementioned types of ovarian cancer there are many other subtypes of ovarian cancer. Identification and designation of the signature molecules for each of the different subtypes of EOC is the current focus of research which facilitates the identification of prognostic markers to aid in the improvement of targeting ovarian cancer specifically. Recent evidence has proven that high-grade serous ovarian cancer also arises from the fallopian tube due to deletion of Dicer, a microRNA synthesis molecule, and PTEN (Kim et al. 2012). These Dicer and Pten knockout animals showed that primary tumors in the fallopian tube spread to the ovary and metastasize

throughout the abdominal cavity (Kim et al. 2012). This study demonstrates a new way for serous high-grade ovarian cancer to understand the molecular origin and progression of this deadly disease. It also suggests that cancer stem cells to arise from these fallopian tubes and migrate into the ovary to form aggressive tumors.

Markers Developed in Ovarian Cancer Stem Cells

The following markers namely, ALDH, CD44, CD24, CD117 and CD133, are some of the most widely expressed markers in ovarian CSCs (Ponnusamy and Batra 2008). A brief description of these markers is described below.

Aldehyde dehydrogenase (ALDH): Aldehyde dehydrogenase is an enzyme essential in the oxidation of aldehydes to carboxylic acids, thereby converting vitamin A into retinoic acid in the liver. Retinoic acid signaling is suggested to play a role in protecting stem cells from oxidative damage. Studies show that ALDH is a very important ovarian CSC marker (Dyall et al. 2010). ALDH positive cells had the potential to initiate tumors, whereas ALDH negative cells could not. One of the 17 isoforms of ALDH is ALDH1A1 which has been found to be expressed very frequently in ovarian cancer. It is associated with chemoresistance pathways, therefore its activity and expression was found to be increased in chemoresistant ovarian cancer cell lines. There is enough evidence that patients with high ALDH1A1 expression have a poor outcome. One hundred to 500 ALDEFLUOR-positive cells were barely needed to form a tumor, whereas the injection of 100,000 ALDEFLUOR-negative cells resulted in the absence of tumor formation. Reports show that chemoresistant tumors were sensitized to docetaxel and cisplatin in both in vitro and in vivo models of OC upon downregulation of ALDH1A1 expression (Landen, Jr. et al. 2010).

CD44: CD44, which belongs to class I transmembrane glycoproteins, acts as a receptor for hyaluronic acid (extracellular matrix component) thereby promoting migration in normal cells. Also, presentation of chemokines and cytokines to their complimentary receptors is another function of CD44 (Naor et al. 2008). CD44 is a multifunctional receptor involved in various processes such as cell-cell and cell-matrix interactions, signal transmission, differentiation and motility. Both primary and metastatic ovarian tumors harbor this multifunctional marker on the cell surface (Aguilar-Gallardo et al. 2012; Naor et al. 2008).

CD24: CD24 is a cell surface glycoprotein (mucin-like) marker that has been identified as a CSC marker in hematological, pancreatic and liver cancers. The vital role of CD24 has been attributed to the cellular process of metastasis and cell survival. A recent study by Gao et al. (2010) had demonstrated CD24 as a putative CSC maker in ovarian carcinomas.

CD117: CD117, also widely known as c-kit, is a well-known CSC marker. It is encoded by the KIT gene. Chau et al. showed that by the activation of Wnt/β-catenin-ABCG2 signaling, c-Kit mediates resistance to chemotherapy as well as tumor-initiating capacity. It was identified that c-Kit is not merely a marker of ovarian tumor initiating cells but it is also a druggable target (Chau et al. 2012). Only a very small subset of human ovarian tumor cells showed a high expression of c-Kit (Raspollini et al. 2004). Therefore, in order to eradicate ovarian cancer, one has to ideally target the c-Kit expressing stem cells/tumor initiating cells as well as the c-Kit non expressing cells (by using another marker), by means of a combinatorial approach.

CD133: CD133, also known as Prominin, was first identified as a hematopoietic stem cell marker and later it was established as a marker to isolate CSCs from various cancers including ovarian cancer. The PROM1 gene encodes CD133, a five transmembrane glycoprotein. This transmembrane glycoprotein helps in the maintenance of stem cell properties by inhibiting differentiation. Expression analysis of CD133 in 8 normal ovaries, 5 benign ovarian tumors and 41 ovarian tumors revealed that primary ovarian cancer $CD133^{+}CK^{+}$

cells had a greater proliferative and colony forming potential as opposed to the $CD133^{-}$ CK^{+} cells. On the contrary, normal ovaries and benign tumors presented a significant reduction in the expression level of $CD133^{+}$ compared to that of the ovarian carcinoma (Ferrandina et al. 2009).

Combination of markers: Isolation of $CD44^{+}CD117^{+}$ cells using FACS gave rise to less than 0.2% cells from a tumor which had the potential to reproduce the original phenotype on engraftment, whereas the remaining population containing $CD44^{-}CD117^{-}$ cells were non-tumorigenic. Moreover, $CD44^{+}CD117^{+}$ spheroids were resistant to chemotherapy. Reports showed that both $ALDH^{+}CD133^{+}$ cells and $ALDH^{+}CD133^{-}$ cells isolated from human ovarian tumor cells have the ability to form tumors in mice. However in Zhang et al study, $ALDH^{+}CD133^{+}$ CSCs gave rise to very aggressive and poorly-differentiated tumors in less than 4 months, which were comprised of $ALDH^{+}CD133^{+}$, $ALDH^{-}CD133^{+}$, $ALDH^{+}CD133^{-}$ and $ALDH^{-}CD133^{-}$ cells. On the other hand, $ALDH^{+}CD133^{-}$ CSCs initiated well-differentiated tumors in 6–12 months (Zhang et al. 2008).

Molecular Pathways Involved in the Maintenance of Ovarian Cancer Stem Cells

The molecular signaling pathways are the series or cascade of events/molecules involved to regulate cellular activity such as cell fate determination, proliferation, survival, apoptosis, cellular growth, maturation, immune response, differentiation and maintenance of somatic stemness (Reya and Clevers 2005). CSCs are influenced by three major signaling pathways viz. Notch, Wnt and Sonic Hedgehog (Shh), influencing the self-renewal and malignant property of CSCs (Figs. 15.3 and 15.4) (Reya and Clevers 2005).

Notch signaling pathway: Among the three major signaling pathways, notch signaling is the evolutionarily conserved pathway that can influence cellular activity on a context dependent basis. In mammalian cells, the notch family consists of four receptors (Notch1, 2, 3 and 4) and five ligands (Jagged1 and 2, Delta-like1, 3 and 4) (Choi et al. 2008). Notch receptors are type I

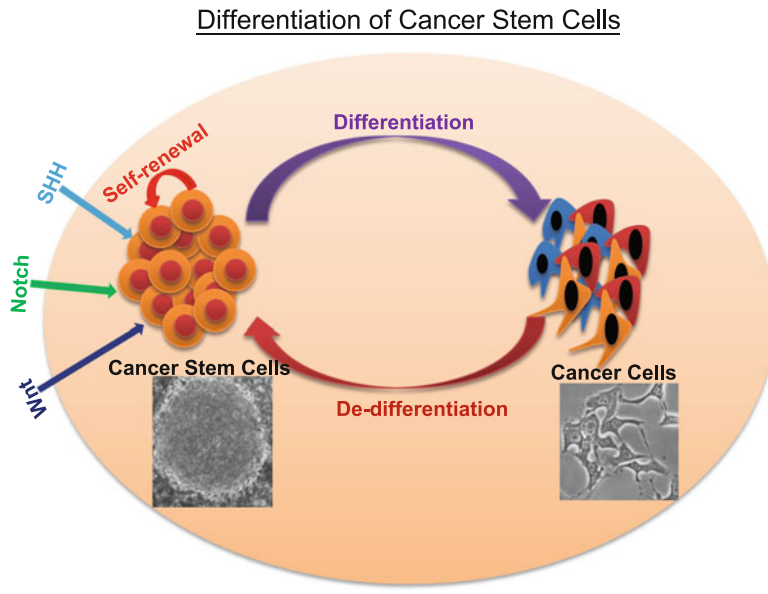

Fig. 15.3 Differentiation of cancer stem cells. Recent evidence showed that self-renewing cancer stem cells are involved in the lineage differentiation. This self-renewal is maintained by different signaling pathways (Shh, Notch and Wnt). Emerging evidence showed that the cancer stem cells also originate from de-differentiation of cancer cells

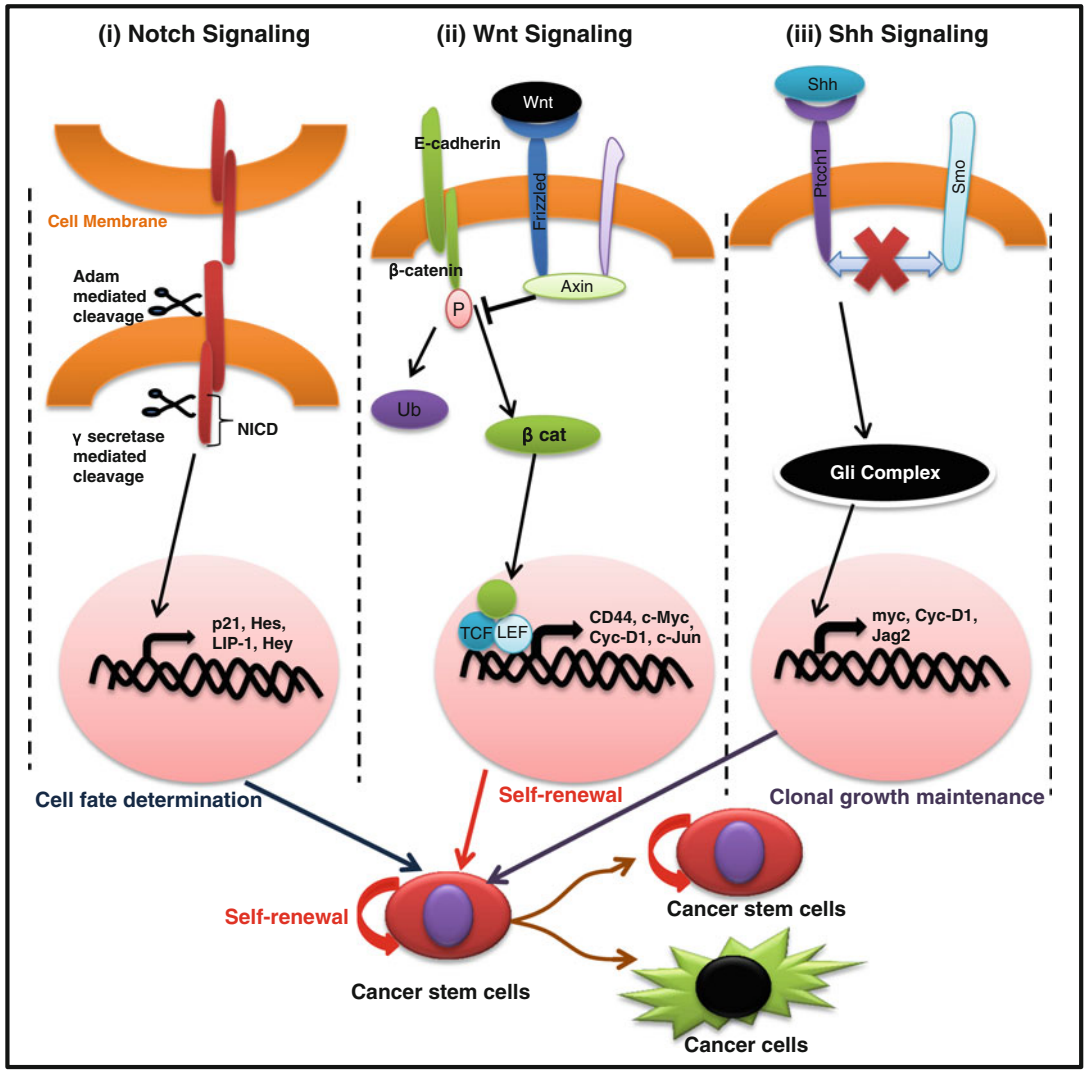

Fig. 15.4 Signaling pathway essential for stem cell fate determination, self-renewal and maintenance of CSC phenotype: (**i**) Notch receptors are activated by jagged and delta-like ligands present in the adjacent cells. Upon activation, the notch receptors present in the cell membrane will undergo two step proteolytic cleavages consecutively mediated by ADAM protease and γ-secretase. Further, the NICD which is released as a result of proteolytic cleavage will lead to translocation in to the nucleus for the transcription of notch-targeted genes such as Hes/Hey, LIP-1 and p21, etc. (**ii**) The WNT/β-catenin signaling pathway regulates cell fate determination and it is primarily involved in the regulation of cell-cell interaction. The WNT/β-catenin signaling will initiate WNT ligand mediated activation of the Frizzled receptor and its co-receptor LRP5/6 (low density lipoprotein (LDL)) receptor related protein. In the absence of the ligand, the multi-protein complex of axin, GSK-3β, APC and CK-1α will recognize the β-catenin and aid in the phosphorylation of the serine and threonine residue in the amino terminal end of β-catenin leading to ubiquitin mediated degradation of β-catenin. However, in the presence of the WNT ligand the Frizzled receptor/LRP5/6 complex will recruit the axin complex, which further leads to disabling β-catenin destruction in the cytoplasm. The unphosphorylated β-catenin will be translocated into the nucleus, thereby allowing transcription of target genes such as CD44, c-Myc, CyclinD1 and c-Jun, etc. (**iii**) In the absence of a Shh ligand, the Patched trans-membrane receptors (PTCH1 and PTCH2) will inhibit Smoothened (Smo) co-receptor, resulting in phosphorylation and cleavage of the GLI family of transcription factors. Upon ligand binding to its receptor, the GLI transcription factor will activate the Shh-targeted genes of myc, Cyclin-D1 and Jag2, etc. Together, these distinct signaling pathways help us to understand the molecular dynamics behind the maintenance, self-renewal and cell fate determination property of CSC

transmembrane proteins consisting of EGF like repeats, a ligand binding extracellular domain (NECD), a transmembrane (TM) domain and an intracellular (IC) domain with nuclear localization sequence (NLS). Interaction between the notch receptor (EGF repeats) with the Delta/Serrate/Lag2 (DSL) domain present in the respective ligands between the neighboring cells will initiate a cleavage event that releases the extracellular domain. Ligand binding also leads to gamma secretase mediated proteolytic cleavage of the transmembrane domain, further releasing the notch intracellular domain (NICD) from the cell membrane. The released NICD will translocate into the nucleus where it interacts with the transcriptional complex CBF1, leading to the transcriptional activation of notch target genes of the *Hes* and *Hey* families. Of the four Notch proteins in mammals, only two notch proteins have been established to be involved in ovarian cancer. Notch 3 signaling has been studied more often in ovarian cancer. It was found to be overexpressed in 20% of ovarian serous adenocarcinomas and is correlated with aggressive tumor growth, tumor progression and poor prognosis. Further the notch 3 amplification of gene expression is also directly correlated with the notch 3 protein expression levels, providing the conclusion that the consequences of gene amplification are responsible for the protein's overexpression. Additionally, it has been shown that certain ovarian cancer cell lines, like SKOV3, OVCAR3 and CaOV3, have higher expressions of notch 3. Also, its respective ligand Jagged1 seems to be overexpressed in EOC cells, as well as Jagged1/Notch3 interaction may constitute a juxtacrine loop, promoting proliferation and the dissemination of ovarian cancer cells (Jung et al. 2010). Another member of the notch family, notch 1 was also found to be significantly active in EOC pathogenesis. Studies of the notch 1 pathway have shown it to be associated with ligand Jagged-2 and Delta like-1, which were highly expressed in ovarian adenocarcinomas, when compared with adenomas (Hopfer et al. 2005). Also, overexpression of the notch 1 intracellular domain (NICD) was associated with enhanced growth of ovarian cancer cells. Thus, the downregulation of NICD by siRNA mediated studies showed reduced growth of ovarian cancer cells. These studies indicate blocking or inhibition of the Notch pathway as one of the potentially attractive pathways to be considered for therapeutic approach. Deregulation of the notch signaling pathway is involved in the maintenance of somatic stem cells of ovarian cancer. In a study, McAuliffe et al. had demonstrated that the notch 3 plays a significant role in sensitization of ovarian cancer cells to platinum therapy. In summary, they have shown how notch 3 and its signaling pathway are involved in the maintenance of CSCs and platinum based chemoresistance in ovarian cancer cells. Furthermore, this study shows an important clinical application of an inhibitor specific for the notch pathway (Gamma secretase) in aggressive ovarian cancer patients (McAuliffe et al. 2012).

Shh signaling pathway: The Sonic Hedgehog (Shh) signaling is an important pathway for the maintenance of stem cell homeostasis. All the three Hedgehog (Hh) proteins, namely Sonic Hh (Shh), Desert Hh (Dhh) and Indian Hh (Ihh), binds to the same receptor, PATCHED1 (PTCH1). The Sonic Hedgehog (Shh) signal transduction pathway in adulthood has been shown to be associated with a variety of human malignancies. Hh binding to its cognate receptor PTCH1 leads to the stimulation of transcriptional factor Gli1, 2 and 3, leading to a malignant and aggressive phenotype. Additionally, the hedgehog pathway has been identified in ovarian cancer as one of the potential mechanisms altered during malignant transformation. Earlier analyses of advanced ovarian tumors suggest that 50–60% of the possible manifestations are of the Hh pathway in these carcinomas. As a result of altered Hh, the pathway will lead to protein level alteration in Smo, Ptch1 and Gli1 in ovarian cancer. Hh signaling is abnormally activated in 30% of cancer, including ovarian cancer. Overexpression of Hh, PTCH1 and Gli transcriptional factor were associated and correlated with poor survival of ovarian cancer patients (Taipale and Beachy 2001). Thus, inhibition of Hh signaling may also be an effective therapeutic strategy for treating ovarian cancer patients.

Wnt signaling pathway: The Wnt signaling is also an evolutionary conserved pathway that plays a vital role in the maintenance of a delicate balance between stem cell self-renewal, proliferation and differentiation. It seems that Wnt signaling is tightly regulated by a diverse group of proteins that can engage in cellular polarity, cell cycle regulation and cell-cell adhesion during both the embryonic and adult period (Reya and Clevers 2005). There are 19 mammalian Wnt homologues (ligands expressed in spatial-temporal pattern) and 10 frizzled receptors identified so far in human. The major effector of Wnt signaling is the transcription factor β-catenin. In the absence of Wnt protein, β-catenin interacts with a multi-protein complex comprised of adenomatous polyposis coli (APC), diversin and axin (scaffold proteins). β-catenin is the destructional complex that can be phosphorylated by serine/threonine kinases casein kinase 1(CK1) and glycogen synthase kinase 3β (GSK3β). Thus, the phosphorylated β-catenin is then ubiquitinated by beta transducin repeat containing protein (bTrCP) and degraded by the proteosomal mediated process. Following the Wnt protein binding to the Frizzled family of receptors, it will form a complex with its co receptors LRP-5/6 protein, leading to the prevention of β-catenin degradation. Thus, the cytoplasmic β-catenin is then translocated in to the nucleus where it interacts with transcription factors, T cell factor (TCF)/lymphoid enhancer factor (LEF), leading to transcriptional activation of Wnt target genes such as *cyclin D1, c-myc, peroxisome proliferator-activated receptors (PPARd)* and *twin* . The Wnt signaling pathway is implicated in embryonic development of the ovary, as well as the follicular developmental process (Reya and Clevers 2005). Recent studies have shown the implication of abnormal and aberrant Wnt signaling in many cancers and in ovarian cancer development (Gamallo et al. 1999). However, in ovarian cancer, the aberrant activation of Wnt signaling is not fully understood. Compelling evidence of CTNNB1 gene mutations (which encodes for β-catenin) have been identified in 30% of endometrioid tumors, implicating the association of Wnt signaling with ovarian cancer pathogenesis (Gamallo et al. 1999; Lee et al. 2003). Furthermore, the nuclear positivity of β-catenin in high grade serous ovarian carcinomas has been linked with the poor survival of ovarian cancer patients (Lee et al. 2003). A recent study by Rosen et al. (2010) has addressed the association of low membranous β-catenin expression and high mitotic count as poor prognostic factors and was linked with early recurrence of ovarian endometrioid carcinoma. Thus, deregulation of β-catenin may also play a vital role in the pathogenesis of ovarian cancer.

Ovarian Cancer Stem Cell Markers as Specific Targets

Considerable progress has been made to identify signaling intermediates that are specifically involved in the development of tumor initiation and the development of resistance for the available therapeutic regimens. CSCs are thought to be the major players for tumor initiation as well as development, tumor relapse and the development of therapeutic resistance. Moreover, the signaling pathways that are specifically activated in CSC populations are not well understood. Thus, the process of elimination of CSCs remains to be a major challenge in the scientific community. Currently, the focus on molecular targeted therapies has been driven by extensive research on CSC molecular pathways and their role in tumor initiation, progression metastasis and tumor recurrence. The specific targets to be discussed in this chapter are CD44, CD133, CD117, ALDH1 and CD24.

CD44 is overexpressed on the surface of primary and metastatic ovarian cancer tissue and its expression was associated with poor survival and the development of chemoresistance to therapy (Zhang et al. 2008). Moreover, patients whose tumors were positive for CD44 had a mean survival of 25 months as compared to patients with CD44 negative tumors whose mean survival was 52 months. A specific form of CD44, CD44s is responsible for binding of ovarian cancer cells to mesothelium and is found to be overexpressed in 38–51% of ovarian tumors. Thus, CD44 positive

cells can be considered as one of the molecular therapeutic targets in ovarian CSCs. Several target therapies, like antibody mediated cytotoxicity, have been developed specifically to different isoforms of CD44 under clinical trials in head and neck cancer (Platt and Szoka 2008). Those antibodies were still in early phase trials and require further exploration. According to the National Comprehensive Cancer Network (NCCN), platinum and taxane-based chemotherapy are the standard therapeutic care for advanced epithelial ovarian cancer patients. Based on these therapeutic regimens, specifically in ovarian cancer, a micro or nanoparticle based drug carrier (delivery vehicle) that has low toxicity and can attach efficiently on the tumor surface increases the bioavailability of the drug. Li et al. have developed a unique method to produce a cisplatin loaded microparticle (Hyplat) (Reya and Clevers 2005) that contains large amounts of cisplatin coupled with hyaluronan which is a natural ligand for CD44. Thus, hyaluronan would serve as a scaffold for cisplatin and as a molecular target that specifically binds to CD44 targeting ovarian CSCs. Another study by Orian et al. in 2010 also showed that hyaluronic based conjugates with paclitaxel can be useful for treating ovarian cancer patients. These drugs had been assessed for the therapeutic effects in the mouse xenograft model system, however, its application in human ovarian cancer needs to be explored.

In recent studies it was observed that the overexpression of CD133 was well correlated with poor survival of ovarian cancer. In ovarian cancer, Bapat et al. showed that single cell clones isolated from the ascitic fluid of advanced ovarian cancer patients, influenced stem cell like properties and were found to be tumorigenic both in vitro and in vivo. Further, Bapat et al. have shown that CD133+ cells were capable of forming tumors in a xenograft model system with reduced tumor latency and increased tumor volume as compared to $CD133^{-}$ cancer cells. Additionally, the $CD133^{+}$ cells showed asymmetric division as a tool for CSCs to maintain their appropriate progeny (Bapat 2010; Ferrandina et al. 2009). Based on this context, several recent studies are aimed at targeting CD133 as a novel therapeutic molecule to be explored for targeting CSCs. One study has demonstrated that CD133 antibody conjugated with a potent cytotoxic agent, monomethyl auristatin F, has been shown to significantly inhibit growth and enhanced apoptosis of hepatocellular and gastric cancer cell lines. Thus, the development of an antibody (AC133) conjugated with drugs against the CD133 antigen will provide the opportunity to eliminate a potentially drug-resistant cancer subpopulation in CD133 positive tumors (Smith et al. 2008).

CD117 exhibits its normal expression on the surface of all hematopoietic stem and progenitor cells, mast cells, melanocytes, interstitial cells of cajal of the digestive tract, germ cells and neurons. Aberration in these receptor tyrosine kinases was associated with hematological and non-hematological defects. To be specific, CD117 is overexpressed in a variety of human neoplasms, including ovarian carcinoma, breast cancer, colon cancer, gastrointestinal stromal tumors, testicular seminoma, lung cancer, acute myeloid leukemia (AML) and melanoma (Chau et al. 2012). CD117 expression has been reported in 40% of ovarian carcinomas and ovarian cancer cells with the CD117 phenotype are highly tumorigenic, chemoresistant and possess CSC-like properties. In ovarian cancer, Zhang et al. have isolated and characterized ovarian cancer initiating cells (OCICs) from primary ovarian serous adenocarcinoma. These OCICs showed their ability to self-renew and had the capability to reestablish their original tumor phenotype in both in vitro and in vivo model systems. Furthermore, these OCICs were isolated using CD44 and CD117 specific antibodies. A subpopulation of 100 cells positive for both CD44 and CD117 are capable of propagating tumors in xenograft models, whereas CD44 and CD117 negative cells failed to show the tumorigenic response. Thus, CD117, along with CD44, has been identified as an attractive candidate for therapy (Zhang et al. 2008).

A recent study by Gao et al. had demonstrated CD24 as a putative CSC maker in ovarian carcinomas. Interestingly, the same group established primary ovarian cancer cell lines from serous and

mucinous cystadenocarcinomas. The isolated cancer cells were shown to exhibit a CSC-like phenotype and characteristics such as quiescence, resistance to chemotherapy and tumor initiating capacity, which were shown to be enhanced in CD24+ cells as compared to $CD24^-$ cells. In addition, the $CD24^+$ cells were found to be associated with increased expressional levels of other stem cell related genes such as *Notch1, Notch4, Oct3/4, Beta-catenin, Nestin and Bmi-1*. Nearly 1% of cells positive for CD24 were also found to co-express both CD133 and CD117. More recently, several clones were isolated from different regions of ovarian tumor (cell clones from the periphery and center of the tumor cell mass). They identified that cell clones isolated from the tumor periphery were enriched with CSC (side population cells) which positively co-expressed CD24 and CD117 markers that are associated with chemoresistance (Gao et al. 2010).

Increased expression of ALDH1 has been associated as a candidate stem cell marker in various cancers (breast and prostate) apart from ovarian cancer (Deng et al. 2010). The increased expression of ALDH1 has been associated with enhanced chemoresistance and poor prognosis of 50% of epithelial ovarian cancer patients. Several studies have shown the co-expression of ALDH1 with CD44 (ALDH1+CD44+) and CD133 (ALDH1+CD133+) in primary ovarian carcinomas. Further, shRNA mediated knockdown of ALDH1 have shown to sensitize ovarian cancer cells to chemotherapeutic agents. Different studies have recently, demonstrated the combined expression of CD133 and ALDH1 as distinct ovarian stem cell markers. They isolated CSCs from both ovarian cancer cell lines and primary ovarian tumors that lack CD133 expression, further combined with fluorescence activated cell sorting (FACS) with ALDH1. ALDH1-positive cells were capable of generating tumors *in vivo,* whereas the ALDH1-negative cells failed to initiate tumors in mice. Several independent groups have also reported that ALDH1 activity is significantly associated with poor clinical outcomes in patients of serous ovarian cancer (Deng et al. 2010). Thus, ALDH1 expression is a positive prognostic stem cell marker in ovarian cancer.

Therapeutic Implications of Ovarian Cancer Stem Cells

CSCs are highly resistant to radiation and chemotherapy, therefore successful elimination of cancer requires more effective therapies to combat this subpopulation of cells. Currently, certain anticancer therapies, such as chemotherapy, radiation therapy, hormonal ablation therapy and immunotherapy, are employed to eliminate rapidly growing differentiated cancer cells, thus reducing tumor burden but potentially leaving behind cancer-initiating cells or CSCs (Zhang et al. 2008). In case of ovarian cancer, the first line standard of treatment is the combined use of cisplatin and paclitaxel; however, it is almost inevitable that this standard care will leave drug resistance. According to the cancer stem cell hypothesis, the reason for the failure of standard care and the development of the therapy resistance phenotype of cell was mainly attributed to the failure of the therapeutic agent to target the tumor progenitors.

CD44 expression has been associated with poor survival and the development of chemoresistance in ovarian cancer patients. Several antibodies were developed to target the various isoforms of CD44 and they have been tried in a phase I clinical trial but they were not able to produce a positive clinical outcome. In an alternative approach to target the receptor tyrosine kinase, there have been CD117 phase II clinical trials treating ovarian cancer patients with the tyrosine kinase inhibitor Imatinib mesylate (Schilder et al. 2008). Therapeutic approaches targeting CD117 using Imatinib mesylate proved to be useful in the GIST and CML types of cancers. The use of imatinib, either as a single agent or in combination with taxene, has been proven to be effective. Unfortunately, a subset of patients had stable disease, thus providing inconsistent evidence for this therapy (Matei et al. 2008). Furthermore, the use of a docetaxel and imatinib based combination was also shown to have significant therapeutic efficacy in the treatment of platinum-resistant ovarian cancer patients. All of these combination and trials failed because the clinical trials

were performed in non-selective patients. Only 30% of patient tumor cells were positive for CD117, making it a CSC-specific therapeutic target (Matei et al. 2008).

Similar to CD117, CD133 is also a well-documented cell surface marker expressed in both normal and cancer stem cells. Preclinical studies by Xu et al. and Ferrandiana et al. have demonstrated that monoclonal antibodies raised against CD133 could be used for isolating ovarian CSCs, which are believed to be causative for the initiation of metastatic spread and the development of a therapeutic resistant phenotype. Furthermore, the monoclonal antibody CC188 conjugated with a NIR dye was examined in the primary tumors of ovarian cancer patients, coupled with a specialized immunofluorescence microarray technique. Tissue microarray analysis revealed a positive staining for ovarian cancer tissues and a low percentage of staining intensity in normal cells. A similar study was also carried out in an in vivo model system for the development of anti-CD133 conjugated dye as an imaging based therapeutic target. CD24 is also a cell surface mucin like glycoprotein expressed on a variety of epithelial cancers, including ovarian cancer. Furthermore, CD24 knockdown studies have implied an antitumor effect in both in vitro and in vivo experiments (Su et al. 2009). In a recent study, ovarian CSCs were isolated based on the positivity for CD24 from two ovarian cancer cell lines, CAOV3 and TOV21G. The isolated cancer cells were tested for drug resistance for two chemotherapeutic drugs such as cisplatin and doxorubicin. The results revealed that $CD24^{+}$ cells had CSC-like properties that are more resistant to anti-tumor drugs and more susceptible to the lysis of natural killer cells (Koh et al. 2012). Thus, identifying potential targets against CD24 might be a potential therapeutic approach against ovarian cancer. ALDH1, a putative ovarian CSC marker whose overexpression has been linked with the development of a more resistant phenotype, suggests a possible role of ALDH1 in the induction of chemoresistance in ovarian CSCs. Several ALDH inhibitors are currently clinically available and one is disulfiram (Liu et al. 2012). More importantly, disulfiram is a FDA approved drug, so it has been safely used in patients along with the combination of other chemotherapeutic drugs, implying the possible tolerance of normal stem cells to these kinds of combination and drugs.

Conclusion and Future Perspectives

In this book chapter, we have discussed the studies on the sub population of committed progenitor cells or cancer stem cells pertaining to ovarian cancer. In order to understand the biological function and pathological role of ovarian cancer stem cells, we have discussed specific cell surface markers for CSC characterization. We have also elaborated on the molecular signaling (Wnt, Shh and Notch) responsible for the self-renewal process of CSCs. In addition, the molecules developed to explore the significance of CSC and the cellular signaling pathways involved in the maintenance of the CSC phenotype are also helpful in the selective eradication of ovarian cancer stem-like cells. Finally, we discussed the specific targets of ovarian cancer stem cells which are involved in the futuristic therapeutic implication of anti-cancer therapy.

Over the past few years, many investigations of ovarian CSCs have been carried out at various places around the world, yet there is still a need to explore very extensively in this area of research. To increase our understanding of ovarian CSCs, the following challenging steps must be carried out. As cell lines do not mimic the exact nature of a tumor, it is always advantageous to study the properties of CSCs in human tumors. This would enable specific therapeutics against ovarian CSCs since the nature of these mutated cells could be studied in a naturally dysfunctional niche. Further, there is a need to study the characteristics of these CSCs in an in vivo progression model of ovarian cancer (animal model portraying the different grades of ovarian cancer). This will help prevent limitations of the ovarian CSCs studies to the cell line and encourage the studies in mice models for a better understanding of the ovarian CSCs. Moreover, as the availability of human tumors is less, mice models could be a substitute

Table 15.1 Pre-clinical agents targeting Notch, Hh and Wnt pathways

Inhibitor	Category	Mechanism of action	Clinical trail	Pathway involved	References
RO4929097, MK0752	γ-secretase inhibitor	Inhibition of Notch cleavage mediated by γ-secretase	Phase-I	Notch	Tolcher et al. (2012), Krop et al. (2012)
OMP-21M18 Anti-DLL4 (monoclonal antibody)	Antibody mediated inhibition of Notch signaling	Interfere with ligand receptor interaction of notch pathway	Phase-I	Notch	Yan (2011)
GDC 0449	Small molecule Smo inhibitor	Inhibits the activity of hedgehog-ligand cell surface receptor PTCH and/or Smo-antagonist action	Phase-II	Hh	Lin and Matsui (2012), LoRusso et al. (2011)
IPI 926	Cyclopamine derived inhibitor	Antagonistic action	Phase-II	Hh	Szkandera et al. (2013)
PF 04449913	Smo inhibitor	Antagonistic action	Phase-I	Hh	Hadden (2013)
IWP compounds-Small molecule inhibitor-interfering with Wnt synthesis	Small molecule inhibitor	Inhibits the porcupine activity(antagonistic action)	–	Wnt	Chen et al. (2009)
ICG-001	Small molecule inhibitor	Selectively inhibit the binding of CBP-β-catenin protein-to-protein interaction	Currently in phase I trial in colorectal cancer patients	Wnt	de Sousa et al. (2011)
NSC668036	Small organic inhibitors of the PDZ domain	Specifically inhibits Wnt 3A induced signaling by interrupting the Frizzled (Fz)- Dishevelled (Dvl) interaction	–	Wnt	Shan et al. (2005)

so that the availability of primary tumors (from mice) could be increased and replace the cell line models entirely. Also, the expression of different CSC markers should be studied in various grades of ovarian cancer and should be correlated with the progressive stages of ovarian cancer. To enhance the growth of CSCs, an intraperitoneal injection should be carried out in mice, as the peritoneum would be a suitable environment for the propagation of ovarian CSCs. The aforementioned points will help reach the goal of targeting ovarian CSCs, and thereby eradicating or reducing the burden of the tumor.

Though there are several markers attributed to various cancers, there is still a lot of ambiguity prevailing in choosing the right markers to strictly isolate CSCs. Therefore, a lot of research is required in this area to either identify precise CSC markers which could be used to faithfully isolate the CSC population from the rest of the tumor cells. Thus, therapies failing to target the CSC population will lead to tumor recurrence and therapy resistant stem cells, which ultimately results in the formation of a more aggressive or advanced type of tumor, leading to death. The emerging concept of CSCs and their impact on progression of cancer is tightly regulated by various signaling pathways such as notch, Wnt/β--catenin and Hedgehog. Therefore, an ideal therapy is to effectively abrogate differentiated tumor cells as well as cancer stem cells. At the same time, the therapy should have a minimal effect on the normal cells with low toxicity and side effects. The specific inhibitors (Imatinib mesylate, Disulfiram, Sunitinib malate, Sorafenib, Cyclopamine, Gefitinib, Cl1033 and tamoxifen) developed for inhibiting the signaling involved in the CSC initiation would be a more effective and selective model of killing the CSC population. The various inhibitors being used in clinical trials and their involvement in notch, Hedgehog and Wnt pathways are discussed in Table 15.1 (Tolcher et al. 2012; Krop et al. 2012; Yan 2011; Lin and Matsui 2012; Szkandera et al. 2013; LoRusso et al. 2011; Hadden 2013; Chen et al. 2009; de Sousa et al. 2011; Shan et al. 2005). Apart from these inhibitors, certain CSC-specific biomarkers, such as CD133, ALDH1, are more potent in the identification of these CSC subpopulations. Recently, the tumor antigen-like MUC4 mucin was identified in the ovarian cancer stem cell population (Ponnusamy and Batra 2008). MUC4 can help target molecule-based, specific targets for the CSC populations. Therefore, developing targeted therapy toward the CSC population would be the best way to eradicate the CSCs present in a tumor without affecting the normal stem cell population.

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Part III

Hematopoietic Stem Cells

Hematopoietic Stem Cells and Bone Regeneration

16

Reeva Aggarwal, Vincent J. Pompili, and Hiranmoy Das

Contents

Abstract

The mechanisms of therapeutic regeneration of osseous tissue in field of orthopedics still remain elusive. Not only cytokines, growth factors, terminally differentiated cells, and mechanical factors play role in etiology of bone functioning but also the numbers, and functionality of stem cells play critical role in bone regeneration and maintenance of bone health. Thus, regeneration of bone tissue using stem cells promises an efficient therapeutic approach for lost or traumatized bone tissue. This chapter emphasizes the factors regulating plasticity of hematopoietic stem cells to trans-differentiate into unconventional cell lineages and osteoblastic lineage. We discuss about the therapeutic application of stem cells of hematopoietic and non-hematopoietic origin for regeneration of bones in the preclinical and clinical setting. We also provide evidences of the use of hematopoietic stem cells for bone regeneration, particularly in osteoporotic bone diseases.

Introduction

Bone loss is one of the major public health issues and poses major morbidity and socioeconomic burden. Pathological degeneration, traumatization or nutritional deficiencies lead to unhealthy skeletal alterations and loss of integrity. These factors have been exacerbated due to

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rise in sedentary and ageing population. Orthopedic surgeries that involve replacement by an implant, tissue engineering with biocompatible scaffolds or bone grafting are rapidly on rise but some diseases such as non-union fractures, osteoporosis still remain untreatable. Thus, cell-based therapy may be considered as a strategy aimed at replacing, repairing, or enhancing the biological function of a damaged tissue or system by autologous or allogenic transplantation. In this chapter, we will briefly describe the cellular and molecular components in normal and osteoporotic bones. We will describe the existing treatments for osteoporosis and use of emerging stem cell therapy in osteoporotic bone disease.

Evolving concepts on stem cell plasticity challenges the previous views of the stem cell being destined to particular traditional lineages. Previously, adult stem cells such as hematopoietic stem cell (HSC)s were believed to give rise to hematopoietic lineage only, however, it has been reported by others and our lab that HSCs could also give rise to cells of non-hematopoietic lineages, such as muscle cells, vascular cells and osteoblasts (Asahara et al. 1997; Das et al. 2009a; Aggarwal et al. 2012). Not only HSCs, other stromal cells residing in the bone marrow were also found to give rise to several nontraditional cell types. In normal and pathological condition it was found that the stem cells egress from their resident tissue, specifically from bone marrow, circulate and migrate to the site of the injured tissue for repair. At the site of injury, these migrated stem cells could differentiate into the host organ, or fuse with the existing cells and may self-renew to generate more stem cells repertoire. As discussed later in this chapter, our lab has demonstrated that in vivo transplantation of $CD34^+$ cells, derived from hematopoietic stem cells, into an osteoporotic mouse was able to augment bone formation and inhibit bone resorption by affecting the activities of the bone cells (Aggarwal et al. 2012). Thus, stem cell biology may need to be viewed in different perspective to harness the full regenerative potential.

Hematopoietic Stem Cells and Their Lineages

HSC differentiate in vivo to mature blood lineages, which are generally categorized as lymphoid, myeloid and erythroid-megakaryocytic lineages. These progenitor cells then give rise to white blood cells and red blood cells by the process called hematopoiesis. In pre-natal life, hematopoiesis takes place in fetal liver and then shifts to bone marrow in the post-natal life. Rather, HSCs were shown to originate from either hemangioblasts or yolk sac or AGM regions in fetal life. Other progenitor cells such as endothelial progenitor cells were also shown to emerge from hemangioblasts. HSCs undergo self-renewal activities and give rise to either long-term self-renewing HSCs (LT-HSCs) or short-term self-renewing HSCs (ST-HSCs) and committed progenitor cells. LT-HSCs self-renew for life-time of the host while ST-HSCs self-renew for relatively short time (8 weeks) (Morrison and Weissman 1994). Since HSCs constitute only 0.05% of the total bone marrow cell population, these cells were isolated based on their surface markers and their self-renewal capacities, was shown by using limiting dilution assays. HSCs are isolated based on the expression of their surface markers (CD133 and/or CD34). CD133 or AC133 is cell surface marker that represents primitive HSC population and is believed to play a central role in asymmetric division that represents true stemness (Das et al. 2009a). CD133 is mostly expressed on premature HSC population that are $CD34^-$ Lin^- population and at later stages of HSC maturation, CD133 is co-expressed with CD34. This latter population is capable of giving rise to $CD34^+$ cells (Gallacher et al. 2000). CD34, a negatively charged transmembrane glycoprotein, was reported to have role in HSCs adhesion, homing in murine and human studies. It is of importance to current and past studies in our lab whereby we observed the maintenance of CD34 expression after ex vivo nanofiber expansion and also efficient bone marrow homing in animal models of osteoporosis,

myocardial and hind limb ischemia (Das et al. 2009a, b; Aggarwal et al. 2012). However, some reports suggested that HSCs may have heterogeneous expression of CD34 and those had low expression of CD34, were able to cause long-term reconstitution in vivo CD34 expression may be related to cell cycle status and represent a relatively committed progeny as it was found to be very low or undetectable in differentiated myeloid or lymphoid lineages (Morel et al. 1996; Sato et al. 1999; Nakamura et al. 1999). The major functions of CD34 are yet to be fully elucidated.

HSCs have been extensively studied for their transdifferentiation potential based on the evidences from in vitro and in vivo data. Although, HSCs give rise to blood cells in vivo, the former were also shown to give rise to endothelial progenitor cells (EPC), smooth muscle cells, neural cells or bone cells. Our laboratory and others have shown that HSCs could give rise to endothelial cells and smooth/skeletal muscle or osteoblastic cells in vitro when cultured in optimum conditioning media (Asahara et al. 1997; Das et al. 2009a, b; Aggarwal et al. 2012)

Transdifferentiation of Hematopoietic Stem Cells

Relevant to the context here, transdifferentiation of HSCs to osteogenic lineages is still largely unknown. Identification of a putative osteogenic stem cell that would self-renew and differentiate to osteogenic lineages is a field of vast interest to clinicians, biomedical engineers and basic research scientists. Osteoblastic cells were believed to originate only from bone marrow stromal cells, or mesenchymal stem cells (MSCs) defined by their adherence to the plastic culture dish in vitro. MSCs and whole bone marrow were shown to differentiate to adipocytes, chondrocytes and other cell types in vivo and in vitro (Pittenger et al. 1999). Indications of existence of single stem cells that can give rise to hematopoietic stem cells and non-hematopoietic stem cells was demonstrated by serial transplantation of single bone marrow cell, suggesting an existence of long term self-renewing stem cell that could give rise to various lineages of different organ systems (Krause et al. 2001; Jiang et al. 2002). Given, the micro-environmental effects, stem cells of one origin may trans-differentiate to cells of different lineages. Such as, our lab showed that CD34 positive cells differentiate towards osteoblasts in vitro under the influence of ascorbic acid (AA), β-glycerophosphate (BGP) (Aggarwal et al. 2012)

Transcriptional Factors in Osteoblastic Differentiation

Osteogenesis is a process of primary cell differentiation towards osteoblast lineage. Several extrinsic and intrinsic factors influence this process, including hormones and growth factors, which activate osteoblast specific transcription factors and signaling molecules as mentioned below (Fig. 16.1).

Core Binding Factor Alpha1

Core binding factor alpha1 (Cbfa1or Runx2/AML3; runt-related homeodomain/acute myeloid leukemia gene 3) belongs to the family of core binding factors that play key roles in hematopoiesis and osteogenesis. This family of transcription factor consists of DNA binding domains that have high degree of homology to murine runt-related transcription factor 2 (Runx 2) (a drosophila pair-rule gene product). Other members of the Cbfa family are Cbfa2 (AML1) and Cbfa3 (AML2). Cbfa1 was found to be most abundant in osteoblasts and is also present in thymocytes and T cells. It was shown that inherited mutations of Cbfa1 result in severe impairment of osteogenesis in human; this defect is called cleidocranial dysplasia. Absence of Cbfa1 in mice blocked the differentiation processes from the mesenchyme and lacked bone formation, and thus no ossification was observed. Heterozygous Cbfa1 mice showed bone defects similar to human suffering from cleidocranial dysplasia. In osteoblasts, Cbfa1 was found to bind to promoter regions of the osteocalcin gene, collagen type1a, bone sialoprotein

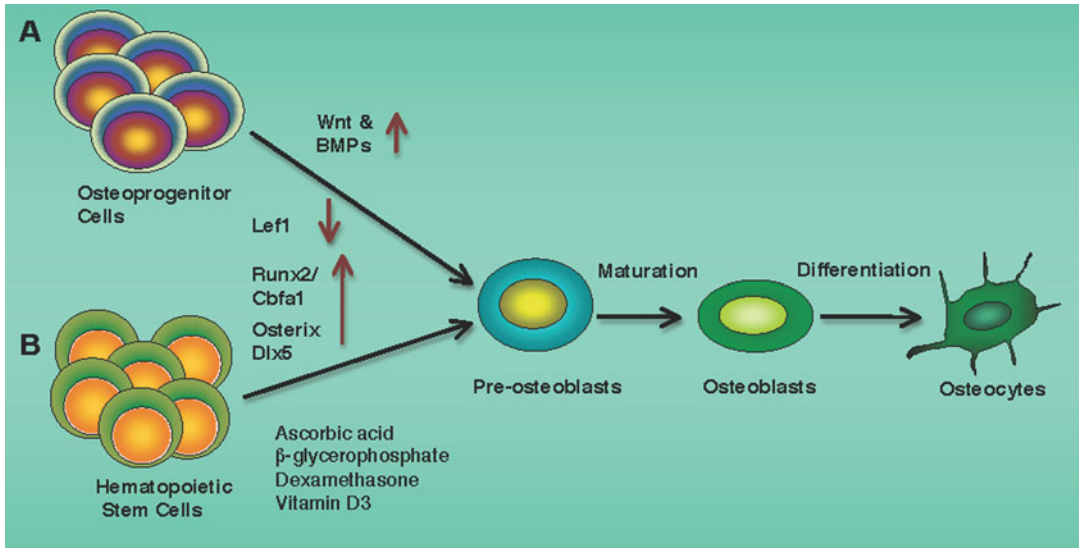

Fig. 16.1 Transcriptional regulation of HSC transdifferentiation. (**a**) Differentiation of osteoprogenitor cells. The commitment signal is initiated via BMPs and Wnt signaling molecular binding to the membrane receptors of osteoprogenitor cells. These signals mediate downregulation of transcription factor, such as Lef1, and upregulation of master regulators such as Runx2/Cbfa1, Osx, and Dlx5. These factors induce preosteoblastic differentiation and mediate cellular commitment towards maturation and differentiation towards osteocytes. (**b**) Hematopoietic stem cells when induced in vitro towards osteogenic lineage by using differentiation factors, such as, ascorbic acid, β-glycerophosphate, dexamethasone, and vitamin D also showed similar pattern in expression of regulatory transcription factor as observed in osteoprogenitor cells. Wnt: wingless; BMP: bone morphogenetic protein; Lef1: lymphocyte enhancer factor1; Runx2: runt-related transcription factor 2; Cbfa1: core binding factor alpha1; Osx: osterix; Dlx5: distal-less homeobox 5

and osteopontin (Ducy and Karsenty 1995). However, activation of Cbfa1 requires binding of bone morphogenetic protein (BMP2) to its cell membrane bound receptors that subsequently activates the cytoplasmic signaling molecules such as mothers against decapentaplegic (Smad)s, which enter the nucleus and complex with Cbfa1. Cbfa1 is critical for the induction of differentiation of stem cells to osteoblasts, however, in vitro differentiation of myoblast cell line (C2C12) could not be induced by activation of Cbfa1 alone (Lee et al. 1999). It is believed that the other transcription factors also play critical role in osteoblastic differentiation. It was suggested that although cbfa1 is a potent inducer of differentiation of progenitor cells to osteochondrogenic lineages, other transcription factors such as Osterix, is potentially involved in the guiding of osteochondrogenic cells towards osteogenic lineages.

Osterix

Osterix (Osx) is a zinc finger domain containing transcription factor, expressed in developing bones. In embryonic and post-natal life, Osx was shown to promote osteoblastic differentiation and formation of new bone. Osx was shown to be required for both endochondral and intramembranous ossification, and acts as a downstream factor of Cbfa1 gene (Nakashima et al. 2002). In Osx null mice, Cbfa1 expression was detected in the stem cells of mesenchymal origin in Osx null mice, however, stem cells were unable to differentiate to osteoblasts or deposit bone matrix, hence no bone formation occurred, and mice died at birth (Nakashima et al. 2002). It was also found that potent bone inducing proteins such as bone morphogenetic protein 2 (BMP2) induces expression of osterix, via induction of distal-less homeobox 5 (Dlx5), which binds to the homeodomain sequences on the proximal region of the osx

promoter. Furthermore, it was suggested that Cbfa1 is involved in the commitment of progenitor cells to osteochondro progenitor, however, Osx may have a role in segregation of osteoblasts from osteochondro progenitor cells and plays an essential role in genetic programming of osteocytes and osteoclasts. In post-natal life, Osx null mice showed hyper mineralized bones in the metaphysial region with limited bone marrow cells and smaller and lower number of osteoclast cells. Thus, since bone formation was affected in Cbfa and osterix null mice, these transcription factors were considered critical for healthy bone development in vivo.

Interestingly, Osx overexpression in stem cells induces them to differentiate towards hematopoietic lineages instead of osteoblastic lineages. It was shown that higher levels of Osx in stem cells indeed lead to upregulation of CD34, hematopoietic stem cells markers, and GATA1 (marker of erythroid lineages), even though the stem cells were cultured in the known osteoblastic conditioning media containing ascorbic acid, β-glycerophosphate, and dexamethasone. Conversely, overexpression of homeobox B4 (HoxB4), a well-known transcription factor of HSCs, lead to osteogenic differentiation of stem cells of embryonic origin, evident by the upregulation of osteoblast specific proteins, such as osteocalcin, bone sialoprotein, and collagen type I. Also, expression of Osx at low level induced transcription of HoxB4, which perhaps may support the idea that cell-cell interaction between HSCs and pre-osteoblasts is required for hematopoiesis. On the other hand, higher levels of HoxB4 in adult CD34 cells may be an underlying cause of pathological mineralization in arteries via expression of bone specific genes such as bone sialoprotein. Thus, these studies definitely point towards a possibility of a close relationship in origin and development of bone and blood lineages at an earlier stage of commitment.

Lymphocyte Enhancer Binding Protein1

Lymphocyte enhancer binding protein1 (Lef1), also known as T cell factor (TCF) is a DNA binding high mobility group (HMG) transcription factor that associates with β-catenin and acts as nuclear effectors in Wnt pathways, essential for maintaining bone homeostasis (van Genderen et al. 1994). However, Lef1 was shown to have less important role in osteoblastic differentiation unlike Cbfa1 and osterix. Although Lef1-deficient mice were found smaller in size and have numerous skeletal deformities compared to littermate controls, however, they still have osteoblasts and mineralized skeletal structures (van Genderen et al. 1994; Galceran et al. 2004), Lef1 regulates osteoblastic differentiation by controlling the expression of extracellular matrix proteins, which are essential for matrix mineralization and terminal osteoblastic differentiation. The lack of proliferation capacity may be due to the fact that these cells are already destined to osteoblastic fate (Kahler et al. 2006). Also, mutations in Wnt pathways such as Wnt3a, Wnt10b, their receptors Lrp6, inhibitors dickkopf (Dkk1, Dkk2) were shown to affect osteoblast functioning, bone formation, and total bone mass (Bennett et al. 2005; Holmen et al. 2004; Morvan et al. 2006). Lef1 is also expressed in lymphocytes and their progenitor cells, such as hematopoietic stem cells (HSCs). Lef1 has been shown to regulate the self-renewal activities of HSCs and the dysregulation of the expression level has been linked to malignancy. However, studies on the murine blood precursors has shown that Lef1 expression is limited to precursor cells only and is turned off at the late stage of lymphocytic development. Similarly, high expression of Lef1 was observed in the pre-osteoblastic, undifferentiated cell lines (MC3T3-E1). Lef1 was found to be downregulated upon osteogenic induction in vitro. It was found that overexpression of Lef1 (almost 140-fold) inhibited the terminal osteoblastic differentiation as was evident by the expression of late bone specific proteins such as alkaline phosphatase, osteocalcin and calcium incorporation. However, reports showed that both upregulation or downregulation of Lef1 affected the proliferation rate of the pre-osteoblastic cells (MC3T3-E1) (Kahler et al. 2006) The evidences of similar activities of Lef1 in two different types of progenitor cells indicate that progenitor cells could be induced towards different lineages, or there is

a possibility that these progenitor cells have a common origin. Furthermore, genetic studies are needed to address specific questions regarding the effects of transcriptional factors in Wnt signaling pathways, which controls progenitor cell differentiation towards osteoblastic lineages and their maturation. The transcription factors and their roles were described above to establish an understanding of the osteoblastic origin and their differentiation from stem cells into mature functional osteoblasts. The above-mentioned transcription factors, reviews the current literature that may provide evidences of stem cell plasticity such as HSCs and their transcriptional regulation dictated by the microenvironment.

To understand the regenerative potential of bone, understanding of cellular components of the bone is important. Bone is being recognized as a regenerative organ as it harbors stem cells in its anatomical structures, such as periosteum (outer most layers of the bones), and bone marrow, however, its regenerative potential declines with age, and is influenced by other risk factors. Major cellular compartment of bone consists of the osteoblasts (bone forming cells) and osteoclasts (bone resorbing cells), and an imbalance in their cellular activities results in various bone disorders such as osteoporosis.

Bone Degenerative Disorder: Osteoporosis

Osteoporosis is a major health problem, affecting almost 75 million people worldwide, especially women and elderly people (WHO 1994). It is a chronic disease that is characterized by low bone density, brittle bones and is caused due to an imbalance of bone formation and resorption activities of the bone cells. This skeletal disorder increases the risk of skeletal fractures, high morbidity and mortality. Multiple factors such as genetics, age, hormonal, drug usage, and environmental factors are responsible for osteoporosis. From various reports it is evident that osteoporosis may also be developed by an age related decline in number, and functionality of stem cells (that give rise to osteoblasts) in rodents, primates, and humans, confirmed by in vitro colony forming assays, mineralization, and alkaline phosphatase staining. Certain pathological conditions or age may induce the differentiation of osteoblastic progenitor cells towards adipocytes rather than osteoblasts (Moerman et al. 2004). Thus, due to the potential limitations of body's own stem/progenitor cells to maintain bone homeostasis, stem cell based alternative therapies are being investigated to provide long-term self-renewing source of bone forming cells. In this section we will summarize the function of osteoblastic and osteoclastic cells and current therapies for osteoporosis followed by emerging new cell-based therapies.

In adult skeleton, bone forming cells or osteoblasts make up to 4–6% of all the bone, whereas, osteocytes make up to 90–95% of the bone. However, all these cells play distinct roles in the initiation and regulation of mineralization of bone matrix. Osteoblasts are mononuclear cells that are recruited to the site of bone formation mainly from stem cells of mesenchymal origin via osseous vasculature. Once, osteoblasts reach the bone surface or bone-remodeling sites, osteoblastic cells produce bone matrix and get mineralized. Later osteoblasts either get embedded within the matrix to become osteocytes or die by apoptosis, thereby releasing signals for resorption, which activates bone resorbing (osteoclasts) cells that targets their removal of dead osteocytes. Osteocyte cell death also occurs in association with pathological conditions, such as osteoporosis leading to skeletal fragility; which results in the loss of the ability to sense microdamage and signal repair. Thus osteocyte viability plays a significant role in maintaining healthy bone.

Osteoclasts or bone resorbing cells are specialized multinucleated cells, derived from the mononuclear monocytic/macrophage precursors, and comprise 1–2% of bone. These mononuclear cells differentiate and fuse to form multinuclear osteoclastic cells in presence of the two differentiating factors, receptor activator of nuclear factor kappa- β ligand (RANKL) and macrophage colony-stimulating factor (MCSF). Bone resorption by osteoclast requires a unique polarization of its cytoskeleton to form ruffled bordered

membrane over the bone matrix. In addition to ruffled border membrane, contact with the bone also causes the reorganization of actin cytoskeleton to form sealing zone or actin ring with the help of integrins. These acidified compartments enables the osteoclast for resorptive purposes by release of vesicles that contain matrix metalloproteases (MMPs), cathepsin K and hydrochloric acid (HCl) to dissolve the underlying type1 I collagen in the bone matrix (Teitelbaum 2000).

Current Therapies for Osteoporosis

As indicated earlier, metabolic disorders such as osteoporosis results from the imbalance between bone resorption and formation, understanding and restoring the balancing mechanisms are important for the development, efficacy, and long-term benefits of therapeutic agents. Current available treatments are largely anti-resorptive, which help to inhibit the resorptive activities of the osteoclasts, however, simultaneously suppress bone formation thus reducing their bone remodeling and thus lowering the overall performance of the drug. So far, only one anabolic treatment mediated via parathyroid hormone is FDA approved for osteoporotic patients and once patients are off- the medication, the osteoporotic symptoms re-appear (Kawai et al. 2011). Thus, it has become paramount to identify molecules and/or therapeutic strategies that could regulate both resorption and formation in synchrony. Newer research suggests that cell therapy may be an option that may restore the balance between bone formation and resorption, and restore the normal bone homeostasis. This section will highlight the current treatments in clinic and development of newer therapies for osteoporosis.

Antiresorptive Drugs

Bisphosphonates are potent inhibitors of bone resorption, and were shown to increase bone mineral density (BMD) and reduce the risk for osteoporotic fractures with reduction in bone turnover in numerous clinical trials. Bisphosphonates chemically bond to the calcium hydroxyapatite in the bone and thus decrease bone resorption by blocking the function and survival of osteoclasts. For example, in Fracture Intervention Trial (FIT), postmenopausal women with low femoral neck BMD were enrolled in randomized, double blind, placebo controlled multi center studies, were shown to have significantly reduced rates of vertebral fractures when compared to placebo controls (Black et al. 1996). However, potential adverse effects (many women with subtrochanteric or typical fractures) of bisphosphonates after a treatment for continued long period of time has been reported. Newer antibody based anti-resorptive drugs, such as denosumab are still under investigation. It is a human monoclonal antibody against the RANK ligand and thus prevents its binding to its receptor RANK, on the osteoclasts and its precursor cells, and thus, has been claimed to reversibly inhibit osteoclast-mediated resorption. In contrast to bisphosphonates, denosumab acts by blocking the formation, function, and survival of osteoclasts. The major concerns of denosumab were related to an increase in overall incidence of cancer, infection or skin problems, as RANK and RANKL are expressed on the members of the lymphoid family. In a separate study, increased incidences in osteonecrosis of jaw (ONJ) caused by both of the above-mentioned anti-resorptive therapies were reported. However, no cases of ONJ were reported in patients on selective estrogen receptor modulators (SERMS) (Nalliah 2012).

SERMS are non-steroidal synthetic compounds that mimic estrogen effects in tissue-specific manner. Since, estrogens causes increase in bone density and inhibits bone turnover, estrogen deficiency in postmenopausal women increases bone turnover and resorption, leading to bone loss. Thus, hormone replacement therapy reverses bone loss in early and late phases of postmenopausal period. However, use of certain SERMS for bone therapy may exert cancerous effects on extra-skeletal tissues, such as breast and uterus, raising concerns for their long-term safety (Rodan and Martin 2000).

Other antiresoprtive drugs such as odanacatib selectively and reversibly inhibits cathepsin K, a cysteine protease expressed in osteoclasts, degrades type I collagen. However, it was found that effects on the bone formation markers of this drug were lesser compared to other anti-resorptive drugs, such as bisphosphonates, and their potential side effects are still unknown (Bone et al. 2010).

Anabolic Drugs

Anabolic drugs were designed to increase bone mineral density by stimulating bone formation and increasing bone remodeling. There are number of anabolic therapies, including bone morphogenetic proteins (BMP 2, 7), insulin-like growth hormone (IGH), vascular endothelial growth factor, parathyroid hormone (PTH), statins, and strontium fluoride. Anabolic agents, such as PTH have the ability to restore bone mass, restoring bone homeostasis, thus lowering the risk of fractures, more than that of anti-resorptive agents. PTH and its analogs, BMP2 and BMP7 were one the FDA approved anabolic drugs for the treatment of osteoporosis. Low levels of PTH directly increase bone mass stimulating osteoblastic survival and activities. Indirectly, PTH regulates skeletal growth factors that induces IGH synthesis, inhibits sclerostin expression (antagonist of BMPs), and activates Wnt signaling pathways. The limitation of PTH are that they need to be administered everyday (Jilka 2007). PTH was shown to increase BMD as well as bone strength in osteoporotic women and men. Statins, a class of lipid lowering drugs, used for the treatment of cardiovascular disease was also shown to reduce the risk of fractures by stimulating the production of BMPs and endothelial nitric oxide synthase (eNOS). However, inhibition of osteoblastic eNOS did not prevent statins in bone formation (van't Hof and Ralston 2001). Newer drugs such as, monoclonal antibody (AMG 785), to sclerostin (antagonists of BMP), induced a dose dependent increase in the bone formation markers, as well as decrease in the bone resorption markers. However, other effects are largely unknown as potential drugs are still under investigation. Newer research has shown that semaphorin 3A (Sema 3A), an axon guidance molecule, expressed by osteoblasts, has a distinct osteoprotective effects in osteoporotic murine models. Sema 3A was shown to have crucial role in bone formation by osteoblast cells, and at the same time limiting the migration, and activities of osteocalsts to the bone formation sites through inhibiting the expression of cytoskeletal protein RhoA and immunoreceptor tyrosine-based activation motif (ITAM) (Hayashi et al. 2012). Interestingly, it was pointed that as mice ages, their serum level of Sema 3A reduced and this could be one of the factors for bone loss due to age. Thus, Sema 3A could be used as a potential bone formation biomarker. Above studies direct towards a possible development of therapies that would restore coupling of bone cells (Hayashi et al. 2012).

Stem Cell Therapies for Bone Regeneration in Osteoporosis

Stem cells have taken a center-stage of the field of regenerative medicine, owing to their self-renewal capacity and ability to differentiate towards various lineages, depending on their potency. In particular, two types of stem cells have been used in bone related pathologies: embryonic stem cells (ESCs) and adult stem cells (ASCs). Adult stem cells particularly comprise of hematopoietic stem cells (HSCs), mesenchymal stem cells (MSCs) and could be derived from various tissues such as bone marrow, umbilical cord blood (UCB), fat tissue or peripheral blood. In particular, UCB-derived stem cells, such as MSCs and HSCs transplantation are promising for various systemic and local disorders. Adult stem cells have been documented to present minimal graft vs. host disease and are multipotent in nature. ESCs are totipotent and were shown to be tumorigenic in vivo and are bound by ethical issues. Recently, it was shown in an osteoporotic individual that MSCs transdifferentiate towards non-bone forming cells, such as adipocyte by switching towards the expression of adipocytic transcriptional factor peroxisome proliferator-activated receptor gamma (PPARγ) from cbfa1. MSCs were tested for their ability to restore bone formation in

murine models of fracture and bone pathologies, however, only few studies have focused on their ability to restore skeletal bone micro-architecture in osteoporotic conditions. Potential drawback of using MSCs, in vivo studies were the lack of bone marrow homing markers, which would be needed to guide the MSCs to mobilize towards their target site for bone regeneration. Recently, a study showed that the use of MSCs in combination with overexpression of homing receptors (CXCR4; ligand of stromal-derived factor 1, SDF1) and osteogenic transcriptional factor (Cbfa1) (Lien et al. 2009). Although, this approach increases stem cell's capacity of bone marrow engraftment but techniques, such as transfection of MSCs were of potential concerns. MSCs could also be derived from adipose tissue by liposuction techniques. It was shown that adipose tissue-derived MSCs (AT-MSCs) transduced with CXCR4 showed enhanced capacity of in vitro differentiation towards osteoblastic lineages and low engraftment abilities were overcome by transduction of CXCR4 (Bobis-Wozowicz et al. 2011). There are limited examples of HSC-mediated regeneration of bones. One of the major limitations of HSCs derived from UCB is low number of available stem cells for the clinical applications. Various protocols for ex-vivo expansion of the stem cells are being extensively explored but have relatively lower expansion rates, which preclude the possibility of their use in clinical applications. Our laboratory has established a nanofiber-based ex vivo expansion technology. We reported that human UCB-derived HSCs could be expanded substantially on nanofiber-coated plates supplemented with cytokine cocktail in serum-free media within a very short period of time. The expanded stem cells were shown to differentiate towards various lineages (Das et al. 2009a; Aggarwal et al. 2012). UCB and adipocyte tissue are being investigated as potential sources of adult stem cells, as they are easy to harvest, no ethical concerns, less discomfort to the donor, and almost no hospital stay time. Furthermore, stem cells could be used in variety of ways to treat the bone related disorders. First, isolated stem cells could be directly transplanted into the injured tissue, thereby allowing the stem cells to be differentiated in vivo into any lineage. Second, stem cells could be transfected with osteoinductive genes to direct towards osteoblastic lineages, and transplanted into the injured part of the body. Third, stem cells could be differentiated in vitro into desired lineage under the influence of certain cytokines, growth factors, and then transplanted into the patients. Most of the stem cell-based therapies used non-hematopoietic stem cells and use of hematopoietic stem cells is still being investigated for bone related disorders. Recently, we have shown that transplantation of nanofiber-expanded $CD34^+$ cells ameliorates bone formation and suppresses the bone resorbing activities of the osteoclastic cells. Nanofiber-expanded $CD34^+$ cells constitutively express high levels of pro-migratory (CXCR4) and pro-adhesive (LFA-1) thus circumventing the need of transduction and enabling efficient homing into bone marrow. We also found that nanofiber-expanded $CD34^+$ cells when induced with osteoblastic induction media were able to upregulate osteoblast related genes (such as bone morphogenetic proteins, Type I collagen, osteocalcin) and differentiate into osteoblastic lineages in vitro. Moreover, we showed that transplantation of these cells into a murine model of osteoporosis increases the serum levels of bone formation markers such as osteocalcin, and simultaneously decrease the serum levels of resorptive chemokines such as monocyte chemotactic protein (MCP-1). Systemic delivery of $CD34^+$ cells increases bone remodeling and concurrently inhibits osteoclastic differentiation and activities in osteoporotic mice. Thus, it is possible that $CD34^+$ cells offer an osteoprotective role by synergistic mechanisms that may help to regenerate bone and inhibit the pathological resorptive activities of the osteoclasts (Fig. 16.2) (Aggarwal et al. 2012).

Conclusions and Future Directions

The potential of stem cell based therapies for various bone degenerative diseases is attractive because the existing treatments suppress the disease symptoms, and are unable to regenerate bone tissues at genetic and cellular level to offer longer disease free periods. The demographic

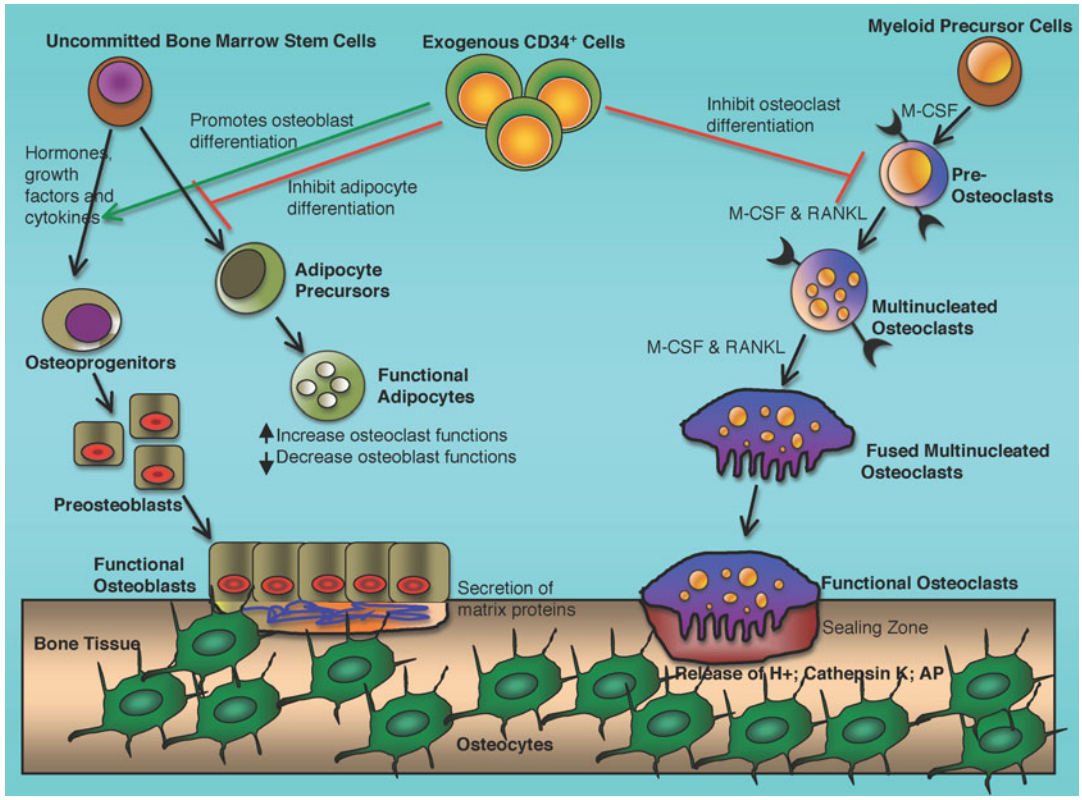

Fig. 16.2 Mechanisms of exogenous CD34⁺ stem cell functions. Exogenous CD34⁺ cells inhibit differentiation of stromal cells towards adipocytic lineages by downregulating the expression of adipocytic master regulator (PPAR γ) in the uncommitted bone marrow stem cells. Thus, bone marrow stromal cells differentiate towards their destined osteoblastic lineages via upregulation of the osteoblast specific transcriptional factors such as Cbfa1/Runx2. Exogenous CD34⁺ cells concomitantly inhibit the differentiation and impair functions of progenitor cells of monocytic-macrophagic lineages, which differentiate towards osteoclasts that resorb the bone matrix and decreases the bone mineral density

challenges presented by the aging population worldwide emphasize the need for innovative research and approaches to address the issues of skeletal restoration. Osteoporosis raises substantial risk of fractures with age. A continuous blood supply is essential to regenerate functional bone tissues, as degeneration of vessels and bones are evident in osteoporosis. So far, vascular supply restoration was shown to be achieved by using stem cells of hematopoietic and non-hematopoietic origins at preclinical levels. Essentially, it was been shown that adult stem cells and embryonic stem cells are able to differentiate into bone cells in vitro and in vivo. However, lot of work still has to be done to understand the mechanistic basis of differentiation and their functionality. These mechanistic studies not only, help us to reveal the origin of the stem cells, but hopefully mirror their functionality in vivo and to find a possible cure for life-long disease free survival. Also, tumorigenic potential in long-term studies, and in vivo fate of therapeutic stem cells after transplantation is largely unknown, and need to be determined prior to therapeutic consideration.

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Children with Solid Tumors: Identification of Hematopoietic and Endothelial Progenitor Cells as Biomarkers

17

Julie Mund and Jamie Case

Contents

Abstract

Pediatric brain tumors are the second most common form of childhood cancer behind hematological malignancies, with close to 3,400 new cases diagnosed each year. Pediatric solid tumors, including brain tumors, have high angiogenic potential, thus increasing their ability for growth and metastases. Blood vessels are a key element to the growth of a tumor, making the endothelial and hematopoietic cells that support angiogenesis ideal for use as a biomarker, potentially able to diagnosis and monitor the progression of disease. In this chapter, data from pediatric solid tumor studies will be analyzed and reviewed to determine which current biomarkers have the most potential for influencing treatment and/or outcome. Flow cytometry has advanced significantly over the past decade, thus allowing for enhanced characterization of these circulating cell subsets, with the most recent clinical studies incorporating these newer sophisticated techniques. Moreover, utilizing multiparameter flow cytometry, a new frontier of pediatric solid tumor diagnosis and monitoring is now being explored to better understand the role of circulating cells in malignancies and the incorporation of successful biomarkers into the clinic.

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Introduction

Over the past 20 years, pediatric solid tumors have remained steady in their diagnosis, following an increase throughout the 1980s (Pollack and Jakacki 2011). These pediatric patients have increased survival and improved outcomes compared to adult cancer patients (McGregor et al. 2007). Pediatric solid tumors grow by utilizing angiogenesis, that is, the formation/sprouting of new blood vessels from endothelial cells and bone marrow derived cells that represent already existing vascular structures, thereby further nourishing and feeding the growing tumor. Furthermore, tumor growth and metastasis can also depend on the ability of the tumor cells themselves to secrete an assortment of different molecules, which in turn entice circulating and resident pro-angiogenic cells in the tumor microenvironment to promote angiogenesis (Mancuso and Bertolini 2010).

The process of angiogenesis is crucial for the maintenance of solid tumors, thus anti-angiogenic therapies have been developed for the treatment of these malignancies. Anti-angiogenic therapies have been utilized to target different cell subsets by manipulating the receptors on the outside of the cells for the specific cell type they are targeting, such as vascular endothelial growth factor (VEGF), which has been shown to be increased in patients with cancer (Jain et al. 2006). Endothelial and hematopoietic cells have long been characterized, with both peripheral blood (PB) and bone marrow (BM) having been collected for the processing of specific cellular fractions to try to identify those involved in active angiogenesis within the body.

Monitoring of these tumors and their subsequent growth rates through the use of PB or BM mononuclear cells (MNCs) via flow cytometry has been attempted for decades on adult patients, with very little known about the corresponding pediatric population.

Within the last decade, advances in both the machinery/software and antibodies available for commercial purchase, multiparameter flow cytometry has now become a viable way to analyze complex cellular populations present in PB, BM and tissue. Current technology allows for up to 7–10 different antigens being analyzed together without needing individual tubes, which therefore permits the identification of interconnected populations that reside within the cellular fraction. Recent adult and pediatric clinical studies have shown the promise of these new biomarkers for the diagnosis, characterization and monitoring of disease progression. Some clinical trials have been successful, while others have shown the issues that remain with the development of biomarkers, the main one being a lack of a consensus definition for the cellular types that includes functional data along with the phenotypic data to correctly verify the cell of interest.

The Cellular Populations of Interest in Pediatric Solid Tumors

Circulating endothelial cells were first described in 1997 and were reported to express the stem cell markers CD34 and Flk1 (also known as vascular endothelial growth factor receptor 2 (VEGFR2) or KDR in human subjects), as well as the pan hematopoietic marker CD45 and CD31, an endothelial marker (Asahara et al. 1997). Following culture for 7 days, these cells grew out into spindle shapes and were able to uptake DiI-labeled acetylated low-density lipoprotein (ac-LDL) (Asahara et al. 1997). Another study later showed that these cells were in fact not endothelial, but a colony of cells that contained a cluster of angiogenic T cells ($CD3^+CD31^+CXCR4^+$) at the center (Hur et al. 2007). This finding therefore verified that multiple hematopoietic cell subsets are important modulators of postnatal vasculogenesis and can correlate with cardiovascular disease (Hur et al. 2007).

Later, true, bona fide endothelial progenitor cells, termed endothelial colony forming cells (ECFCs), that had proliferative potential, the hallmark cobblestone morphology, and were capable of forming tube-like structures on a collagen matrix, were described (Ingram et al. 2004). However, no circulating cell could be identified phenotypically, as these cells (i.e., ECFCs) grew

out in culture from a MNC fraction. Since the discovery of circulating endothelial cells, investigators on both sides of the angiogenesis field, specifically oncology and cardiovascular disease, have wanted to use all of the various cell subsets to correlate disease with a biomarker. Unfortunately, these cells were previously incorrectly identified as endothelial progenitor cells, therefore leading to confusion within the fields and causing an inability to compare data across multiple studies which incorporated various conflicting phenotypes (Mund and Case 2011; Prater et al. 2007; Timmermans et al. 2009).

Recently, the true, bona fide endothelial progenitor cell population (i.e., ECFCs) were phenotypically characterized by multiparameter flow cytometry ($CD31^{+}CD34^{bright}CD45^{-}AC133^{-}CD14^{-}CD41a^{-}CD235a^{-}$LIVEDEAD Violet^{-}) and functionally verified using colony forming assays, to ensure the correct cell population is in fact being selected (Estes et al. 2010a; Mund et al. 2012). This newly standardized method should now add clarity to future studies, and thereby allow for the comparison between various fields of research.

Circulating progenitor cells are bone marrow derived hematopoietic precursor cells that have also been studied to monitor angiogenesis (Bertolini et al. 2006; Duda et al. 2007; Mancuso et al. 2001). These cells are increased in adult patients with various solid tumors, including breast cancers (Mancuso et al. 2001, 2006; Shaked et al. 2008). Interestingly, a population of circulating progenitor cells previously reported as being homogeneous (and of endothelial origin), has recently been identified as being a heterogeneous mix of hematopoietic stem and progenitor cells with either pro-angiogenic or non-angiogenic qualities (Estes et al. 2010b; Pradhan et al. 2011). These newly, phenotypically and functionally identified circulating hematopoietic stem and progenitor cells (CHSPCs; $CD31^{+}CD34^{bright}CD45^{dim}AC133^{+/-}CD14^{-}CD41a^{-}CD235a^{-}$LIVEDEAD Violet^{-}) were shown to be decreased in patients with severe peripheral artery disease, and increased in patients with cancer (Estes et al. 2010b; Pradhan et al. 2011). The pro-angiogenic cell subset of the CHSPCs (pro-CHSPCs; $CD31^{+}CD34^{bright}CD45^{dim}AC133^{+}CD14^{-}CD41a^{-}CD235a^{-}$LIVEDEAD Violet^{-}) were able to induce significant tumor growth in a murine model of melanoma, whereas the parent population containing both fractions and the non-angiogenic cell subset of the CHSPCs (non-CHSPCs; $CD31^{+}CD34^{bright}CD45^{dim}AC133^{-}CD14^{-}CD41a^{-}CD235a^{-}$LIVEDEAD Violet^{-}) did not alter the size of the tumor (Estes et al. 2010b).

To date, most clinical trials of anti-angiogenic therapies in oncology have been focused on adult malignancies (Jain et al. 2006). This has led to a notable gap in the knowledge of the mechanisms behind the more robust pediatric solid tumors, which are known to be highly angiogenic. Although the use of progenitor cell populations as potential biomarkers has been around for over a decade, very little clinical trial data on pediatric solid tumor patients has been published, with the few studies that are available, having shown mixed results (DuBois et al. 2012; Pradhan et al. 2011; Taylor et al. 2009).

Clinical Trials Using Multiparameter Flow Cytometry

Initially, the use of flow cytometry to analyze cellular populations in pediatric solid tumor patients was actually describing the cells of the tumor itself, not the circulating cells or bone marrow derived cells. In an interesting study that characterized the cells of Ewing sarcomas found in pediatric patients, it was determined that they expressed the stem cell marker CD34 (Yaniv et al. 2007). This is an incredibly important determination as it is routine for stem cells to be harvested from the PB or BM for autologous transplantation at a later stage, with those harvested cells being separated by their CD34 expression. This therefore creates a new perspective on Ewing sarcoma and autologous transplantation post treatment, as it was previously thought that by selecting $CD34^{+}$ cells, the tumor cells could be removed from the healthy BM (Vogel et al. 2000). However, there is still controversy over whether these few cells that can make it back into the patient following transplantation, could

cause adverse effects (reviewed in (Shimoni and Korbling 2002)).

In 2009, a groundbreaking study looking at circulating cellular populations in pediatric solid malignancies identified populations that could be used as biomarkers (Taylor et al. 2009). In summary, PB was collected from pediatric patients with localized or metastatic disease, as well as from healthy age matched controls. The MNCs were subsequently isolated and stained with various combinations of antibodies (CD45, CD34, VEGFR2 (KDR), CD31, CD146 and 7AAD, a viability dye), to identify either circulating endothelial cells (CECs; $CD45^{-}CD31^{+}CD146^{+}7AAD^{-}$) or $VEGFR2^{+}$ bone marrow derived progenitor cells ($VEGFR2^{+}$-BMD progenitors; $CD45^{dim}CD34^{+}VEGFR2^{+}7AAD^{-}$). The $VEGFR2^{+}$-BMD progenitors were also shown to uniformly expressed CD133.

In patients with metastatic disease, $VEGFR2^{+}$-BMD progenitors were shown to be significantly higher than that observed in the healthy control samples (median 1.5% (0–10.3%) and 0.3% (0–1.6%) respectively) (Taylor et al. 2009). Even more interesting was that at initial diagnosis, patients who had very highly elevated levels of $VEGFR2^{+}$-BMD progenitors, had metastatic disease, not localized. This is the first time a population of cells in pediatric solid tumor patients has been shown to correlate with severity of disease.

The CECs described in the above mentioned study did not have any significant differences between either the patient populations or the patients and the healthy controls. This could be in part due to their definition of a CEC, and the rarity of such cells in most individuals. Interestingly, a phenotypic sub-population of BMD progenitor cells ($CD45^{-}CD34^{+}7AAD^{-}$) was found to not significantly express VEGFR2, which is contradictory to the generally accepted endothelial progenitor cell phenotype of $CD45^{-}CD34^{+}VEGFR2^{+}$ cells (Asahara et al. 1999; Dimmeler and Zeiher 2000).

One subject that needs to be looked at critically with respect to this study is the common switching between $VEGFR2^{+}$-BMD progenitors and $VEGFR2^{+}$-BMD endothelial progenitor cells for the apparent same population. It may seem like semantics, but for the future of biomarker studies, it is imperative that the cells be identified by both their phenotype and function. It has now been reported that true circulating endothelial progenitor cells are $CD45^{-}$, therefore making these above mentioned cells (i.e., $VEGFR2^{+}$-BMD progenitors), a set of hematopoietic progenitors, potentially similar to the recently described pro-CHSPCs (Estes et al. 2010b; Mund et al. 2012).

Additionally, VEGR2 has been reported to not be able to be titred, which is extremely important for all multiparameter flow cytometry studies (Estes et al. 2010a). The authors also state that all of the antibodies were titered except for VEGFR2, which was "used at the manufacturer's recommendation" (Taylor et al. 2009). Therefore, without a leveling off of the mean peak of channel fluorescence (MPCF), an increase in MPCF at each concentration is observed. Concisely, the more of the antibody that is added, the more positive cells can be detected because of non-specific staining that is concentration dependent. This makes VEGFR2 unreliable for multiparameter flow cytometry panels, and can add additional confusion to the results, as most studies are done over a longer duration of time.

Pediatric patients with osteosarcoma have also been studied using endothelial biomarkers to follow disease progression (DuBois et al. 2012). This study was the first to analyze CECs in osteosarcoma, even though angiogenesis, which is measured mainly through serum VEGF levels, plays a large role in the pathogenesis and prognosis of the tumor (Kaya et al. 2000, 2002; Lin et al. 2011). In this particular study, CECs were characterized as $CD146^{+}CD31^{+}CD45^{-}CD133^{-}$, and circulating progenitor cells (CPCs) were defined as $CD146^{+}CD31^{+}CD45^{-}CD133^{+}$. Unfortunately, no significant difference was observed in CEC levels between patients and the healthy control samples.

Interestingly, the only significant difference that was observed was when the CD146 antibody was removed from the CEC antigen panel and replaced by VEGFR2. This subsequently modified CEC definition then showed significant lowering of these $VEGFR2^{+}$ CECs in osteosarcoma patients

(median 725 cells/mL) when compared to normal healthy controls (median 2,230 cells/mL) (DuBois et al. 2012). The authors give some potential explanations for their seemingly negative results. First, they decided not to use CD34, which is a popular marker routinely used for the identification of other progenitor cell populations, and secondly, they had a relatively small sample size (n = 18), which could account for the negative results.

In 2011, another novel paper was published looking at circulating progenitor cell populations in pediatric patients with various solid tumors (Pradhan et al. 2011). Following a previously published protocol (Estes et al. 2010a), pro-CHSPCs, non-CHSPCs, ECFCs, and mature circulating endothelial cells (CECs) were identified in the PB of both healthy children and children with malignancies (Pradhan et al. 2011). The purpose of the small pilot study was to see if the cellular biomarkers previously described, had any use for determining the diagnosis or efficacy of cancer treatments. This study found that mature CECs were significantly higher in healthy controls versus patients at both baseline and day 21. However, no difference in CEC levels was observed in the patients between baseline and day 21. Furthermore, the ECFC population was shown to be significantly higher in patients at day 21 as compared to healthy controls, which was hypothesized to be indicative of the mobilization of these cells in response to treatment (Pradhan et al. 2011).

The most interesting piece of data obtained from the Pradhan et al. (2011) study was that it demonstrated that patients with cancer had a higher ratio of the two CHSPC populations (i.e., pro-:non-CHSPCs), that had been previously reported as a homogeneous population (Duda et al. 2007). The median ratio of the pro:non-CHSPCs was 2.12 (0.34–5.95) at baseline for the patients, while the healthy controls had a median ratio of 1.23 (0.24–1.54) (Pradhan et al. 2011). Following treatment, the median ratio for the patients dropped to 1.59 (0.21–8.75) (Pradhan et al. 2011). This is the first time that the two subsets had been reported in any human malignancy. While the CHSPC parent population has been reported many times in cancer research, it was never subdivided into the two phenotypically and functionally different cell subsets (Duda et al. 2007; Jain et al. 2006; Willett et al. 2009).

Conclusions

Pediatric solid tumor patients are now being studied for the development of an accurate biomarker for both diagnosis and to aid in the efficacy of treatment. The importance of these biomarkers lays in their potential use for a better clinical outcome by allowing a representative view of the cells in circulation. Newly identified populations of both circulating endothelial and hematopoietic progenitor cells will allow for more trials to have consistent cellular definitions that are defined in terms of both phenotype and function. Older studies have contributed many important discoveries, but it is now time for consensus definitions to be reached to allow and improve the ability for the knowledge to be used across multiple areas of research.

Based on the most recent research findings, it has been shown that an increase in a ratio of specific circulating cell types (pro:non-CHSPCs) is associated with an increase in active angiogenesis. If other researchers are able to duplicate the data in different fields, this knowledge could be used to further both oncology research and cardiovascular disease. Interestingly, these two fields share an important link because cancer survivors have been shown to have vascular complications later in life, which is particularly important in survivors of pediatric cancers (reviewed in (Mulrooney et al. 2012)).

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Pediatric Neuroblastoma: Combined Treatment with Monoclonal Antibody and Cytokines Preceded by Hematopoietic Stem Cell Transplantation

Mehmet Fevzi Özkaynak

Contents

Abstract

The high expression of GD2 in neuroblastoma and its restricted distribution in normal tissues make anti-GD2 monoclonal antibodies suitable for immunotherapy. A chimeric human–murine anti-GD2 monoclonal antibody called ch14.18 has shown activity against neuroblastoma in preclinical studies and early-phase clinical trials. This activity was enhanced when ch14.18 was used in combination with granulocyte–macrophage colony-stimulating factor (GM-CSF) and interleukin-2 (IL-2) to augment antibody-dependent cell-mediated cytotoxicity. The feasibility of administering ch14.18 in combination with GM-CSF, IL-2, and isotretinoin during the early post autologous hematopoietic transplantation period has been shown in two sequential phase 1 Children's Oncology Group (COG) studies. These studies paved the way for the most recent COG study (ANBL0032), a randomized, phase 3 study, that tested whether adding immunotherapy (consisting of ch14.18 with GM-CSF and IL-2) to isotretinoin therapy, as compared to the use of isotretinoin alone, improves the survival of children with high-risk neuroblastoma who are in remission after intensive multimodal therapy which included myeloablative therapy with stem-cell rescue. Immunotherapy with ch14.18, GM-CSF, and IL-2 was associated with a significantly improved outcome (20% higher event-free survival) as compared with

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standard therapy in patients with high-risk neuroblastoma who achieved minimal residual disease status after intensive multimodal therapy. The standard of care for high-risk neuroblastoma has changed and now includes immunotherapy.

Introduction

Neuroblastoma is the cancer of the sympathetic nervous system. Nearly half of the patients with neuroblastoma present with high-risk features and have poor outcome despite aggressive therapy. The standard of care has been surgery, chemotherapy, high-dose chemotherapy with autologous peripheral blood stem cell transplantation (ASCT), radiotherapy and isotretinoin administration. During the last decades, one of the major progresses has been the establishment of the role of ASCT and isotretinoin (Stram et al. 1996). In Childrens Cancer Group study (CCG-3891) of 379 patients, the 5-year event-free survival (EFS) for patients randomly assigned to ASCT was significantly higher ($30 \pm 4\%$) than those randomly assigned to chemotherapy ($19 \pm 3\%$) ($p = 0.04$). All patients who completed cytotoxic therapy with ASCT without disease progression were then randomly assigned to receive a 6 month course of isotretinoin versus observation. Isotretinoin was given as 160 mg/m^2/day for 2 weeks/month for 6 months, and began approximately 3 months following ASCT. The 3 year event free survival, from the time of the second –isotretinoin- randomization was $46 \pm 6\%$ for the 130 patients who received isotretinoin, as compared to $29 \pm 5\%$ for the 128 patients randomized to no further therapy ($p = 0.027$) (Matthay et al. 1999). The report by Yu et al. in 2010 on the role of immunotherapy in high-risk neuroblastoma has been the latest breakthrough (20% higher event-free survival) in the management of one of the most frustrating childhood cancers (Yu et al. 2010). Immunotherapy is now considered to be the part of the standard of care in North America for patients with high-risk neuroblastoma.

The story of immunotherapy goes all the way back to the end of the nineteenth century when Paul Ehrlich envisioned antibodies as 'magic bullets' that would specifically trace and kill microbes and tumor cells (Schwartz 2004). Ehrlich's theory gained momentum in 1975, when Kohler and Milstein described the generation of murine antibodies (mAbs). Murine antibodies quickly became an important diagnostic tool, however, their development as therapeutic agents was hampered by the immune response of the patients, which readily inactivated mAbs. Only after technical advances in antibody generation in the 1980s, mAbs became available as valuable treatment tools. US FDA approval of the first chimeric (murine and human) antibody, rituximab for the treatment of B-cell non-Hodgkin's lymphoma took place in 1997.

The search for a target on neuroblastoma cells started in 1980s. Reisfeld and Gillies (1996), showed that disialoganglioside GD2 is strongly expressed on neuroblastoma cells in early 1980s (Schulz et al. 1984). The group at La Jolla led by Reisfeld and Cheung at New York developed the first mAbs against GD2. The rationale for the clinical use of anti-GD2 antibody is based on the widespread expression of GD2 on neuroblastoma cells, whereas, the restricted expression on normal tissues such as the peripheral nervous system – hence, the neuropathic pain associated with the treatment-, skin melanocytes and the cerebellum, which is protected by the blood–brain barrier (Svennerholm et al. 1994). GD2 is strongly expressed on the surface of human neuroblastoma cells and there is little intra- or intertumor heterogeneity. Anti-GD2 antibody localizes to human GD2-positive tumors in animal models and in humans and activate complement and mediates antibody-dependent cellular cytotoxicity (ADCC) with monocytes, neutrophils, natural killer and lymphokine-activated killer cells. Murine monoclonal antibodies 14.G2a and 3F8 were the first to be tested in clinical studies for neuroblastoma. Gillies formed a chimeric construct (ch14.18) using genes for the murine 14.G2a variable region's heavy and light chains and human constant region genes for IgG1 heavy chain and kappa light chain (Mueller et al. 1990) Thus,

ch14.18 has antigen-binding properties comparable to those of 14.G2a, but physical properties and complement- and Fc receptor-binding properties comparable to those of the human Ig chain. Ch14.18 has been the antibody used in the most recent CCG and COG immunotherapy studies that have changed the standard of care in neuroblastoma. Currently, a phase 1 study of humanized anti-GD2 antibody (HU14.18K322A) is being conducted at St. Jude Research Hospital. In addition, a humanized fusion protein, hu14.18-IL2, has recently been studied in the pediatric phase I and II setting in COG. The hu14.18-IL2 fusion protein consists of the humanized 14.18 anti-disialoganglioside (GD2) monoclonal antibody genetically linked to two molecules of human recombinant interleukin-2.

Phase 1 and 2 Studies with Anti-GD2 Antibodies

Cheung et al. (1987) from Memorial Sloan Kettering Cancer Center (MSKCC) reported their phase 1 experience with the murine IgG3 monoclonal antibody (MoAb) 3F8, specific for the ganglioside GD2. 3F8 was administered intravenously to 17 patients with metastatic GD2 positive neuroblastoma or malignant melanoma at doses of 5, 20, 50, and 100 mg/m^2. Serum 3F8 levels achieved were proportional to the dose of 3F8 infused. However, serum antimouse antibody levels did not increase with the amount of 3F8 administered. Toxicities included pain, hypertension, urticaria, and complement depletion. All acute side effects were controllable with symptomatic therapy. No long-term side effects were detected in patients observed for more than 14 months. None of the 17 patients received any antitumor therapy post-antibody treatment. Antitumor responses occurred in seven of 17 patients. These ranged from complete clinical remissions to mixed responses. MSKCC group later showed the persistence of GD2 expression in refractory or recurrent neuroblastoma patients suggesting that complete antigen loss is an uncommon event after 3F8 treatment (Kramer et al. 2001). Of 62 patients who had refractory or recurrent neuroblastoma following 3F8 treatment, 61 (98%) tested positive for GD2 reactivity by bone marrow immunofluorescence (n=51) or tumor immunohistochemistry (n=10). Yu et al. conducted a phase I trial with ch14.18 in 10 patients with refractory neuroblastoma and one patient with osteosarcoma (Yu et al. 1998). They received 20 courses of ch14.18 at dose levels of 10, 20, 50, 100, and 200 mg/m^2. The most prevalent toxicities were pain, tachycardia, hypertension, fever, and urticaria. Most of these toxicities were dose-dependent and rarely noted at dosages of 20 mg/m^2 and less. Although the maximum-tolerated dose was not reached in this study, clinical responses were observed. These included one partial and four mixed responses and one stable disease among 10 assessable patients. Biologic activity of ch14.18 in vivo was shown by binding of ch14.18 to tumor cells and complement-dependent cytotoxicity of posttreatment sera against tumor target cells. An anti-ch14.18 immune response was detectable in seven of 10 patients studied.

Since the efficacy of the anti-GD2 antibody is dependent on the number of effector cells, investigators were quick to add cytokines to anti-GD2 antibody therapy. MSKCC group reported their results with 3F8 and GM-CSF (Kushner and Cheung 1989). GM-CSF (2–20 ng/mL) increased ADCC by 93–267% at limiting dilutions of 3F8 (1 μg/mL). With most GD2-positive cell lines tested, this effect translated into a tenfold or greater augmentation in 3F8 efficiency at mediating ADCC. Their data suggested that this cytokine may have the potential of amplifying 3F8 antitumor activity in patients by increasing effector cell numbers and by priming granulocytes for greater cytotoxicity. Kushner et al. reported their phase 2 experience of MSKCC with 3F8 and GM-CSF (Kushner et al. 2001). Patients were eligible for 3F8/GM-CSF if intensive therapy had not eradicated potentially lethal neuroblastoma. One cycle consisted of GM-CSF (subcutaneous) on days 1–5, 11, and 12, and GM-CSF (2-h intravenous [IV] infusion) followed after a 1-h interval by 3F8 (1.5-h IV infusion) on days 6–10 and 13–17. GM-CSF was dosed at 250 μg/m^2/day on days 1 through 7 and at 500 μg/m^2/day on days 8 through 17. 3F8 was dosed at 10 mg/m^2/day

(100 mg/m^2/cycle). 3F8 was given with an opiate and an antihistamine. Patients without progressive disease or elevated human antimouse antibody titers were allowed to be treated again beginning 3 weeks after completion of a cycle. Among 19 patients treated for neuroblastoma resistant to induction therapy, 12 of 15 had complete remission (CR) of bone marrow (BM) disease, and three others who had less than partial responses achieved prolonged progression-free survival. Among patients treated for recurrent neuroblastoma resistant to retrieval therapy, five of 10 had CR in BM. The 15 patients treated for progressive disease fared poorly, although two had scintigraphic findings suggestive of a short-term response. Side effects were limited to readily manageable pain and, less commonly, rash of short duration; hence, patients were treated as outpatients. 3F8/GM-CSF was well tolerated and showed promise for treatment of minimal residual disease in the BM. MSKCC investigators recently showed that GM-CSF–induced granulocyte activation in vivo is associated with improved patient outcome (Cheung et al. 2012). Patients with neuroblastoma received multiple treatment cycles, each consisting of 3F8 plus subcutaneous (SC) GM-CSF. Peripheral-blood (PB) samples from 151 patients were collected on day 0 and day 4 of cycle 1. PB from a subgroup of 35 patients had intravenous (IV) instead of SC GM-CSF during cycle 4. Samples were analyzed by flow cytometry for CD11a, CD63, CD87, and CD11b and its activation epitope CBRM1/5. Comparing cycle 1 day 4 PB samples with day 0 PB samples, five of five activation marker–positive granulocytes were significantly higher. The change in frequency and mean fluorescence intensity of CBRM1/5-positive granulocytes correlated with progression-free survival (PFS; $p=0.024$ and $p=0.008$, respectively). A multivariable analysis identified increasing CBRM1/5-positive granulocytes and missing killer immunoglobulin-like receptor ligand as positive independent prognostic factors for PFS, whereas second-line cyclophosphamide-based therapy before protocol entry negatively influenced outcome. Thirty-five patients who received SC GM-CSF at cycle 1 and IV GM-CSF at cycle 4 had significantly less CBRM1/5 activation after IV GM-CSF. In contrast, 63 patients who received SC GM-CSF at both cycles had comparable CBRM1/5 activation. This activation was more apparent when GM-CSF was given by the SC route instead of IV route.

Hank et al. (1990) showed that ADCC can be augmented in cancer patients following in vivo IL-2 therapy. In these studies, mAbs with relative tumor specificity were used to target immunologically activated effector cells in an in vitro ADCC assay. The mAbs included 3F8 and 14.G2a, which are both specific for neuroblastoma and melanoma and recognize ganglioside GD2, and mAB ING-1, a mouse-human chimeric antibody with constant regions from human IgG1 and kappa chains and variable regions from a mouse mAb that binds to a broad range of human adenocarcinomas. Each of these mAbs was able to mediate ADCC with fresh effector cells and antibody binding targets. When peripheral blood mononuclear cells were obtained from cancer patients prior to and following in vivo therapy with IL-2, a significant increase was noted in ADCC activity by peripheral blood mononuclear cells obtained following IL-2 therapy. Inclusion of IL-2 in the medium during the cytotoxic assay with mAbs further boosted ADCC. The total activity seen was often greater than the sum of the independent lymphokine activated killer (LAK) activity and standard ADCC activity.

Frost et al. (1997) reported on the CCG experience using murine monoclonal antibody, 14.G2a with IL-2. The hypothesis was that 14.G2a which recognizes GD2 and facilitates ADCC, when administered with IL-2 can increase ADCC by enhancing the activity and number of circulating lymphocytes. Thirty-three pediatric patients with GD2 positive malignancies, ranging in age from 2 to 17 years (median, 9.9 years), received IL-2 and 14.G2a in this Phase I/IB study and were monitored for toxicities and response to therapy. Seven of these patients also received GM-CSF. The maximum tolerated dose (MTD) of 14.G2a with IL-2 was 15 mg/m^2/day. The most prevalent Grade 3–4 toxicities were generalized pain (42%) and fever without documented infection (52%). IL-2 was thought to be the causative agent

in most cases of fever. Toxicities attributed to 14.G2a included pain, allergic or anaphylactic reactions, and rash. Human antimouse antibodies were demonstrated in 9 of 21 evaluated patients. One patient with neuroblastoma had a partial response, and one patient with osteosarcoma had a complete response. Immunocytology demonstrated that the number of neuroblastoma cells in bone marrow decreased in three patients.

All of these results have set the stage for clinical trials by combining in vivo treatment with IL-2, GM-CSF and anti-GD2 antibodies. In order to minimize the risk of human anti-murine (HAMA) or human anti-chimeric (HACA) antibody development while maximizing the anti-neuroblastoma effect in minimal residual tumor setting, investigators have chosen post-ASCT setting to assess the efficacy of immunocytokines. Ozkaynak et al. (2000) conducted a phase I study of ch14.18 with GM-CSF in children with neuroblastoma immediately after ASCT. In this CCG study, patients became eligible when the total absolute phagocyte count was greater than 1,000/μL after ASCT. Ch14.18 was infused intravenously over 5 h daily for four consecutive days. Patients received GM-CSF 250 μg/m^2/day starting at least 3 days before ch14.18 and continued for 3 days after the completion of ch14.18. The ch14.18 dose levels were 20, 30, 40, and 50 mg/m^2/day. GM-CSF IV as a 2-h infusion was continued during the days of ch14.18 infusion and for 3 days afterward. The same dose of GM-CSF could be administered as a subcutaneous injection on an outpatient basis. In the absence of progressive disease, patients were allowed to receive up to six 4-day courses of ch14.18 therapy with GM-CSF. Nineteen patients with neuroblastoma were treated. A total of 79 courses were administered. No toxic deaths occurred. The main toxicities were severe neuropathic pain, fever, nausea/vomiting, urticaria, hypotension, mild to moderate capillary leak syndrome, and neurotoxicity. Three dose-limiting toxicities were observed among six patients at 50 mg/m^2/day: intractable neuropathic pain, grade 3 recurrent urticaria, and grade 4 vomiting. Human antichimeric antibody (HACA) developed in 28% of patients. The MTD was 40 mg/m^2/day for 4 days when given in this schedule with GM-CSF. Gilman et al. (2009) then went on to add IL-2 to the ch14.18 and GM-CSF combination. In this phase 1 COG study, patients were given ch14.18 for 4 days at 28-day intervals. They received IL-2 during the second and fourth courses of ch14.18 and GM-CSF during the first, third, and fifth courses. The MTD was determined based on toxicities occurring with the second course. After the determination of the MTD, additional patients were treated to confirm the MTD and to clarify appropriate supportive care. Twenty-five patients were enrolled. The MTD of ch14.18 was determined to be 25 mg/m^2/day for 4 days given concurrently with 4.5×10^6 U/m^2/day of IL-2 for 4 days. IL-2 was also given at a dose of 3×10^6 U/m^2/day for 4 days starting 1 week before ch14.18. Two patients experienced dose-limiting toxicity due to ch14.18 and IL-2. Common toxicities included pain, fever, nausea, emesis, diarrhea, urticaria, mild elevation of hepatic transaminases, capillary leak syndrome, and hypotension. No death attributable to toxicity of therapy occurred. No additional toxicity was seen when cis-retinoic acid (cis-RA) was given between courses of ch14.18. No patient treated at the MTD developed HACA. It was concluded that ch14.18 in combination with IL-2 was tolerable in the early post-ASCT period and cis-RA can be administered safely between courses of ch14.18 and cytokines. Although this was a phase 1 trial, the observed 3-year overall survival of 78% was consistent with a benefit attributable to immunotherapy compared to historical controls, a benefit which was later confirmed with a randomized trial as described below.

The hu14.18-IL2 fusion protein consists of the humanized 14.18 anti-disialoganglioside (GD2) monoclonal antibody genetically linked to two molecules of human recombinant IL-2 (Reisfeld and Gillies 1996). The monoclonal antibody component of hu14.18-IL2 recognizes and binds to the GD2 disialoganglioside that is highly expressed on neuroblastoma. The IL2 component binds to and activates both NK and T cells via their IL2 receptors; while the Fc end remains intact and triggers ADCC. The hu14.18-IL2 fusion protein was tested in the phase I pediatric

setting within COG. Twenty-eight subjects were enrolled (27 with recurrent/refractory neuroblastoma and 1 with melanoma) (Osenga et al. 2006). An MTD of 12 mg/m^2 was established. The dose limiting toxicities included hypotension and allergic reactions which were predicted based on the known toxicities of the GD2 monoclonal antibody and IL2. Subsequent COG phase II study assessed the antitumor activity of hu14.18-IL2 in two strata of patients with recurrent or refractory neuroblastoma (Shusterman et al. 2010) Hu14.18-IL2 was given intravenously (12 mg/m^2/daily) for 3 days every 4 weeks for patients with disease measurable by standard radiographic criteria (stratum 1) and for patients with disease evaluable only by [(123)I]metaiodobenzylguanidine (MIBG) scintigraphy and/or bone marrow (BM) histology (stratum 2). Thirty-nine patients were enrolled (36 evaluable). No responses were seen in stratum 1 (n = 13). Of 23 evaluable patients in stratum 2, five patients (21.7%) responded; all had a complete response (CR) of 9, 13, 20, 30, and 35+ months duration. Grade 3 and 4 nonhematologic toxicities included capillary leak, hypoxia, pain, rash, allergic reaction, elevated transaminases, and hyperbilirubinemia. Two patients required dopamine for hypotension, and one patient required ventilatory support for hypoxia. Most toxicities were reversible within a few days of completing a treatment course. In this study, these patients were genotyped for KIR, HLA, and FcR alleles to determine whether KIR receptor-ligand mismatch or specific FcγR alleles were associated with antitumor response (Delgado et al. 2010). DNA samples were available for 38 of 39 patients enrolled: 24 were found to have autologous KIR/KIR-ligand mismatch; 14 were matched. Of the 24 mismatched patients, 7 experienced either complete response or improvement of their disease after hu14.18-IL2 therapy. There was no response or comparable improvement of disease in patients who were matched. Thus KIR/KIR-ligand mismatch was associated with response/improvement to hu14.18-IL2 ($p = 0.03$). There was a trend toward patients with the FcγR2A 131-H/H genotype showing a higher response rate than other FcγR2A genotypes ($p = 0.06$). These analyses indicate that response or improvement of relapsed/refractory NBL patients after hu14.18-IL2 treatment is associated with autologous KIR/KIR-ligand mismatch, consistent with a role for natural killer cells in this clinical response. Based on these phase 1 and 2 study results, hu14.18-IL2 is further being tested in children with nonbulky high-risk neuroblastoma.

Phase 3 Studies with Anti-GD2 Antibodies

These phase 1/2 studies paved the way for phase 3 trials. MSKCC group reported their experience with 3F8 alone (Cheung et al. 1998). Thirty-four patients were treated with 3F8 at the end of chemotherapy. Most had either bone marrow (n = 31) or distant bony metastases (n = 29). Thirteen patients were treated at second or subsequent remission (group I) and 12 patients in this group had a history of progressive/persistent disease after ASCT; 21 patients were treated in first remission following N6 chemotherapy (group II). Before 3F8 treatment, 23 patients were in complete remission, eight in very good partial remission, one in partial remission, and two had microscopic foci in marrow. Twenty-five had evidence of neuroblastoma by at least one measurement of occult/minimal tumor (iodine 131-3F8 imaging, marrow immunocytology, or marrow reverse-transcriptase polymerase chain reaction [RT-PCR]). Acute self-limited toxicities of 3F8 treatment were severe pain, fever, urticaria, and reversible decreases in blood counts and serum complement levels. There was evidence of response by immunocytology (six of nine), by RT-PCR (seven of 12), and by 131-3F8 scans (six of six). Fourteen patients were alive and 13 (age 1.8–7.4 years at diagnosis) were progression-free (40–130 months from the initiation of 3F8 treatment) without further systemic therapy, none with late neurologic complications. A transient anti-mouse response or the completion of four 3F8 cycles was associated with significantly better survival. They concluded that despite the high-risk nature of stage 4 neuroblastoma, long-term remission without ASCT can be achieved

Table 18.1 Immunotherapy treatment schema: Schedule of overall ch14.18, GM-CSF, IL2 and 13cisRA

Course 1	Course 2	Course 3	Course 4	Course 5	Course 6
Ch14.18	Ch14.18	Ch14.18	Ch14.18	Ch14.18	
GM-CSF	IL-2	GM-CSF	IL-2	GM-CSF	
13cisRA	13cisRA	13cisRA	13cisRA	13cisRA	13cisRA

Ch14.18: 25 mg/m^2 × 4 days, q 4 weeks
Courses 1,3,5: GM-CSF 250 mcg/m^2 × 14 days, starting 3 days before ch14.18
Courses 2,4: IL2 3.0 × 10^6 IU/m^2 × 4 days on week one, IL2 4.5 × 10^6 IU/m^2 × 4 days on week two with ch14.18
13cisRA: 160 mg/m^2 × 14 days

with 3F8 treatment. Lack of a contemporaneous control arm is the weakness of this report.

German investigators reported on their experience with ch14.18 (Simon et al. 2004). Stage 4 patients older than 1 year who completed initial treatment without an event were eligible. Ch14.18 was administered at a dose of 20 mg/m^2/day over 5 days in six cycles every 2 months. Patients who did not receive ch14.18 served as controls. Of 334 assessable patients, 166 received ch14.18, whereas, 99 received a 12-month low-dose maintenance chemotherapy (MT) instead, and 69 had no additional treatment. During 695 ch14.18 cycles, fever (55% of cycles), abnormal c-reactive protein without infection (35%), cough (24%), rash (22%), and pain (16%) were the main side effects. Univariate analysis found similar event-free survival (EFS) for the three groups (3-year EFS, 46.5 ± 4.1%, 44.4 ± 4.9%, 37.1 ± 5.9% for patients treated with antibody ch14.18, MT, and no additional therapy, respectively; log-rank test, $p = 0.314$). For overall survival (OS), ch14.18 treatment (3-year OS, 68.5 ± 3.9%) was superior to MT (3-year OS, 56.6 ± 5.0%) or no additional therapy (3-year OS, 46.8 ± 6.2%; log-rank test, $p = 0.018$). Separate univariate analysis of patients with ASCT revealed no difference between patients with ch14.18 treatment and no additional consolidation. Multivariate analysis failed to demonstrate an advantage of antibody treatment for EFS and OS. Simon et al. concluded that compared with oral maintenance chemotherapy and no consolidation treatment, ch14.18 had no clear impact on the outcome of patients. This report however, suffers from several design flaws. First of all, these were sequential treatment protocols spread over several years. By definition, patients who were treated on the low-dose consolidation protocol did not receive ASCT. However, not all patients assigned to no-consolidation or ch14.18 treatment underwent ASCT. The ASCT assignment was left to the discretion of the treating physician. Patients were not stratified by prognostic factors. Isotretinoin was not administered. Interestingly however, Simon et al. (2011) recently re-analyzed their data with a longer observation time, and found that ch14.18 treatment seemed to have prevented late relapses. The median observation time was 11.11 years. The 9-year event-free survival rates were 41 ± 4%, 31 ± 5%, and 32 ± 6% for ch14.18, maintenance chemotherapy (NB90 MT), and no consolidation, respectively ($p = 0.098$). In contrast to their earlier report, ch14.18 treatment improved the long-term outcome compared to no additional therapy ($p = 0.038$). The overall survival was better in the ch14.18-treated group (9-y-OS 46 ± 4%) compared to NB90 MT (34 ± 5%, $p = 0.026$) and to no consolidation (35 ± 6%, $p = 0.019$). Multivariable Cox regression analysis revealed that ch14.18 consolidation improved outcome compared to no consolidation, however, no difference between NB90 MT and ch14.18-treated patients was found.

The definitive study on the role of immunotherapy in neuroblastoma was conducted by COG (Yu et al. 2010). Patients with high-risk neuroblastoma who had a response to induction therapy and ASCT were randomly assigned, in a 1:1 ratio, to receive standard therapy (six cycles of isotretinoin) or immunotherapy (six cycles of isotretinoin and five concomitant cycles of ch14.18 in combination with alternating GM-CSF and IL-2) (Table 18.1). Eligible patients had high-risk neuroblastoma, defined

Table 18.2 Immunotherapy treatment schema: Treatment schema for courses with GM-CSF (28 days per course)

Day	0	1	2	3	4	5	6	7	8	9	10–13	24
GM-CSF	X	X	X	X	X	X	X	X	X	X	X	Begin
ch14.18				⇑	⇑	⇑	⇑					Course 2&4
13cisRA											⇑	⇑

Table 18.3 Immunotherapy treatment schema: Treatment schema for courses with IL2

Day	0	1	2	3	4–6	7	8	9	10	11–13	14–27	28
IL2	X	X	X	X		X	X	X	X			Begin courses 3&5
Ch14.18						⇑	⇑	⇑	⇑			
13cisRA											⇑	⇑

strictly by the COG and confirmed by means of review of clinical, pathological, and biologic features by the COG Neuroblastoma Biology Study Committee and local institutions, before study enrollment. Other eligibility requirements were an age at diagnosis of under 31 years; completion of induction therapy, ASCT, and radiotherapy; achievement of at least a partial response at the time of evaluation before ASCT; ASCT performed within 9 months after the initiation of induction therapy; enrollment between day 50 and day 100 after the final ASCT; absence of progressive disease; and adequate organ function and a life expectancy of at least 2 months. Patients in the standard-therapy group received oral isotretinoin given at a dose of 160 mg/m^2/day, divided into two daily doses, for 14 consecutive days within each of six consecutive 28-day cycles. Patients received IV ch14.18 at a dose of 25 mg/m^2/day for four consecutive days during each of five consecutive 4-week cycles. During the last 2 weeks in each of the five cycles, they also received oral isotretinoin at a dose of 160 mg/m^2/day; this dose of isotretinoin was also given by itself during the final sixth cycle (Table 18.1). During cycles 1, 3, and 5, SC GM-CSF (Leukine, Berlex) was given daily at a dose of 250 μg/m^2/day for 14 days, starting 3 days before ch14.18 was started (Table 18.2) During cycles 2 and 4, interleukin-2 (Proleukin, Chiron) was given, by means of continuous IV infusion, for 4 days during week 1 at a dose of 3.0×10^6 IU per square meter per day, as well as for 4 days during week 2 at a dose of 4.5×10^6 IU per square meter per day, concurrent with ch14.18 (Table 18.3). Event-free survival and overall survival were compared between the immunotherapy group and the standard-therapy group, on an intent-to-treat basis. A total of 226 eligible patients were randomly assigned to a treatment group. In the immunotherapy group, a total of 52% of patients had pain of grade 3, 4, or 5, and 23% and 25% of patients had capillary leak syndrome and hypersensitivity reactions, respectively. With 61% of the number of expected events observed, the study met the criteria for early stopping owing to efficacy. The median duration of follow-up was 2.1 years. Immunotherapy was superior to standard therapy with regard to rates of event-free survival (66 ± 5% vs. 46 ± 5% at 2 years, $p = 0.01$) (Fig. 18.1a) and overall survival (86 ± 4% vs. 75 ± 5% at 2 years, $p = 0.02$ without adjustment for interim analyses) (Fig. 18.1b).

Patients with biopsy-proven residual disease after ASCT were eligible for enrollment but not for randomization and were nonrandomly assigned to receive immunotherapy. They were excluded from the primary efficacy analysis. Twenty-five patients were nonrandomly assigned to undergo the immunotherapy regimen because of biopsy-proven residual disease after ASCT. The 2-year estimates for event-free survival and overall survival were 36 ± 10% (16

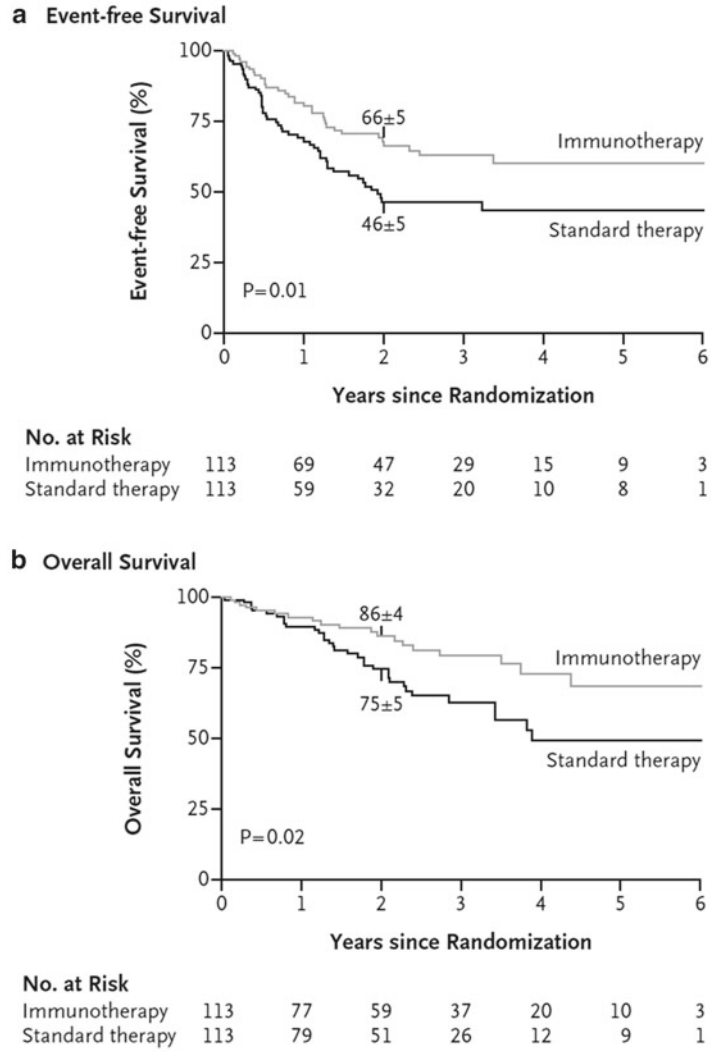

Fig. 18.1 Kaplan–Meier curves by treatment group

events) and 76 ± 9% (10 deaths, all disease-related), respectively. The median duration of follow-up among the patients who did not have an event was 3.6 years (range, 1.0–6.7). All 25 patients were over 18 months of age at diagnosis, and 23 had INSS stage 4 disease; 6 tumors showed MYCN amplification, 16 had unfavorable histologic features, and 12 were diploid. A total of 21 of the 25 patients had a partial response before ASCT; only 1 of the 25 had undergone two ASCTs (rather than one). These findings have confirmed that immunotherapy has a somewhat limited role in patients with more than minimal residual disease.

It has been noted that systemic anti-GD2 treatment is not effective against CNS disease because of limited central nervous system (CNS) availability from the blood–brain barrier (Svennerholm et al. 1994). Kramer et al. (2010) evaluated the addition of compartmental intrathecal antibody-based radioimmunotherapy (cRIT) in patients with recurrent metastatic CNS neuroblastoma following surgery, craniospinal irradiation, and chemotherapy. Twenty one patients treated for recurrent neuroblastoma metastatic to the CNS, received a cRIT-containing salvage regimen incorporating intrathecal (131)I-monoclonal antibodies targeting GD2 or B7H3 following surgery

and radiation. Most patients also received outpatient craniospinal irradiation, 3F8/GMCSF immunotherapy, 13-cis-retinoic acid and oral temozolomide for systemic control. Seventeen of 21 cRIT-salvage patients were alive 7–74 months (median 33 months) since the CNS relapse, with all 17 remaining free of CNS neuroblastoma. One patient died of infection at 22 months with no evidence of disease at autopsy, and one of lung and bone marrow metastases at 15 months, and one of progressive bone marrow disease at 30 months. The cRIT-salvage regimen was well tolerated, notable for myelosuppression minimized by stem cell support (n = 5), and biochemical hypothyroidism (n = 5). One patient with a 7-year history of metastatic neuroblastoma is in remission from MLL-associated secondary leukemia. This is a significant improvement compared to published results with non-cRIT based approaches where relapsed CNS neuroblastoma has a median time to death of approximately 6 months.

The side-effect profile of immunotherapy is quite different than of chemotherapy regimens. Most of the side-effects of immunotherapy are acute i.e., neuropathic pain starting in a matter of minutes after the initiation of ch14.18 infusion, and they can be life-threatening e.g. anaphylaxis, broncospasm. Because severe neuropathic pain is relatively common, it is recommended that a continuous infusion of IV morphine be initiated with the start of ch14.18 administration. The serious side-effects, such as capillary leak syndrome has been more frequently observed with the IL-2 rather than the GM-CSF courses. Because of the acute onset of relatively serious side-effects, the administration of immunotherapy requires very close observation and at times ICU care. However, almost all of the side-effects are transient and somewhat short-lived. Severe neuropathic pain improves in matter of hours to days after the end of the last dose of ch14.18. Infrequently, diplopia has been observed which may not resolve for weeks to months. Within the framework of the German Collaborative Neuroblastoma Study NB97, Kremens et al. (2002) reported on 85 children with high-risk neuroblastoma who received ch14.18. Symptoms of a parasympathetic deficit corresponding to internal ophthalmoplegia, i.e. mydriasis and accommodation deficit, were found in 10 patients. They were uni- or bilateral, began after the termination of ch14.18 infusion and improved or disappeared in all surviving children. They did not reappear or worsen upon repeated ch14.18 infusions. It is concluded that during the treatment with ch14.18, reversible symptoms of parasympathetic denervation of the eye may occur, however, this does not warrant termination of the treatment. The toxicities seen with this immunotherapy regimen were expected and were primarily attributable to antibody binding to GD2 expressed on normal nerve cells, to cytokine-mediated capillary leak, or to hypersensitivity reactions associated with antibody and/or cytokines. These toxicities may also reflect the proposed mechanism of action of this combination; namely effector functions induced by the mAb, including complement activation, and distinct pathways of ADCC mediated by NK cells, neutrophils and monocytes. Upon the request of FDA, in preparation for Biologic License Application for ch14.18, COG is currently conducting another study on 100 patients with high-risk neuroblastoma undergoing immunotherapy identical to the COG ANBL0032 protocol as described above, and data on all types of toxicities –grade 1 through 5- are being collected.

While laboratory analyses are not yet complete, preliminary data for COG ANBL0032 study suggest that there is less of a HACA response in patients within 100 days of ASCT than have seen in prior trials of ch14.18 when ch14.18 was given in the relapse setting. To date, of 111 patients that received the ANBL0032 immunotherapy regimen (for which we have serum specimens from at least two time points), only six patients have shown any detectible HACA response (OD > 0.3), and only three have shown a strong HACA response (OD > 0.7). This is most likely from the substantial suppression of the adaptive immune response from the induction and consolidation treatment administered shortly before the initiation of ch14.18 regimen in the ANBL0032 population.

In conclusion, this is a new era in pediatric oncology. An immunotherapy approach has been shown to be efficacious in high-risk neuroblastoma

patients with minimal residual disease. The standard of care of high-risk neuroblastoma has changed. However, despite this progress, one-third of the high-risk neuroblastoma patients still succumb to their disease despite the use of chemotherapy, surgery, ASCT, radiotherapy and immunotherapy with isotretinoin. Whether the use of hu14.18-IL2 and/or new cytokines such as IL-15 or other effector cell engineering techniques will improve upon the current immunotherapy results remain to be seen.

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Autologous and Allogeneic Hematopoieitic Cell Transplantation: Risk of Second Malignancies

19

Bryan Trottier and Linda J. Burns

Contents

Abstract

With improved measures for supportive care and an increasing number of hematopoietic cell transplants being performed for both malignant and non-malignant disorders, many patients are surviving for longer periods of time following transplantation. Unfortunately, second malignancies in survivors are well-described complications that often carry a very poor prognosis and thus are devastating for patients and their families. The magnitude of risk ranges from 4- to 11-fold that of the general population. The types of second malignancies, current understanding of their pathogenesis, prognosis and available therapies are detailed here.

Introduction

The field of hematopoietic cell transplantation (HCT) continues to evolve, offering potentially curative therapy for individuals with fatal hematologic conditions. Through advances in treatment regimens and supportive care, the number of long-term HCT survivors, as well as the median duration of survival, can be expected to grow over the coming years. For individuals who survive transplant-related and disease-related mortality early on, increasingly recognized late complications await. Of these late complications, second malignancies following HCT unfortunately portend poor prognoses and treatment options carry significant risk-benefit ratios for the patient.

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Second malignancies following HCT fall into three categories: (1) post-transplant lymphoproliferative disease (PTLD), (2) therapy-associated myelodysplastic syndrome/acute myeloid leukemia (t-MDS/AML) and (3) solid cancers. Each subset demonstrates unique latency periods in relation to time since transplant. To this point, the vast majority cases of PTLD occur within the first year after transplant (Landgren et al. 2009) while t-MDS/AML predictably follow a 2–5 year latency period (Pedersen-Bjergaard et al. 2009). Unfortunately, the most recent long-term follow-up of HCT patients demonstrates an ever-growing incidence of solid malignancies occurring with time since transplant when compared to the general population (Rizzo et al. 2009).

Post-Transplant Lymphoproliferative Disease (PTLD)

PTLD represents a spectrum of lymphoid malignancies, largely Epstein-Barr virus (EBV) driven, occurring as a result of chronic immunosuppression in allogeneic-HCT patients. Following primary infection, EBV infects host B-lymphocytes using altered viral antigen production to evade cytotoxic T-cell mediated recognition and destruction (Cohen 2000); the end-result is latent infection similar to other members of the herpes virus family. In the post-HCT setting, a number of factors impair cytotoxic T-cell surveillance, ultimately allowing for viral reactivation. By definition, the very purpose of allogeneic HCT is to destroy and replace the recipient's immune system; any acquired immunity to control latent viral infections is sacrificed during the transplant process. For this reason, PTLD is seen exclusively in the allogeneic population of transplant recipients. Full donor immune reconstitution can take upwards of a year post-transplant. Additionally, to prevent both donor graft rejection and prevent/treat graft versus host disease (GVHD), varying degrees of immunosuppression are necessary, further impairing cytotoxic T-cell function.

During this time of impaired T-lymphocyte function, viral proteins encoded by EBV act as oncogenes within B-cells, leading to unchecked cellular proliferation and ultimately lymphomatous transformation (Cohen 2000). Based upon this line of reasoning, the great majority of observed PTLD cases are expectantly B-cell lymphomas and EBV-mediated; however, a much smaller fraction of T-cell/NK-cell-based lymphomas and EBV negative cases do occur (Curtis et al. 1999).

The World Health Organization (WHO) officially recognizes four forms of histologically-defined PTLD: (1) early lesions characterized by reactive B-cell proliferation, (2) polymorphic PTLD demonstrating nodal architecture effacement, (3) monomorphic PTLD marked by nodal destruction through monoclonal cellular proliferation and (4) Hodgkin lymphoma-type PTLD (Swerdlow et al. 2008). All PTLD forms may be EBV-associated; diffuse large B-cell lymphoma (DLBCL) represents the single most common form of monomorphic PTLD malignancy (Swerdlow et al. 2008).

With a growing understanding of PTLD's pathogenesis, epidemiologic studies focused on incidence and risk factors have been ongoing for the last several decades. Landgren et al. recently published an update on the largest, ongoing retrospective analysis of PTLD following HCT (Landgren et al. 2009). Reviewing 26,901 patients from the Center for International Bone Marrow Transplant Research (CIBMTR) and Fred Hutchinson Cancer Research Center (FHCRC) registries, the overall incidence of PTLD was noted to peak approximately 2–3 months after transplant with a sharp decline seen after 12 months (see Fig. 19.1). Polymorphic PTLD comprised the majority of cases within the first year while cases of monomorphic disease occurred years, even upwards of a decade, later. Reasons for the sharp decline observed after 12 months are hypothesized to correlate with return of cytotoxic T-cell immune reconstitution (Lucas et al. 1998). Late-occurring (greater than 12 months post-HCT) PTLD remains poorly understood, possibly due to a separate pathogenesis and set of risk factors, as many cases are EBV-negative.

Regarding risk factors for the development of PTLD, Landgren et al.'s updated review analyzed

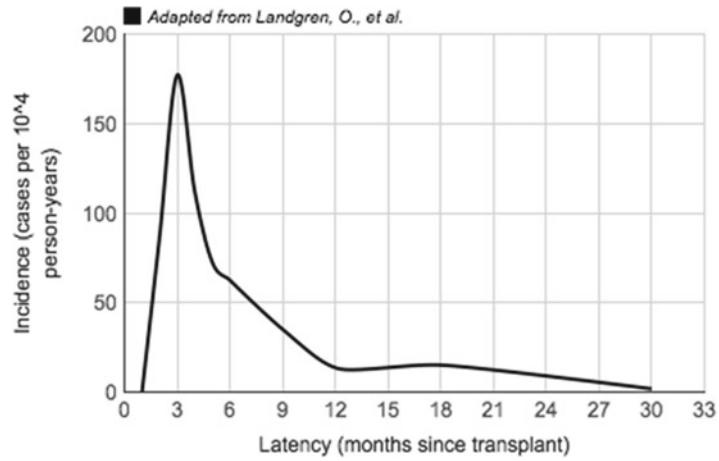

Fig. 19.1 Latency and post-transplant lymphoproliferative disease incidence

previously identified risk factors and identified several new risks as well. Previously identified risk factors included the use of unrelated or human leukocyte antigen (HLA) mismatched graft, T-cell depletion of donor marrow, use of antithymocyte globulin (ATG) or anti-CD3 antibody for prevention or treatment of GVHD as well as acute GVHD grades II through IV (Curtis et al. 1999). Newly identified risk factors included age greater than 50 years and second transplantation (Landgren et al. 2009). The use of ATG and T-cell depleted grafts has consistently represented the strongest risk factors for the development of PTLD (Landgren et al. 2009; Curtis et al. 1999). Pooling these risk factors, individuals with three or more risk factors experienced a 110 relative risk increase for the development of PTLD compared to those patients with no risk factors; the cumulative incidence with three or more risk factors was 8.1% compared to 0.2% for those with no identified risk factors. The use of total body irradiation (TBI) has not been identified as a risk factor.

Taking these identified risk factors into account, many transplant centers perform EBV surveillance using polymerase chain reaction (PCR) testing on high-risk individuals. Van Esser et al. retrospectively analyzed the incidence of EBV reactivation and PTLD development in 85 EBV-seropositive patients undergoing T-cell depleted allogeneic HCT and 65 EBV-seropositive patients receiving an unmanipulated allogeneic HCT (van Esser et al. 2001). The incidence of EBV reactivation, defined as >50 copies EBV genome/mL, occurred in 65% of patients receiving a T-cell depleted graft compared with 31% receiving an un-manipulated HCT. While the overall positive predictive value of detectable EBV reactivation was low at 39%, increasing levels of EBV copy correlated with increasing positive predictive values. Yet despite surveillance, EBV viremia does not confirm the diagnosis of PTLD and several cautions must be addressed. First and maybe most importantly, EBV reactivations occur without ensuing PTLD. Additionally, EBV quantification suffers a large degree of inter-laboratory variability making standardization difficult; therefore, Gulley and Tang (2010) recommend monitoring EBV kinetics measured as doubling time to further best identify patients with imminent EBV-driven PTLD.

Guided by risk factors and EBV surveillance, providers additionally need to be able to gauge the level of clinical suspicion based on presentation. Several features of PTLD separate it from other forms of non-Hodgkin lymphoma (NHL) seen in the general population. PTLD presents in an aggressive fashion, often with advanced stage and extra-nodal manifestations at the time of diagnosis. Presentations vary from asymptomatic to constitutional complaints to lymphadenopathy and organ involvement/dysfunction. Similar to NHL and other malignancies, the role of positron emission tomography/computed tomography (PET/CT) is evolving, but small case series point out its ability to better detect

organ involvement for staging as well as response to therapy (Bianchi et al. 2008).

Treatment options ultimately concentrate on restoring balance between latently infected B-lymphocytes and impaired cytotoxic T-cell function. Attempts to provide return of cytotoxic T-cell function focus on four broad areas: (1) reduction of immunosuppression, (2) antiviral therapy, (3) donor lymphocyte infusions and (4) EBV-specific cytotoxic T-cell infusions. Unlike recipients of solid organ transplantation, HCT patients do not appear to benefit from reductions in immunosuppression. Post-HCT, patients already suffer from impaired immune function and reductions in immune suppression come with the risk of graft rejection and/or GVHD. Additionally, antiviral therapies have not proven largely beneficial as the targets are oncogenic B-lymphocytes. In a small case series of five patients, donor lymphocyte infusions (DLI) ultimately resulted in fatal, immune-mediated respiratory failure in two patients and GVHD within the remaining survivors (Papadopoulos et al. 1994). In an attempt to reduce the risk of GVHD from non-specific donor lymphocytes, Heslop (2009) engineered EBV-specific cytotoxic T-cells for short-term restoration of immune surveillance. Used in both high-risk preventative as well as treatment settings, 80% remission rates with treatment have been reported.

With limited, risky options aimed at returning cytotoxic T-cell function, other treatments options focus on reducing the burden of disease within B-lymphocytes. Using EBV PCR as PTLD surveillance, Van Esser compared the use of single agent rituximab in a prospective cohort series involving 17 patients (van Esser et al. 2002). Using an EBV viral load of 1,000 copies as indication of developing PTLD and an indication to treat, when compared with a historical cohort series, the use of rituximab demonstrated a reduction in PTLD incidence from 49 ± 11% to 18 ± 9% with no mortality seen in the treated group compared with 26 ± 10% in the historical group. For patients who fail to respond to single-agent rituximab, infusional cyclophosphamide, doxorubicin, vincristine and prednisone (CHOP) chemotherapy remains the standard of care. A paucity of comparative data regarding response rates and outcomes exists regarding HCT-related PTLD. When used as salvage therapy following immunosuppression reduction and rituximab, CHOP salvage chemotherapy induced a complete remission (CR) in 5 and a partial remission in 2 of 11 treated patients within a small case series (Trappe et al. 2007). Four of five complete responders remained in CR at a median duration follow-up of 44.2 months while non-responders (stable and progressive disease) died at the time of study close from PTLD-related complications.

Poor prognostic factors for PTLD include advanced stage at the time of diagnosis, central nervous system involvement, late-occurring PTLD and EBV-negative PTLD. Diminished drug delivery beyond the blood brain barrier has hampered efforts to improve central nervous system (CNS)-PTLD. Whole brain radiation as well as intrathecal agents such as rituximab and methotrexate represent potential treatment options. With regards to late-occurring PTLD and EBV-negative PTLD, an entirely separate pathogenesis may be at play; current therapies may be ineffective due to tumor biology or underlying reasons as yet to be determined.

Therapy-Associated Myelodysplastic Syndrome/Acute Myeloid Leukemia (t-MDS/AML)

Following the initial reports of t-MDS/AML post autologous HCT in 1994, subsequent studies reported actuarial or cumulative incidences ranging from 1% to 24% with a wide range of follow-up (Pedersen-Bjergaard et al. 2009). Furthermore, there is concern that the incidence may rise due to the increasing age that patients undergo transplantation with the advent of nonmyeloablative and reduced-intensity conditioning regimen. t-MDS/AML represents a major cause of nonrelapse mortality post-autologous HCT, particularly for patients with lymphoma as compared with other diseases including multiple myeloma or germ cell tumors (Hake et al. 2007; Barlogie et al. 2008; Kollmannsberger et al. 1999).

Physicians must not assume that MDS/AML diagnosed post allogeneic transplantation is secondary to relapse of the patient's pre-transplant disease. Although more commonly seen following autologous HCT, t-MDS/AML has also been reported following related-donor and unrelated donor HCT. Baker and colleagues reported that 2 of 1,407 patients developed t-MDS/AML after related-donor and 2 of 772 patients after unrelated donor HCT in recipient cells (Baker et al. 2003). These newly developed diseases had morphologically and cytogenetically distinct abnormalities compared with the patients' underlying AML or chronic myeloid leukemia, inconsistent with disease relapse. Furthermore, leukemia may also arise following allogeneic transplantation in donor cells, so-called "donor cell leukemia (DCL)." First recognized in 1971, DCL was initially felt to be rare but additional reports suggest the incidence may be as high as 5% of all post-transplant "relapses" (Wiseman 2011).

Patients with t-MDS/AML post-transplant typically present with peripheral blood cytopenias and multilineage dysplasia on bone marrow examination. Criteria for diagnosing t-MDS/AML include: (1) significant marrow dysplasia in at least two cell lines, (2) peripheral cytopenias without alternative explanations, and (3) blasts in the marrow (Gilliland and Gribben 2002). Historically, two classes of chemotherapy agents have been associated with t-MDS/AML, topoisomerase II inhibitors and alkylators. Topoisomerase II inhibitor-associated t-MDS/AML typically has a short latency period of 2–3 years with chromosome gene rearrangements involving the *MLL* gene at 11q23. In contrast, alkylating agent-associated t-MDS/AML has a longer latency of 5–8 years and is associated with abnormalities of chromosomes 5 and/or 7 (del 5q, del 7q).

There has long been discussion among physicians caring for patients as to whether post-HCT t-MDS/AML is secondary to therapy received prior to transplant or if the transplant itself is to blame. Most likely, it is a combination of factors; thus, numerous studies have attempted to identify specific risk factors. A number have been reported, including increased age, therapy with increasing cumulative doses of alkylators and topoisomerase II inhibitors, TBI-based conditioning regimens, peripheral blood stem cells as the graft source, stem cell mobilization with etoposide, four or more chemotherapy regimens prior to transplant, difficult mobilization of adequate numbers of stem cells, and delayed platelet and neutrophil engraftment (Kalaycio et al. 2006). More recently, fludarabine, particularly at a cumulative dose of >150 mg/m^2, has been associated with t-MDS/AML (Carney et al. 2010; Waterman et al. 2012). It is unclear which factors are most important as the patient population, heterogeneous underlying diseases and conditioning regimens, as well as the strength of each factor's association varies among studies.

In addition to evidence that pre-transplant and transplant therapies (including priming chemotherapy and the conditioning regimen) contribute to post-transplant t-MDS/AML, there is evidence that the engraftment process itself may also contribute. It has been hypothesized that the proliferative stress of engraftment may lead to the expansion of pre-leukemic clones from stem cells with genotoxic damage secondary to exposure from prior therapies. Along these lines, shortened telomere length, a measure of proliferative stress, has been reported in patients who developed t-AML/MDS (Chakraborty et al. 2009).

Finally, in both de novo and therapy-associated AML, a variety of inherited polymorphisms and acquired mutations have been identified that may play a role in t-MDS/AML susceptibility (Rund et al. 2005). These include cell signaling genes and transcription factors in the "leukemia pathway" as well as genes involved in drug metabolism and DNA repair. Whether any or all of these genes contribute to t-MDS/AML arising in the post-transplant is under investigation.

The prognosis of t-MDS/AML following transplantation is very poor with a median survival of 6–12 months. In a report from the Dana Farber Cancer Institute, 13 of 41 patients who developed MDS following autologous HCT for non-Hodgkin lymphoma underwent allogeneic HCT and all died of transplant-related complications (11 patients) or relapse (2 patients) with a median survival of only 1.8 months (Friedberg et al. 1999). Clearly,

strategies to decrease the risk of t-MDS/AML post transplant need to be implemented. Interventions should include avoiding leukemogenic agents, including radiation, whenever possible during therapy, performing transplants earlier in the disease course when warranted to avoid excess therapy and consideration of alternative approaches other than autologous HCT for those patients with difficulty mobilizing stem cells. As genetic polymorphisms that confer a risk of t-MDS/AML are identified and confirmed in the future, an individualized approach to patients at high risk may be possible.

Solid Tumors

In comparison to PTLD and t-MDS/AML, secondary solid cancers demonstrate a latency period of approximately 3–5 years followed, more importantly, by a steadily increasing risk associated with time since transplant (see Table 19.1). Because the post-HCT cohort is so heterogeneous with respect to age, most investigators have utilized standardized incidence ratios (SIR) focusing on observed to expected ratios when compared to the general population. Expected cases for the general population can be approximated using the Surveillance Epidemiology and End Results (SEER) database for age-appropriate incidences of cancer.

Risk factors for the development of a second solid malignancy across the majority of studies focus on age at transplantation and use of radiation for conditioning. Children are at particularly high risk from radiation-induced damage for reasons not entirely clear, but multiple long-term duties involving Hodgkin lymphoma and other hematologic malignancies confirm this association (Dores et al. 2002). Rizzo et al. (2009) published results from the largest, retrospective patient cohort from the combined CIBMTR-FHCRC database. Within this cohort of 28,874 patients, young patients (less than 10 years of age) and the use of TBI were associated with an excess absolute risk (EAR) of 43.2 and 76.40 after 1 and 5 years post-transplant time, respectively. A trend in decreasing EAR with increasing age at time of transplantation was noted in association with the use of TBI. Specific cancers associated with the use of TBI included breast, thyroid, brain and CNS, bone and melanoma. The presence of chronic GVHD was associated with the development of squamous cell carcinomas (SCC). Across multiple studies, younger age at the time of transplantation as well as the use of TBI has been associated with the development of second malignancies (Curtis et al. 1997; Baker et al. 2003; Bhatia et al. 2001). Regarding timing and onset, comparable cumulative incidences of 2.5%, 5.8% and 8.8% have been observed at 10, 15 and 20 years following HCT, respectively (Rizzo et al. 2009).

Reviewing specific examples of post-HCT solid malignancies, the European Group for Blood and Marrow Transplantation (EBMT) Late Effects Working Group retrospectively reviewed 68,936 patients who had undergone allogeneic and autologous transplantation (Cohen et al. 2007). Thirty-two cases of thyroid cancer (23 papillary and 9 follicular) were identified. In comparison with the European population, the SIR for transplant patients was 3.26 (95% CI 2.23–4.60) for the development of thyroid cancer. Similar to other second malignancies, age at time of transplant (0–10 years) was identified as the strongest risk factor in multivariate analysis when compared to older patients (>20 years). So strong was younger age as a risk factor that the relative risk, 24.61 (95% CI 4.45–136.25), was greater than the sum of other identified risk factors: use of conditioning irradiation (RR 3.44, 95% CI 1.41–8.37), female sex (2.79, 95% CI 1.34–5.79) and chronic GVHD (RR 2.94, 95% CI 1.21–7.15). Considered a cancer of older patients in the general population, thyroid cancers in the HCT population occurred at a median age of 23.5 years and a median latent period of 8.5 years following transplant.

Female HCT survivors not only are at increased risk of thyroid cancer compared to the general population, but they are also at increased risk of breast cancer. Friedman et al. (2008) analyzed a combined cohort comprised of FHCRC and EBMT patients who had undergone allogeneic transplantation and observed 52 cases of breast cancer among 3,337 patients surviving

Table 19.1 Second solid tumors and demographics, incidences and risk factors

Cancer	Median age	Median time from transplant	5-year incidence	10-year incidence	15-year incidence	20-year incidence	25-year incidence	Risk factors	Reference
All	NS	NS	NS	2.5% (95% CI: 2.0–3.0)	5.8% (95% CI: 4.3–7.0)	8.8% (95% CI: 6.2–12.3)	NS	Younger age at transplant, TBI	Rizzo, J.D., et al.
Thyroid	23.5 years (range: 8.8–52.2)	8.5 years range: 0.6–18.5)	NS	NS	NS	NS	NS	Younger age at transplant, TBI, female sex, chronic GVHD	Cohen, A., et al.
Breast	47.5 years (range: 25.5–65.8)	12.5 years (range: 5.7–24.8)	NS	0.8% (95% CI: 0.5–1.2)	NS	4.6% (95% CI: 3.1–6.7)	10.8% (95% CI: 6.8–15.8)	Time from transplant, younger age at transplant, TBI	Friedman, D.L., et al.
BCC	47.9 years (range: 12.6–72.3)	7.9 years (range: 0.5–30.2)	1.4% (95% CI: 1.0–1.7)	2.5% (95% CI: 2.0–3.0)	4.0% (95% CI: 3.3–4.8)	6.5% (95% CI: 5.3–7.7)	8.4% (95% CI: 6.8–10.2)	Time from transplant, younger age at transplant, TBI, white race, chronic GVHD	Leisenring, W., et al.
SCC skin and mucosa	48.9 years (range: 17.4–72.1)	6.3 years (range: 0.3–24.8)	1.0% (95% CI: 0.7–1.3)	1.4% (95% CI: 1.1–1.9)	2.2% (95% CI: 1.7–2.8)	3.4% (95% CI: 2.6–4.3)	5.5% (95% CI: 4.1–7.3)	Time from transplant, younger age at transplant, acute GVHD, chronic GVHD	Leisenring, W., et al.

BCC basal cell carcinoma, *GVHD* graft versus host disease, *NS* not stated, *SCC* squamous cell carcinoma, *TBI* total body irradiation

greater than 5 years. Compared to the general population, the SIR of observed to expected malignancies was 2.2 (95% CI 1.7–2.9) for female HCT survivors. Breast cancers occurred at an earlier age, with a median age of 47.5 years. Multivariate Cox regression analysis determined that time from transplantation, TBI-containing conditioning regimens and age at time of transplantation were all independent risk factors for the development of breast cancer. Adjusted hazard ratios rose with time since transplantation when compared to the 5–9.9 year follow-up period: 10–14.99 years (HR 2.7, 95% CI 1.2–6.4), 15–19.99 years (HR 5.1, 95% CI 1.9–13.6) and >20 years (HR 10.8, 3.2–36.1). Accordingly, the cumulative incidence of breast cancer in patients surviving greater than 25 years was 11.0% (95% CI 7–16%), rising to 17% (95% CI 9–26%) for patients who had received TBI. But similar to other solid cancer risks, age at time of transplantation was also a highly significant risk factor with an adjusted hazard ratio of 9.5 (95% CI 1.8–51.1) and SIR of 25.0 (95% CI 12.5–50.1) for female patients younger than 18 years at the time of transplantation.

Focusing on skin and mucosal cancers, Leisenring et al. (2006) analyzed risk factors for the development of these post-HCT malignancies from a retrospective cohort study of 4,810 patients in the FHCRC database. One hundred fifty-eight patients developed basal cell carcinomas (BCC) while 95 patients developed SCCs (tongue, tonsil, vocal cord, esophagus, genitourinary tract and skin); 58 patients had one more than one occurrence. Significant risk factors for the development of BCCs were age at transplantation, TBI-containing regimen, white race and chronic GVHD; age at time of transplant and both acute and chronic GVHD were significant risk factors for the development of SCCs.

Prognosis of second solid malignancies has not been well studied with respect to the general population. Factors such as low overall incidence and a heterogeneous population make such comparisons difficult. Reviewing solid malignancies in a cohort of patients from the University of Minnesota, Baker et al. (2003) determined that 42% of patients who developed a solid cancer died from that malignancy, but ultimately mortality from a given cancer likely relates more to the nature of the cancer.

Treatment at the present time does not appear to differ for respective cancers when compared to those developing within the general population. However, as with any effort behind successful screening campaigns, long-term follow-up clinics focusing on the continuing health of HCT survivors have concentrated their attention on early detection. An international collection of hematopoietic transplant experts has recently updated and published the most recent recommendations for screening and preventative practices for HCT-survivors (Majhail et al. 2012). Specifically regarding screening for breast cancer, the panel recommends initiation of mammography at 25 years of age or 8 years after receiving radiation. Protecting the skin against ultraviolet light and frequent oral health examinations, in addition to heightened surveillance for second cancers, rounds out updated screening efforts in addition to a healthy lifestyle.

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Part IV

Neural Stem Cells

Treatment of Injured Spinal Cord: Engraftment of Neural Stem Cells

20

Martin M. Mortazavi, Nimer Adeeb, Aman Deep, and R. Shane Tubbs

Contents

Abstract

Spinal cord injury is one of the main causes of disability in the young population. Based on the associated pathological changes, many modalities of treatments, including stem cells and non-stem cells transplantation have, been trialed with very promising results. The route of delivery and engraftment of these cellular transplants is an important determining factor in the functional outcome, and should be chosen as to be safe and efficacious on human patients. In this chapter we will discuss the pathology of spinal cord injury, the potential cellular therapy, and the routes of delivery.

Introduction

In 1944, an article was published by Woolsey et al. (1944) reporting the first spinal cord transplantation in known history. A 16 years-old male was shot in his right shoulder with the bullet reaching the upper border of the fourth thoracic vertebra leading to complete loss of motor and sensory function below the level of injury. Following laminectomy, the injured spinal cord was completely transected and replaced with a cadaver spinal cord fixed in 10% formaline for 12 days, and cleaned and sterilized with running and distilled water and 70% alcohol. No improvement in the patient's condition was noted, and the patient died almost 4 months after the surgery. Autopsy showed exceptional preservation of the transplanted cord, though with restricted regeneration and

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limited tissue reaction. No explanations or related conclusion on the microscopic findings could be made, although the preservation was related to the preoperative use of formalin.

Spinal Cord Injury

The world wide annual incidence of spinal cord injury (SCI) is 15–40 cases per million. The incidence is ~ 12,000 cases in the United States. Of these, 4,000 die before reaching the hospital and 1,000 during hospitalization (Sahni and Kessler 2010). Most of these injuries occur in otherwise healthy and young patients, and are mainly due to fracture or dislocation of the vertebral column (Profyris et al. 2004). Based on gross findings, SCI can be classified into four groups: (1) solid cord injury; (2) contusion/cavitation; (3) laceration; and (4) massive compression. Contusion is the most common, and the solid injury is the least common type. However, these findings carry no significant differences in the consequent histological changes (Norenberg et al. 2004).

Histological Changes

Extensive studies have been done on different animal models to reproduce and study the effects of various mechanisms of injury on the spinal cord. Based on their findings, the histological changes following SCI can be divided into four main phases: immediate hyperacute, acute, intermediate, and late phases (Norenberg et al. 2004). It can also be divided into primary and secondary injury (Profyris et al. 2004). In the following text, we will discuss the histological changes with focus in the neural cells. Glial and oligodendrocytes changes associated with SCI have been described in the next chapter.

The immediate hyperacute phase is caused by the primary insult of injury, and usually takes place within the first 1–2 h of injury. During this phase, the initial insult, whether it is a contusion, compression, shearing, or stretching of the spinal cord, will lead to disruption of the neural and endothelial tissue. This is associated with hemorrhagic necrosis that is mainly localized in the gray mater and the center of the cord. The localization is due to the high vascularity of the gray mater and the epicentric movement of the injured tissues, which, in turn, places the most damage on the centrally located cells, and the least on the subpial ones. Moreover, at the site of injury, myelinated axons exhibit more pathological injury than unmyelinated ones. This is because the longitudinal force (especially in spinal cord contusion) stretching the fibers is concentrated at the nodes of Ranvier. In many cases, however, no abnormalities are seen following the initial trauma, and most of the consequent changes occur due to secondary injury (Norenberg et al. 2004; Profyris et al. 2004).

Following the first 3 h of injury, the secondary phase begins. This phase can be further divided into acute phase (hours to 3 days), intermediate (days to weeks), and late phase (weeks to months) (Norenberg et al. 2004). However, as most of the processes that occur during the secondary injury are interconnecting, we do not prefer the use of the subdivision.

During the secondary injury, expanding of the hemorrhagic sites appears early, and is related to cellular death which is precipitated by acute necrosis and subacute apoptosis. Inflammatory response is an important determinant in this process. It starts during the first day of injury, and is initiated by the release of the chemical mediators that attract the early inflammatory cells (i.e. neutrophils) to the site of injury. Neutrophils release inflammatory mediators and free radicals that will exacerbate and accelerate the secondary phase of injury (Norenberg et al. 2004). Necrosis starts as a wave that spreads in centripetal and rostro-caudal directions from the site of primary injury. This necrosis occurs via various mechanisms, including, infarction, excitotoxicity, and reperfusion injury (Profyris et al. 2004).

Infarction, which begins during the primary injury phase, occurs early due to disruption of the vascular bed, which, in turn, interrupts the blood perfusion to the neural tissue. Thereafter, inflammatory changes associated with vasospasm, thrombosis, and neurogenic shock play important role during the secondary phase. The resulted

hypoperfusion is associated with inhibited oxidative phosphorylation and the glycolytic pathways, and lead to loss of energy production and consequent necrosis. *Reperfusion* of the neural cells during this stage will exacerbate cellular death. This is due to reactive oxygen species (ROS) formation from the ischemic endothelial cells. This, added to the ROS produced by the inflammatory cells, will cause direct damage and necrosis to the reperfused cells. *Excitotoxicity* is initiated with the accumulation of the glutamate within the extracellular spaces at the sites of injury. This accumulation is mainly due to defected absorption, excessive release from the damages cells, and exocytosis of the glutamate synaptic vesicles. This glutamate will lead to overactivation of the neural depolarization by the activation of the glutamate receptors. This persistent depolarization will create ionic and osmotic imbalance across the plasma membrane that will cause water influx and consequent lyses. It also leads to excessive calcium influx into the cell and the activation of the auto-destructive calcium-dependent enzymes (Profyris et al. 2004).

Apoptosis begins as early as 6 h following injury, and spreads in a wave similar to that in necrosis. During the early phase, almost any cell type can be involved. Later on, the oligodendrocytes and myelinated cells are predominantly involved (Profyris et al. 2004). This programmed cell death occurs due to the secretion of inflammatory mediators and the extravasasion of toxic substances following the injury (Almad et al. 2011). Some authors, however, deny the presence of apoptosis during SCI in humans (Norenberg et al. 2004). The above mentioned processes, although extending through the following phases, comprise the main components of the acute phase of the secondary injury.

Over the ensuing days and weeks, more inflammatory cells will invade the site of injury in order to clear the debris and initiate the process of healing via neural fibrosis or gliosis. This starts with accumulation of the myelin and oligodendrocytes debris followed by activation and migration of microglia and macrophages which phagocyte these debris. At this early stage, the phagocytosis may enhance the regenerative process. Moreover, microglia may contribute, via the secretion of various cytokines including IL-1β, Il-6, and TNFα, to facilitate neural protection and regeneration. However, overtime, progressive maturation of the glial scar, followed by migration and proliferation of the astrocyte, inhibits the regeneration and remyelination of the neuronal cells (Norenberg et al. 2004; Profyris et al. 2004).

At the same time, in an attempt to maintain the viability of the remnant tissue and slow the progression of tissue loss, increased number of blood vessels can be noticed at the site of injury. This is mostly due to combination of tissue loss and preservation of the vascular structure, and the secretion of angiogenic factors in response to the inflammatory process (Norenberg et al. 2004).

Axonal disruption starts as early as few minutes following SCI, as described above. The periaxonal swelling leads to rupture and peeling of the surrounding myelin, which can be observed in the extracellular space 24 h after SCI. This process is accompanied by wallerian degeneration (WD) that last for years, and form the major component of the late secondary injury. WD is mainly characterized by degeneration and disruption of the axonal function and it usually extend in cranial and caudal directions from the initial site of injury (Norenberg et al. 2004; Profyris et al. 2004).

Late changes also include the formation of mesenchymal scar, formed by fibroblast and collagen fibers, and mostly stimulated by the injured glia limitans of the subpial space. Schwannosis, in which the injured spinal tissues are replaced by Schwann cells, is also seen at late stages in many patients with SCI. Other complications, including cysts and syrinx formation may also be seen (Norenberg et al. 2004). In a comparison between contusion, dislocation, and distraction SCI, the degenerative changes were most extensively associated with dislocation injury, and least with distraction injury (Choo et al. 2008).

The functional deterioration after SCI can be classified according to the American Spinal Injury Association (ASIA) into complete (ASIA "A"), where there is no sensory or motor function below the level of injury; incomplete (ASIA "B," "C," or "D"), where sensory with or without

varying degree of motor function are lost below the level of injury; with ASIA "E" being functionally normal (Maynard et al. 1997). It is fundamental to note that it does not automatically infer that functionally complete injuries are anatomically complete, which is uncommon, and it can be explained in terms of tissue sparing. Thus, even small preservation (~ 10–15%) and/or regeneration of the lost fibers may be enough to restore meaningful function, and this can be applied most effectively on individuals with functionally incomplete and some with complete injury (Reier 2004). Thus, beside the degree of injury and functional loss, it is vital to identify the degree of anatomically preserved fibers, and the site and extent of injury.

Cellular Replacement and Stem Cells

Based on the above mentioned pathological changes following injury, many methods of treatment have been applied to slow and reverse the progressive derangements. These include pharmacological and non-pharmacological methods (Mortazavi et al. 2011; Mortazavi et al. 2012). Of these treatment modalities, cellular replacement of the damaged neurons and glial cells and of the supporting tissue is the most promising. That is mainly due to serious restrictions that lie within the other modalities and includes restricted capacity for regeneration and repair of damaged spinal nerve cells and tracts, and the limitation in neural plasticity associated with SCI. These also include the permanent neuronal loss and gap formation that complicate the SCI and the extrinsic inhibition that add on the intrinsic restricted regeneration (Curt 2012).

To achieve this goal, replacement of the lost elements of the SCI has gained most attention for clinical research. The transplanted cells should enable regenerating axons to cross barriers, functionally replace lost cells, and/or create an environment supportive of neural repair (Vawda et al. 2012). These efforts are mostly directed towards white matter injury, which carry the biggest burden of the functional disability. However, regeneration of the gray matter has also an important role in restoring proprioception and muscle coordination (Reier 2004). Cellular and paracellular transplantation for SCI include stem cells and non-stem cells transplants. Non-stem cell transplants include olfactory ensheathing cells, schwann cells, peripheral nerve, and genetically modified fibroblasts.

Methods of Cellular Engraftment

In the past, different routes for cellular transplantation into the injured spinal cord have been trialed. However, the fact that most of the recipients were and still are of animal models with limited trials on humans makes it difficult to compare the safety and effectiveness of these methods, and more studies on this field are still required.

Intramedullary Route

Direct intramedullary engraftment represents a classical mode of cellular transplantation in animal models of SCI. This invasive method involves direct access to the site of injury via laminectomy followed by multiple injections of the transplant into the injury epicenter and/or into the parenchyma adjacent to the injury. This route has been applied on animals using different types of human stem cells, including neural stem cells of various origins (Iwanami et al. 2005; Karimi-Abdolrezaee et al. 2006; Hatami et al. 2009; Fujimoto et al. 2012), oligodendrocytes (Sharp et al. 2010), motor neurons (Erceg et al. 2010), and bone marrow stem cells (Himes et al. 2006). The major disadvantage of this method is its invasiveness and the associated risk of causing further harm and trauma to the injured tissue during surgery, risking additional functional deterioration. This method may also compromise the vulnerability of cells which are transplanted into the hostile environment of the injured spinal cord due to released inflammatory and cytotoxic chemokines (Bakshi et al. 2006). Moreover, multiple injections at different points of time are needed, which is always associated with risk of complications due

to anesthesia or the neurosurgical procedure (Neuhuber and Fischer 2010). During surgery, dura mater is often compromised rendering the patient more susceptible to CSF leakage postoperatively, in addition to other postoperative complications such as deep venous thrombosis and pulmonary complications (Paul et al. 2009). All these factors make this route far from optimal for clinical application on human patients. However, there are some clinical trials which have used this method on humans with different stages of SCI. The outcomes of these studies were very promising and considered superior by the authors as compared to other routes including intrathecal route, and with no remarkable complications (Park et al. 2005, 2012). This is especially true in cases of subacute and chronic SCI, and might be related to the limited migration and time window of recovery associated with the intrathecal engraftment. In light of the above mentioned drawbacks, although still unproven over long term in human clinical trials, less invasive methods were investigated.

Intrathecal Route

Intrathecal cellular transplantation via lumbar puncture (LP) was first introduced by De la Calle et al. (2002). Thence after, this technique was adopted as a minimally method to deliver stem cell transplant into injured spinal cord by Bakshi et al. (2004). These authors, in this and later studies (Lepore et al. 2005; Bakshi et al. 2006) using different types of cells including bone marrow stem cells and neural precursor cells, reported on cellular accumulation in large numbers at the site of injury, mainly at the interface of injury and meninges, following transplantation. Except for few cells in the lining of brain ventricles, no other transplanted cells were noticed in intact neural tissues. This selective homing mechanism is mediated by chemotactic signals expressed at the injury site. These signals include SDF-1α and its CXCR4 receptor that are presented on the transplanted cells. Other factors may include platelet-derived growth factor (PDGF), transforming growth factor alpha (TGFα), insulin growth factor (IGF), and hepatocyte growth factor (HGF). This homing process appears to be more active and effective during early stage of injury, evident by more cellular accumulation occurring at this stage. Although more toxic substances and hostile environment are present at this time, the amount of secreted chemokines involved in attracting cells to the injury is increased in the initial phase. Moreover, the healing process associated with glial scar formation will limit the cellular migration and integration at later points of time. Thus, according to the authors, the window of opportunity of intrathecal deliver is limited to the acute and partly the subacute phase of SCI, and not optimal at the chronic level, unless glial scar debridement was initiated first (Neuhuber and Fischer 2010).

This window of opportunity was proven in clinical trials on human patients where intrathecal engraftment showed minimal functional improvement in patients with subacute and early chronic SCI (< 6 months), but failed to show any improvement in patients with late chronic SCI (>6 months) (Callera and do Nascimento 2006; Saito et al. 2008; Pal et al. 2009; Kishk et al. 2010).

When comparing this mode of cellular transplantation with the direct intramedullary injection on animal models with acute and subacute phases of SCI, functional improvement was more remarkable using the latter, and that was even more noticeable in chronic phase injury. Both methods, however, were neuroprotective, resulting in reduction of injury size and greater tissue sparing, in addition to better functional outcomes compared with controls (Neuhuber et al. 2008).

Although this represents a less effective method so far, it limits patient risk, side effects, and cost and can be used to deliver multiple doses of cells. In regard to its limitation in advanced phases of SCI, it is believed that optimization of the LP procedure in the future by further optimization of cell dosage, timing of delivery, and number of deliveries may improve grafting efficiency and thereby functional recovery to levels comparable to direct injection (Neuhuber and Fischer 2010).

Intravascular Route

The systemic delivery of the transplanted cells via intravascular route (intraarterial or intravenous) represents the least invasive, though the least efficacious, method of engraftment. The multisegmental arterial supply to the spinal cord limits the use of intraarterial delivery, as it requires highly selective and technically challenging cannulation of the spinal arteries (Hatami et al. 2009). On the other hand, intravenous delivery is a safer and easier method to apply. Experimental trials on animal models with SCI using intravenous route has shown promising results (Akiyama et al. 2002) with evidences of cellular migration to the site of injury mediated by HGF and stromal cell-derived factor-1 (SDF-1), which peak at day 7 of injury (Takeuchi et al. 2007). Nevertheless, the undisrupted blood–brain barrier (BBB) still represents a limiting factor in the effectiveness of this route. Additional limiting factors include the first-pass effects and trapping of these cells in extraneural tissues such as lung and liver, along with prolonged exposure to the immune cells during circulation (Bakshi et al. 2004; Paul et al. 2009). Although the number of cells accumulating at the site of injury increases with time and is associated with mild functional improvement, most studies have reported markedly decreased engraftment efficiencies as compared to other routes of delivery, keeping in mind that as time passes, more irreversible neural degeneration is expected (Bakshi et al. 2004; Geffner et al. 2008; Paul et al. 2009). Using this route in human has proven some degrees of functional recovery that was mainly consistent in patients with acute and subacute phase injury, and much less effective in chronic phase. That gives this route the same window of opportunity as the intrathecal one (Sykova et al. 2006; Chernykh et al. 2007).

Intraventricular Route

Stem cells engraftment for SCI through the ventricular system of the brain was once a favored method of cellular replacement (Yandava et al. 1999). However, with the development of more effective and minimally invasive modes of deliver, it has been almost abandoned. This method includes direct injection of the transplant cells into a ventricular cavity, followed by cellular migration and integration at the site of injury in the same homing mechanism as the intrathecal route. Although these two routes have comparable functional outcomes, the latter is much less invasive and more reliable for clinical applications (Wu et al. 2002a, b).

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Spinal Cord Injury Engineering Using Neural Stem Cells

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Contents

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Abstract

Overtime, various modalities of spinal cord injury (SCI) treatment have been trialed. Of these, the most attractive is the cellular transplantation of neural and mesenchymal stem cells. Extensive experimental studies have been done to identify the safety and effectiveness of their transplantation in animal and human models. In this chapter, the different sources, isolation and transplantation of these multipotent stem cells, and associated outcomes will be discussed.

Introduction

Stem cells are non-differentiating cells that have high proliferation, differentiation, and self-renewal potentials. The proliferation and self-renewal potential is achieved via the asymmetric division, in which one of the daughter cells becomes further differentiated while the other maintains the characteristics of the cell of origin. In regard to their differentiation capacity, the stem cells can be divided into, totipotent, pluripotent, multi- or oligopotent, and unipotent stem cells. An example of *totipotent* stem cells is the fertilized egg, and they are capable of generating embryonal and extra-embryonal cells. With further specialization, *pluripotent* stem cells are formed, and they are capable of differentiation into any cell of the three germ line (ectoderm, mesoderm, and endoderm). A good example of these cells is the embryonic stem cells and the

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induced pluripotent stem cells. *Multipotent* stem cells are able to differentiate into different types of cells within each germ line. An example of these is the neural progenitors/stem cells. However, there is evidence that these cells are able to trans-differentiate into other germ lines' cells. However, induced trans-differentiation may result in induced carcinogenesis. *Unipotent* stem cells can differentiate into only one type of cells, and include neuroblast and glioblast, which give rise to neurons or glial cells, respectively (Nandoe Tewarie et al. 2009; Sahni and Kessler 2010; Cherian et al. 2011). Both pluripotent and multipotent cells might be used as a source of transplanted cells. For the purpose of cellular replacement, the more differentiated the transplanted cell is, the less risk of consequent teratoma. Thus, differentiation of the applied stem cells is required before transplantation (Sahni and Kessler 2010).

Neural Progenitors/Stem Cells

In 1928, Spanish neuroanatomist Santiago Ramón y Cajal stated in his book on the degeneration and regeneration of the nervous system that "Once the development was ended, the fonts of growth and regeneration … dried up irrevocably". These beliefs were maintained for a long period of time, until the neural stem cells (NSC) were first identified and isolated from striatum of the adult mouse brain by Reynolds and Weiss in 1992 (Tsuji et al. 2011). Now, after two decades of their discovery, along with extensive and prolonged experimental studies, the characteristics, origin, and differentiation of these cells are widely uncovered.

The NSC are one type of the multipotent stem cells, that have the capability of differentiation into multiple unipotent cells, including neural and non-neural tissues (e.g.,oligodendrocytes and astrocytes). This ability to replace the major lost elements of degenerated nervous system gave them a central role in the regenerative attempts and trials. The source of the NSC can be divided according to the developmental level of isolation into embryonic and adult sources. The embryonic source of the NSC includes the embryonic stem cells and the developing central nervous system (CNS), while the adult sources include the adult neural tissue and induced pluripotent stem cells.

hNSC Derived from Fetal Neural Tissues

Isolation, culture, and expansion of the NSC have been widely carried on embryonic rodent brains and spinal cords. For enhancing the growth and expansion of these, different mitogenic factors have also been used, including the epidermal growth factor (EGF) and basic fibroblast growth factor (bFGF).

Isolation and differentiation was also induced on human fetal neural tissue-derived NSC. The major issue was in the proper culture and expansion of these cells before transplantation. For this purpose, different protocols were applied, including the use of recombinant adenoviruses by Sabate et al. (1995). The most significant breakthrough was made by Svendsen et al. (1997). The isolated human NSC (hNSC) were expanded as neurospheres in a growth culture using EGF and bFGF. For the first time, these stem cells were capable of migration and differentiation into the three neural phenotypes (neurons, oligodendrocytes, and astrocytes) after transplantation into rat brains. Nevertheless, cellular markers detected only a very few cellular survival 20 weeks after transplantation. 1 year later, Svendsen et al. (1998) were able to further expand the neurosphere culture and maintain a rapid cellular growth to 1.5 million-folds over a period of 200 days using a different method of sphere separation during NSC passage. In this protocol, instead of mechanical dissociation of the neurospheres, they were divided into quarters using a microscopic technique. This method maintained the cell–cell contacts and permitted a rapid and continual growth of each individual quarter over a period of 14 days. Differentiation of these cells was limited into neurons and astrocytes.

Vescovi et al. (1999) isolated the NSC from human embryonic brain and maintained their self-renewal and prolonged proliferation in a neu-

rosphere culture using EGF and bFGF. In contrast to murine NSC, the use of either of these growth factors would suppress the hNSC proliferation. Transplantation of these cells into rat brain was associated with migration and differentiation, and survival up to 1 year post transplantation. In the same year, Quinn et al. (1999) derived NSC from human fetal spinal cord, and expanded them using combination of EGF and bFGF in a neurosphere forming culture. At later passages, the only cell lines that could be maintained were those with the potential differentiation to astrocytes. According to the authors, this might indicate that spinal cord neural precursors were restricted to the astrocytic lineage under these conditions.

In the same year, Carpenter et al. (1999) added the leukemia inhibitory factor (LIF), EGF, and bFGF to the NSC culture derived from human embryonic brain. Compared to a control group, where only EGF and bFGF were added; the culture exhibited a faster and larger expansion, which was not evident until 50–60 days in culture. The mechanism for this delayed effect might be related to the maintenance of the cell survival beyond the typical NSC lifespan (50–60 days) or might be related to inhibited differentiation of the NSC and consequent maintained proliferation. This effect may also occur using cilliary neurotrophic factor (CNTF). The bFGF also proved to be essential in enhancing the expansion of the hNSC in a concentration-dependant fashion. In contrast, the absence of EGF did not affect the cellular growth and neuron formation, but the long-term effect of its absence was not recorded. This protocol facilitated hNSC growth, renewal, and expansion to as much as 10^7-folds, and maintained the differentiation potential for over 1 year, with unknown ultimate capacity.

Thus, this approach became favorable in obtaining the hNSC for experimental transplantation in later studies., Zhang et al. (2011) used a different method by applying a combination of EGF and brain-derived growth factor (BDGF). The addition of the latter was associated with enhanced proliferation and migration of the hNSC through a PI3K/Akt kinase pathway.

In a study by Ostenfeld et al. (2000), the authors concluded that the hNSC have shorter telomeres than their rodent counterparts and to the embryonic stem cells. However, its exact effect on the cellular replication has not been determined, despite the shortening associated with each replication in some cellular populations, and their absence at certain stage with related slowed growth. Moreover, with the transplantation of 200,000, one million, or two million cells per animal, they found that the smaller the transplant, the more likely to differentiate and extend neuronal fibers and less likely to provoke immune rejection. The authors suggested the existence of an upper limit to the optimal number of transplanted cells. This is in contrast to previous beliefs that high density transplants are more suitable for regeneration.

To study the regional or temporal characteristics with regard to growth and differentiation, Kim et al. (2006) isolated NSC from almost all parts of the CNS including telencephalon, diencephalon, midbrain, cerebellum, pons and medulla, and spinal cord. Based on their study, they found that, compared to the midbrain and hindbrain, the forebrain-derived NSC grew faster and ultimately gave rise to significantly more neurons. There was also a reduction in neuronal emergence from the respective neurospheres over time in culture, except in those derived from cerebellum, which were significantly increased. Moreover, distinctive molecular markers of regional identity were expressed by neurospheres of different compartments and were maintained during long-term passaging in vitro. However, these region-specific markers are not irreversible, and may change in response to local inductive cues.

Transplantation of NSC Derived from Fetal Neural Tissues

Cummings et al. (2005) reported on transplantation of fetal brain-derived NSC into mice with SCI at T9. Immunocytochemistry revealed extensive human cell survival and engraftment within the injured mouse spinal cord, along with migration of the neural cells starting at 24 h after transplantation. The engrafted cells showed less differentiation into astrocytes than neurons and oligodendrocytes.

These neurons and oligodendrocytes showed signs of active synaptic function restoration and effective myelination, respectively. Basso, Beattie, and Bresnahan (BBB) score assessment at 16 weeks post transplantation showed significant locomotor improvement compared to control group.

In the same year, Iwanami et al. (2005) reported on fetal spinal cord-derived NSC transplantation into C5 injured spinal cords of common marmosets at 9 days after injury. Histological study 8 weeks post transplantation showed cellular survival and migration outside the grafted site. Differentiation into neuronal and oligodendrocyte lineage was also seen. Functional recovery assessment using bar grip tests revealed locomotor improvement starting 2 weeks post transplantation. The authors also concluded that concomitant use of scaffolds, the blockage of axonal regeneration inhibitors, and the administration of neurotrophic factors can be effectively use with NSC transplantation.

In the experiment of Tarasenko et al. (2007), NSC were isolated from fetal brain and allowed to expand by the addition of EGF, bFGF, and LIF. These cells were then transplanted into T10 injured spinal cord of rats 9 days after injury. Differentiation into functioning neurons and oligodendrocytes, which have survived for at least 3 months, has been seen. BBB evaluation showed significant locomotor improvement starting 2 weeks after grafting and reaching a plateau 40 days post transplantation. According to the authors, these timing represent an important window for transplantation. Emgard et al. (2009) isolated and transplanted fetal brain-derived NSC into injured spinal cords of rats. These cells showed remarkable proliferation and migration starting 6 h post transplantation and thereafter over the next 3 weeks. They did not assess the locomotor outcome of this transplantation.

hNSC Derived from Adult Neural Tissues

Since Reynolds and Weiss (1992), many studies have attempted to isolate NSC from different areas of adult CNS. These include the subependymal zone (SEZ), the subventricular zone (SVZ), and the hippocampus. Other areas may include the spinal cord, striatum, and neocortex. As in the case of fetal-derived NSC, proliferation of the adult-derived NSC needs the presence of EGF and FGF mitogens. Withdrawal of these growth factors will induce differentiation to neurons, astrocytes and oligodendrocytes in vitro (Akiyama et al. 2001).

Transplantation of NSC Derived from Adult Neural Tissues

In an experimental study by Akiyama et al. (2001), NSC were identified and isolated from adult brain subependymal and subventricular zones. These cells were allowed to expand by applying the mitogens bFGF and EGF, and were then transplanted into dorsal columns of lumbar region of injured spinal cords of rats. Observation of the site of transplant showed NSC differentiation into myelinating schwann cells and, to a lesser extent, oligodendrocytes for 6–8 weeks. This directed differentiation is different from fetal neural tissue-derived NSC, and could be related to intrinsic capacity of the adult progenitor cells and the cellular and extracellular milieu of the transplant zone, including cytokines released from microglia or macrophages.

Using the same protocol, Karimi-Abdolrezaee et al. (2006) isolated NSC form subventricular zone of rats. These cells were then expanded using EGF and bFGF, and transplanted into T6 – T7 region at 2 and 6 weeks after SCI. The addition of EGF, bFGF, and platelet-derived growth factor (PDGF-AA) into the spinal subarachnoid space promoted their survival and proliferation. After 8 weeks of transplantation, the cells were able to proliferate and migrate along the site of injury, and differentiate into myelinating oligodendrocytes and, to a lesser extent, astrocytes. Functional assessment using BBB locomotor rating scale, footprint analysis, and grid walk assessment showed significant locomotor recovery. These findings, however, were only detected in the early (subacute) transplant and not in the late (chronic) transplant. Some scientists prefer the use of NSC over ESC for clinical application, as they might have a lower risk of tumorgenesis. However, some challenges are present in using

these cells including the need for pure populations of differentiated cells, inefficient tracking systems, and moderate cell survival after transplantation. Nowadays, the induced pluripotent stem cells present the promising source of progenitor cells.

hNSC Derived from hESC and hiPSC

Embryonic stem cells (ESC) are derived from the inner cell mass of the blastocyst, an early-stage embryo. These cells were studied extensively over a period of almost two decades before it was isolated from human embryos by Thomson et al. in 1998. It has always been thought that late during fetal development, the ESC start to differentiate and gradually acquire a specific cell type in a unidirectional fashion. However, this was opposed in 2006, when Takahashi and Yamanaka were able to reverse the cellular cycle by reprogramming the somatic fibroblast cells into pluripotent cells using defined pluripotency-related transcription factors (i.e. Oct3/4, Sox2, c-Myc and Klf4). These cells were then called *Induced pluripotent stem cells* (iPSC). The major advantages of these cells include solving the ethical problem related to ESC derivation, and eliminate the need for immunosuppressive factors as they represent an autologous transplant. The major disadvantages include the genetic instability and high teratogenic potential associated with the process of reprogramming and culture, and also endogenous within the cells of origin.

For neural cell replacement within the SCI, the pluripotent stem cells are used as a source of the neural precursors/stem cells, motor neurons, and oligodendrocytes. The derivation of the NSC and the derivation and transplantation of motor neuron and oligodendrocytes from hESC and iPSC have been discussed in details in the previous chapter.

Transplantation of NSC Derived from hESC and hiPSC

Hatami et al. (2009) induced NSC formation from hESC cultured in DMEM/F12 medium supplemented with N2 and exposed to bFGF, retinoic acid (RA), Noggin, sonic hedgehog (SHH), and LIF at different stages. Their protocol resulted in around 50% NSC formation. They were then transplanted along with collagen scaffold into T10 spinal cord hemisection of rats. The transplanted cells migrated and incorporated into the damaged sites of the spinal cord and differentiated into neurons and glial cells, as evident by immunocytochemistry. Significant hindlimb locomotor function recovery assessed by the BBB scoring system compared to the control group was noted 5 weeks post-transplant. They also noted improved sensory response in the study group. No complications were observed on long term observation.

Nori et al. (2011), reported on iPSC reprogramming from adult human dermal fibroblasts using Oct3/4, Sox2, Klf4, and c-Myc. These cells were then induced to form embryoid body (EB) using RA and NSC using bFGF, which were transplanted into T10 SCI sites of mice. Electrophysiological function and functional recovery was monitored using Motor-Evoked Potential (MEP) and Basso mouse scale (BMS) score, which showed progressive improvement of motor function 12 days after transplant and thereafter, followed by a plateau. After 47 days of transplant, histological study showed survival, migration, and differentiation into neurons and glial cells. They also promoted angiogenesis, axonal regeneration, and local-circuitry reconstruction. No complications, including tumor formation, were observed.

Using the same protocol, Fujimoto et al. (2012) induced iPSC reprogramming. After expansion of iPSC-derived NSC cells using bFGF and EGF, they were transplanted into a T10 injured spinal cords of mice. The results were compared with human fetal spinal cord-derived NSC. Hind limb motor function revealed no difference between the two types of cells as compared to control group using the BMS for at least 8 weeks. At 12 weeks, the MEP amplitudes were significantly higher than those of control group. As evident by immunohistochemistry, transplanted cells survived and migrated to both rostral and caudal directions around the lesion site. Differentiation into neurons and glial cells, along with synaptic formation and enhanced

survival of endogenous neurons, were also observed. Moreover, injection of these cells into the motor cortex of the hind limb area showed signs of corticospinal tract (CST) reconstruction but without CST axonal re-extension. To test the theory that lentiviral infection affects the differentiation potential, induced differentiation of infected and uninfected cells were observed and no differences could be detected.

Mesenchymal Stem Cells

As in the case of NSC, the mesenchymal stem cells (MSC) are a type of multipotent stem cells that have the ability to differentiate into any cells within the mesenchymal linage, including osteoblasts, chondrocytes, adipocytes, and stroma (Sandner et al. 2012). They are also able to transdifferentiate into cells of endodermal and ectodermal lineages such as hepatocytes and neurons, respectively (King et al. 2012). However, this does not occur in a quantitatively relevant fashion (Sandner et al. 2012). The MSC can be isolated from adult and neural tissues, with the bone marrow being the major source in adults, and umbilical cord blood in fetuses and neonates. The fact that the MSC can be used as autologous cell grafts that can easily expand from relatively small amounts of bone marrow aspirates, along with their inflammatory and immune-modulating function, makes them an attractive source for cellular transplantation (King et al. 2012; Sandner et al. 2012). Moreover, despite the fact that they can rapidly and extensively be expanded in cell cultures, there is no evidence of tumor production in vivo, and they have a strong capacity to increase tissue preservation, decrease cyst and injury size, and increase recovery of function (Bhanot et al. 2011). They are also capable of producing a number of growth factors in vivo, including NGF, brain-derived neurotrophic factor (BDNF), vascular endothelial growth factor (VEGF), hepatocyte growth factor (HGF), and NT-3, which significantly promotes neuronal survival and functional recovery, with the BDNF being the most important and potent factor. The human MSC (hMSC) can also be genetically modified for further secretion and consequent enhanced functional improvement.

Transplantation of human umbilical cord blood-derived MSC (hUCB-MSC) and human bone marrow-derived MSC (hBM-MSC) has been applied on animal models via direct or intravenous injection. These studies were mostly applied on acute SCI, and were associated with significant locomotor and somatosensory improvement assessed by the BBB scoring system, Somatosensory-Evoked Potential (SSEP), and MEP. It was also reported that hUCB-MSC have greater ex vivo expansion capabilities, faster proliferation, and lower immunogenicity than hBM-MSC (Weiss et al. 2008; Troyer and Weiss 2008). The combined treatment with intrathetcal Taxol, an anticancer drug, and hUCB-MSC may lead to further decrease in astrocytic activation and increased axonal preservation, and consequent enhanced functional recovery (Zhilai et al. 2012).

Experimental Trials on Humans

An experimental study by Pal et al. (2009) included lumbar intrathcal injection of hBM-MSC into 30 patients with complete SCI. Lumbar injection is considered safe, feasible and beneficial as the injected cells migrate eventually to the site of injury. The patients were divided into two groups depending on the timing of injury, into less, or more than 6 months. Over 1-year follow-up, the patients within the first group (< 6 months) showed noticeable, yet variable, improvement of daily activities and quality of life, starting with recovery of bladder and bowel sensation and control, followed by sensory and then motor function. However, these sensory and motor recoveries were not sufficient to elicit a positive electrophysiological response on the MEP, SSEP, and Nerve Conduction Velocity (NCV). Also, no MRI-changes could be noticed. On the other hand, patients within the second group (more than 6 months) failed to show any improvements. No major adverse reaction could be noticed in these patients, and only two patients reported neuropathic pain after the transplant.

Kishk et al. (2010) reported on the hBM-MSC transplantation in 43 patients with various degrees of SCI in the cervical and thoracic areas for a period of 6 months (subacute and chronic phases). These patients received lumbar intrathecal injection monthly for 6 months, along with rehabilitation therapies 3 times weekly. The functional recovery on the American Spinal Injury Association (ASIA) Impairment Scale, ASIA grading of completeness of injury, Ashworth Spasticity Scale, Functional Ambulation Classification, and bladder and bowel control questionnaire were very minimal as compared to the control group, and were noticed almost always in patients with incomplete SCI. Adverse effects included spasticity, neuropathic pain in 24 patients, and encephalomyelitis in one patient with a history of post-infectious myelitis.

In the study of Bhanot et al. (2011), 13 patients with chronic (more than 8 weeks) cervical or thoracic complete SCI were chosen. Each patient received one intramedullary hBM-MSC injection at the site of injury and two lumbar intrathecal injections 1 and 2 weeks after the first injection, respectively. These patients were followed every 3 month with complete neurological evaluation and ASIA scale, and every 6 months with the electrophysiological studies including SSEP, MEP, and NCV. Over 1 year of follow-up, only one patient showed a slight motor recover, 2 patients had patchy improvement in pin prick sensation below the level of injury, and 1 patient subjectively developed sensation of fullness of bladder. None of these patients had serious or persistent complications, but some of them experienced transient manifestations, including, spasticity, fever, malaise, vomiting, tingling or burning girdle sensation.

Park et al. (2012) reported on long-term follow-up of 3 patients out of the original 10 patients with incomplete or complete SCI. Their experiment included intramedulary injection of hBM-MSC into the site of injury more than 1 month (subacute and chronic) following trauma. Seven out of ten patients showed no motor, electrophysiological, or MRI improvement. The other 3 patients, who had residual neurological function, showed motor improvement within the first 6 months of follow-up, and were further evaluated for more than 30 months. Over this period, they had remarkable motor and functional recovery. Electrophysiological study, assessed by SSEP and MEP, and MRI scan also, for the first time, showed significant improvement. This improvement may be related to several factors, including: (1) direct delivery of the transplant into the site of injury is more effective method for SCI recovery compared to intrathecal injection, especially in subacute and chronic phases. The latter, although proven safer, but is associated with more limited migration and time window of recovery. At the same time, their study was the first to apply multiple intramedullary injections: 2 above the cavity and 3 into the cavity, and using the fibrin glue to seal the site of injection. (2) The fact that the 3 patients who showed locomotor recovery already had residual neurological function following injury may indicate that this treatment could be more effective for patients with incomplete injuries rather than complete injuries. No serious complications were reported in any of these patients.

From the previous studies, we can conclude that the use of autologous hBM-MSC represent a safe, feasible, and reliable method of cellular transplantation for SCI treatment. That is the reason why they are the main source of transplant applied to human. However, the use of these cells in subacute and chronic phase of injury showed only minimal promising results, and further studies are needed. (Tables 21.1 and 21.2).

Transplantation of Bone Marrow Stem Cells (BMSC) in Patients with SCI

The BMSC are a mixed cell population including hematopoietic stem cells, mesenchymal stem cells, endothelial progenitor cells, macrophages, lymphocytes, and marrow stromal cells. The use of the entire BMSC can combine the established neuroprotective effect of its components, including the hematopoietic cells and their secretory factors (Mehler et al. 1993; Chong et al. 2002), the stromal cells (Chen et al. 2002, 2005), and the MSC.

Table 21.1 Human cellular transplantation in spinal cord injury in animals

Study	Year	Type of cells	Source of cells	Transplant	Site of transplant	Phase of transplant	Integration and differentiation	Locomotor improvement
hBM-derived SC and MSC transplantation into animals will not be presented as they have been applied on humans								
Akiyama et al.	2001	hNSC	Adult SEZ and SVZ	Rats	T11	Acute injury	Yes	-
Cummings et al.	2005	hNSC	Fetal brain	Mice	T9	Acute injury	Yes	Significant
Iwanami et al.	2005	hNSC	Fetal spinal cord	common marmosets	C5	Acute injury	Yes	Significant
Karimi-Abdolrezaee et al.	2006	hNSC	Adult SVZ	Rats	T6–T7	Subacute and chronic injury	Yes, only in subacute	Significant, only in subacute
Tarasenko et al.	2007	hNSC	Fetal brain	Rats	T10	Acute injury	Yes	Significant
Emgard et al.	2009	hNSC	Fetal brain	Rats	-	Acute injury	Yes	-
Hatami et al.	2009	hNSC	hESC	Rats	T10	Acute injury	Yes	Significant
Nori et al.	2011	hNSC	hiPSC	Mice	T10	Acute injury	Yes	Significant
Fujimoto et al.	2012	hNSC	hiPSC	Mice	T10	Acute injury	Yes	Significant

Spinal cord injury: Acute (up to 4 weeks), Subacute (4–8 weeks), Chronic (more than 8 weeks)

Table 21.2 Human cellular transplantation in spinal cord injury in humans

Study	Year	Type of cells	Source of cells	Transplant	Site of transplant	Phase of transplant	Integration and differentiation	Locomotor improvement
Park et al.	2005	hBMSC	BM	Humans	Intramedullary cervical	Acute	-	Significant
Sykova et al.	2006	hBMSC	BM	Humans	Intravenous and intra-arterial	Acute and chronic	-	Minimal, only in acute
Hwan Yoon et al.	2007	hBMSC	BM	Humans	Intramedullary cervical	Acute, subacute, chronic	-	Moderate, only in acute and subacute
Geffner et al.	2008	hBMSC	BM	Humans	Intrmedulary, intrathecal, and intravenous	Acute and chronic	-	Minimal, only in acute
Pal et al.	2009	hMSC	BM	Humans	Intrathecal lumbar	Chronic injury (<6 m, >6 m)	-	Minimal, only in <6 m chronic
Kishk et al.	2010	hMSC	BM	Humans	Intrathecal lumbar	Subacute and chronic (<6 m)	-	Minimal
Bhanot et al.	2011	hMSC	BM	Humans	Intramedullary cervical and thoracic, Intrathecal lumbar	Chronic	-	Minimal
Park et al.	2012	hMSC	BM	Humans	Intramedullary cervical	Chronic	-	Moderate

Spinal cord injury: Acute (up to 4 weeks), Subacute (4–8 weeks), Chronic (more than 8 weeks)

Park et al. (2005) and Hwan Yoon et al. (2007) reported on direct intramedullary transplantation of BMSC along with subcutaneous injection of granulocyte macrophage-colony stimulating factor (GM-CSF). The latter has been found to induce the growth of different hematopoietic cell lineages and prevent apoptotic cell death of the hematological and neuronal cells. In these studies, the authors also concluded that the GM-CSF had direct effect on the transplanted BMC by enhancing their survival in the spinal cord and activating them to excrete neurotrophic cytokines. These factors were also found to stimulate microglial cells to produce neurotrophic cytokines such as BDNF. The experiment of Park et al. (2005) involved 6 patients with complete cervical SCI within 14 days (acute phase), only 5 received intramedullary transplant and 1 received only GM-CSF injection. Four of the 6 patients (including the one with GM-CSF alone) experienced significant motor and sensory recovery noted 3–7 months postoperatively. No permanent or serious complications were noted, although the patients experienced transient fever and myalgia related to GM-CSF injection. In the other experiment of Hwan Yoon et al. (2007), 35 patients with complete cervical SCI were divided into 3 groups based on the timing of injury into acute (less than 2 weeks), subacute (2–8 weeks), and chronic (more than 8 weeks). Over 10 months of follow-up, noticeable locomotor improvement was noted in the acute and subacute patients to variable degrees, but none in the chronic patients. No permanent or serious complications were noted. Neuropathic pain was only reported in 7 patients.

The significant locomotor improvement noted in the study of Park et al. (2005) may be related to several factors, including: (1) Their study was the only one to apply stem cells therapy in acute spinal cord injury, and based on animal studies, early cellular transplantation is always associated with enhanced recovery. This also can be noticed when their results are compared with those of Hwan Yoon et al. (2007) who use the same protocol but in patients with different phases of injury. (2) The intramedullary route of transplantation is proved to be superior to other routs, including intrathecal injection (see above). (3) The use of the entire BMSC. (4) The concomitant subcutaneous injection of GM-CSF. However, further studies are still needed to confirm the role of these factors.

In a study by Sykova et al. (2006), 20 patients with complete SCI were divided into two groups who received BMSC via intravenous and intra-arterial routes, respectively. Each group contained patients with acute and chronic phase injury. Over a 3 months follow-up period, very minimal motor and sensory recovery were noticed in 5 of 7 acute patients and in 1 of 13 chronic patients. These patients were mainly from the intra-arterial group.

The study by Geffner et al. (2008) included 8 people with acute and chronic SCI who received infusions of BMSC via multiple routes: directly into the spinal cord, directly into the spinal canal, and intravenous. Over 2 years of follow-up, almost all patients experienced variable degrees of functional improvements assessed by ASIA scores, the Barthel Index, Ashworth scores, and bladder function. In none of these patient serious or permanent complications including neuropathic pain and tumor formation were reported.

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Endogenous Regenerative Potential of Neural Stem/Progenitor Cells of the Newborn Brain (An Overview)

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Contents

Abstract

Neonatal hypoxic-ischemic encephalopathy, intraventricular hemorrhage, periventricular leucomalácia, neonatal stroke and chronic hypoxia are the main causes of brain damage and long-term neurological impairments in neonates, representing an enormous burden for patients and their families. Although it has been suggested for a long time that the developing brain has an increased plasticity and a robust regenerative potential, it was only in the last years that an increasing number of studies started to evaluate the endogenous regenerative response of neural stem/progenitor cells in animal models of neonatal brain injury and in anatomopathological studies of the human brain. In this chapter we will discuss how the most common forms of injury can either induce a regenerative response or compromise the function of the neurogenic niches of the newborn brain.

Introduction

The neonatal period, comprising the first 28 days after birth in humans, represents a time of increased vulnerability to central nervous system damage, particularly as a consequence of hypoxemia (decreased partial pressure of oxygen in the arterial blood) and/or cerebral blood flow alterations. The severity and timing of the insult will determine the pattern of brain injury, which reflects the regional susceptibility of different

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regions at a given stage of brain development. Therefore, while perinatal insults especially, but not exclusively, damage the gray matter in term newborns (particularly the thalamus, basal ganglia and perirolandic cortex), the white matter is predominantly affected in premature infants (Volpe et al. 2011).

Another distinguished feature of neonatal brain injury is that important developmental processes, such as neurogenesis and gliogenesis, are still taking place in the brain at the time of injury. Neurogenesis is finely regulated in space and time, so that specific neuronal subtypes are generated within specific locations of the neurogenic niches, in a progressive fashion, through the progressive commitment of neural progenitors, although some degree of plasticity may exist. Indeed, despite the prominent role of cell-intrinsic factors, neurogenesis is tightly controlled by the local environment. In the neurogenic niche, neural stem/progenitor cells (NSPC) are exposed to diffusible factors, matrix glycoproteins and cell-to-cell contacts that control the proliferation, differentiation and migration of these cells and of their progeny (Ihrie and Álvarez-Buylla 2011). Upon a neonatal brain injury, several components of the NSPC niches are altered, including changes in glial and immune cells, blood vessels and oxygen tension. All these changes could result in either the compromise or the stimulation of neurogenesis. Therefore, it is possible to speculate that the endogenous regenerative potential of the developing brain will also be influenced by the type and the timing of the insult. In this chapter, we will give an overview of the endogenous regenerative potential of NSPC of the newborn brain. We will discuss how neurogenesis and gliogenesis occurs during normal brain development and how these processes are regulated by the most common forms of neonatal brain injury.

Neurogenesis in the Developing Human Brain

During the development of the human cerebral cortex, neurogenesis is initiated by the asymmetric division of neuroepithelial cells (neural stem cells) localized in the ventricular zone (VZ, also called germinal matrix or germinal epithelium) at embryonic day 33. As a result, two daughter cells are formed: one will differentiate into a postmitotic neuron or glial cell and the other will maintain the pool of NSPC. Thereafter, neuroepithelial cells will also give rise to radial glial cells (RGC), neuronal and glial progenitors which have an endfoot contacting the ventricular surface and a long radial process that extends from the VZ towards the pial surface, serving as a scaffold for the migration of newborn neurons. RGC will also generate intermediate progenitor cells that populate the embryonic subventricular zone (SVZ), a second site of cortical neurogenesis that appears around 7 weeks of gestational age at the basal border of the VZ, which in turn will give rise to neurons of the upper cortical layers from the 20th to at least the 25–27th gestational weeks. Furthermore, recent observations have shown that the SVZ can be divided after the 20th gestational week in two parts, the inner SVZ and the outer SVZ. The latter contains a population of progenitors that resembles RGC (despite the lack of an apical endfoot), which contributes to enhance the neurogenic capacity of the human embryonic neocortical wall.

Cortical projection neurons, glutamatergic excitatory neurons that extend axons to distant targets, are formed in the neurogenic regions of the dorsal telencephalon and migrate radially to the cortical mantle. On the other hand, cortical interneurons, GABAergic inhibitory neurons that make local connections, are generated in the ventral telencephalon, migrating long distances from the regions where they are formed (mainly the medial and caudal ganglionic eminences) to their final position in the neocortex, although a subpopulation of interneurons might be generated in the SVZ of the dorsal telencephalon in humans (Bystron et al. 2008).

From midgestation (around 20 gestational weeks) until birth, RGC of the VZ will terminally differentiate into astrocytes, as well as will give rise to ependymal cells, oligodendrocytes and to NSPC that persist in the adult SVZ. Accordingly, neural stem cells (also called type B1 cells) of the postnatal SVZ share several common characteristics with RGC, including

the expression of some astroglial markers and the presence of an apical process extending into the ventricular lumen. B1 cells also possess a basal "radial" process that reaches the surface of blood vessels, instead of contacting the pial surface. Therefore, B1 cells are localized in close contact with the cerebrospinal fluid and with blood vessels, being exposed to diffusible factors, basement membrane and extracellular matrix components and vascular growth factors (Ihrie and Álvarez-Buylla 2011). In mammalians, two main neurogenic regions persist in the forebrain after birth: the SVZ and the hippocampal subgranular zone (SGZ) of the dentate gyrus. In the SVZ, in the lateral walls of the lateral ventricles, NSPC continuously give rise to new neurons that migrate to the olfactory bulb, where they replace local interneurons.

However, recent studies have suggested that the production of new neurons in the adult human brain seems to be lower than initially thought, based on studies in rodents. One striking study has analyzed human brain specimens from birth to 84 years, demonstrating that the SVZ neurogenesis decreases drastically over the first 6 months of life. This transition coincides with the appearance of a hypocellular gap between the ependymal layer and a periventricular ribbon of astrocytes in the postnatal human SVZ (Sanai et al. 2011). Although a subset of these astrocytes are proliferative in the adult SVZ, retaining the potential to generate clonal neurospheres and giving rise to neurons, oligodendrocytes and astrocytes in vitro (Sanai et al. 2004), only a small number of immature neurons can be found in the ventral SVZ (but not in the dorsal, lateral, medial or caudal SVZ) in the adult human brain (Sanai et al. 2011; Wang et at. 2011). Indeed, Sanai et al. (2011) observed a rapid decrease in the number of immature neurons in the SVZ of the newborn brain over the first months of life.

Similarly, a robust chain of migrating immature neurons can be observed in the RMS of the newborn brain, up to 6 months of age, but not in older children (2-year-old) or in adults. These neuroblasts migrate from the SVZ, along the olfactory peduncle and olfactory tract to the olfactory bulb, surrounded by glial cells and processes, often in close association with blood vessels. Interestingly, an additional migratory stream, branching from the proximal limb of the RMS in direction to the ventromedial prefrontal cortex (called medial migratory stream, or MMS), was found in the brain of young infants, but not in older children (Sanai et al. 2011). Indeed, only rare neuroblasts can be observed in the human adult RMS, migrating singly or in pairs from the ventral SVZ, but not forming chains (Sanai et al. 2011; Wang et al. 2011).

Therefore, these studies indicate that neurogenesis and neuronal migration occurs robustly in the SVZ of neonates and young infants, representing a potential source of cells for regeneration after an injury. On the other hand, pathological events during the neonatal period could disrupt SVZ neurogenesis, potentially affecting the development of other brain regions, such as the ventromedial prefrontal cortex. In the next topics, we will summarize recent studies that have addressed the effects of several types of brain damage on neurogenesis and gliogenesis in the newborn brain.

Neonatal Hypoxic-Ischemic Encephalopathy (HIE)

Neonatal encephalopathy is a clinical syndrome manifested by neurological symptoms in the first days of life. HIE, the most common cause of long-term neurological impairments in term infants, refers to 30–60% of the cases of neonatal encephalopathy caused by perinatal asphyxia. A minimum set of criteria are necessary for the diagnosis of HIE, such as the presence of metabolic acidosis in umbilical artery blood (pH 7.0 and base deficit >12 mmol/L), and evidence of an acute intrapartum causation, such as abrupt changes in fetal heart rate, neuroimaging evidences of acute hypoxic-ischemic brain injury, or the presence of a sentinel event (e.g., uterine rupture or placental abruption).

The rodent model of HIE consists of permanent unilateral common carotid artery occlusion followed by exposure to systemic hypoxia (8% oxygen-balance nitrogen) for several

minutes/hours in post-natal day 7 rats or, more recently, in neonatal mice. Since then, this model has been widely used for the study of the pathogenesis of HIE, as well as for the preclinical evaluation of potential therapies. The expanded regenerative capacity of the newborn brain has been demonstrated by several studies using the Rice-Vanucci animal model of HIE. It was observed that new neurons are generated in the SVZ, migrating to the striatum in mild cases of HIE, as well as to the neocortex in the more severe cases. These newly formed neurons occupy cell-sparse columns in the cortex (formed as a consequence of neuronal loss), where they stay in close contact with microglial cells and astrocytes. Strikingly, this neurogenic response is not limited to the acute phase of the injury, persisting for at least 5 months, although 85% of these neurons die before maturation, suggesting that the local environment might not be favorable for their survival and integration (Yang et al. 2007).

A subsequent study has also shown that most of the neuroblasts that migrated to the striatum after the injury differentiated into calretinin-positive interneurons, which represent only 0.5% of all striatal neurons. Indeed, the density of calretinin-positive interneurons increased two-fold in the intact and in the damaged areas of the ipsilateral striatum, compared to the contralateral striatum (Yang et al. 2008). Furthermore, a recent study has identified NSPC expressing the transcription factor Emx1 in the dorsolateral SVZ as the origin of virtually all newly generated calretinin-positive interneurons in the hypoxic-ischemic striatum. Given that Emx1-expressing NSPC also produces calretinin-positive interneurons in the intact striatum, it seems that the fate of their progeny in the striatum is very limited and is not changed after the injury (Wei et al. 2011). This restriction is even more important if we consider that in the striatum, most of the neurons are GABAergic medium-sized spiny projection neurons, while less than 5% are interneurons (divided into four populations, based on the expression of calretinin, parvalbumin, somatostatin, or choline acetyltransferase). Thus, the restricted fate of newborn striatal neurons is probably an obstacle for the regeneration of this brain region (Yang et al. 2008).

The Rice-Vannucci model of hypoxia-ischemia was also used to compare the endogenous neurogenic response in immature (postnatal day 9) and juvenile (postnatal day 21) mice. Interestingly, this model induces an increase in neurogenesis only in the striatum of postnatal day 9 mice, suggesting that the immature brain has a great potential for striatal regeneration, although other studies have already observed the generation of newborn neurons in the striatum of adult mice after stroke (Zhu et al. 2009).

Gliogenesis is also affected by the hypoxic-ischemic injury. At the time of HIE, RGC are terminally differentiating into astrocytes. It was shown that the radially oriented pattern of RGC processes is disrupted in a model of acute hypoxic-ischemic injury in the preterm-like brain (in postnatal day 3 rats), correlating in time with the appearance of reactive astrocytes (Sizonenko et al. 2008). However, it is unknown if HIE accelerates the differentiation of RCG into astrocytes and it is still necessary to investigate how the function of RGC is affected by the injury.

Futhermore, it was found that the number of newly generated oligodendrocytes increases both in the injured and in the contralateral corpus callosum in the hypoxic-ischemic brain (Zaidi et al. 2004). However, a retroviral lineage tracing study has demonstrated that the number of SVZ-derived astrocytes increases in the white matter, at the expense of a decreased generation of oligodendrocytes after HIE. A similar effect was obtained in gliosphere cultures of SVZ cells, by the combined administration of transforming growth factor beta 1 (TGF-β1), epidermal growth factor (EGF) and LIF, three factors whose expression is upregulated in the hypoxic-ischemic SVZ (Bain et al. 2010). In addition, one study has described that despite the increase of oligodendrocyte progenitor cells (OPC) in the striatum and cerebral cortex after the injury, SVZ cells contributed minimally to this regenerative oligodendroglial response (Dizon et al. 2010). Therefore, it seems that the generation of new oligodendroglial lineage cells and/or the migration of these cells to the injured white matter could be compromised in

the SVZ, despite the regenerative response of OPC localized outside the SVZ.

Future studies should address the molecular mechanisms regulating the function of NSPC, OPC and their progeny following HIE. Possible targets include cellular (e.g., microglial cells and astrocytes) and extracellular matrix components of the glial scar, which are able to modulate the regenerative response of the neonatal brain by multiple mechanisms, enhancing or inhibiting several aspects of this response, such as the proliferation and differentiation of NSPC, and the migration of their progeny (Ihrie and Álvarez-Buylla 2011).

Astrocytes and microglia play an important role in the orchestration of inflammation in the brain. Microglia cells exhibit an activated phenotype from the first few hours to at least one week after the hypoxic-ischemic insult and the accumulation of activated microglia/macrophages occurs more rapidly following cerebral ischemia in neonatal than in adult animals. In addition, changes in the permeability of the blood–brain barrier and the infiltration of blood-derived immune cells are also important features of HIE-induced inflammation (Vexler and Yenari 2009). Both the resident and infiltrating immune cells, reactive astrocytes and activated endothelial cells produce a broad spectrum of cytokines, chemokines, as well as high levels of nitric oxide and other inflammatory mediators that are known to influence the behavior of NSPC.

Inflammatory mediators that act as important modulators of neurogenesis after HIE are the cytokines leukaemia inhibitory factor (LIF) and IL-6 (Bain et al. 2010; Covey et al. 2011). IL6 expression is upregulated in the hypoxic-ischemic SVZ up to 48 h after the injury. The administration of indometacin, a non-steroidal anti-inflammatory drug, decreases IL-6 expression in the SVZ and reduces the proliferation of SVZ cells after HIE. In addition, IL-6 enhances neurosphere growth, self-renewal and tripotentiality, an effect that can be reverted by indometacin (a non-specific ciclooxigenase inhibitor) or by a ciclooxigenase-2 inhibitor. On the other hand, indometacin has an opposite effect on the regulation of hippocampal neurogenesis, increasing the proliferation of NSPC in the subgranular zone of hypoxic-ischemic animals (Covey et al. 2011).

A recent study has also revealed the role of the glycoprotein osteopontin as an important glial-derived factor that stimulates endogenous repair processes in the neonatal brain. Osteopontin expression is upregulated within the first 12 h after HIE, particularly in astrocytes and microglial cells. Interestingly, osteopontin knockout mice have less proliferating cells in the cerebral cortex and in the hippocampal subgranular zone, and a decreased number of newly formed oligodendocyte precursors and mature oligodendrocytes in the cerebral cortex and hippocampus after HIE. Furthermore, these mice have an increased white matter injury and more pronounced sensorimotor deficits after HIE (van Velthoven et al. 2011).

Astrocytes, microglial cells and infiltranting immune cells can also produce chemokines, moleculaes involved in the recruitment of blood-derived cells to the damaged brain. Some of these chemokines, such as stromal-derived factor 1 (SDF-1) and monocyte chemotactic protein-1 (MCP-1), are involved in the recruitment of neuroblasts to sites of ischemic injury and are important regulators of the endogenous regenerative response (Vexler and Yenari 2009).

Intrinsic mechanisms could also affect the NSPC response to neonatal brain injury. For instance, an interestingly study has observed that endonuclease VIII-like 3 (Neil3), an oxidized base-specific DNA glycosylase, is highly expressed in the neurogenic regions of the perinatal mouse brain and is involved in DNA repair in NSPC. Neil3-deficient mice exhibited a reduced number of newborn neurons in the striatum after HIE. In addition, neurospheres generated from the brain of hypoxic-ischemic Neil3-deficient mice exhibited a decreased NSPC proliferation and a 63% reduction in the neuronal differentiation of their progeny (Sejersted et al. 2011). Finally, although hippocampal neurogenesis is less studied in the Rice-Vannucci model of HIE, it has been demonstrated that the generation of new neurons

increases in the subgranular layer of the hippocampal dentate gyrus after the injury (Bartley et al. 2005).

Perinatal Stroke

Neonatal arterial ischemic stroke has an incidence of around 1–2,800 live births. It is the leading cause of hemiplegic cerebral palsy and is also associated with post-neonatal seizures, cognitive deficits and other neurological impairments (Chabrier et al. 2011).

Comi and colleagues have developed a mouse mode of neonatal stroke that consists of unilateral common carotid artery ligation in postnatal day 12 CD-1 mice. Given that the occlusion of this artery is not sufficient to cause brain infarcts in C57BL/6 mice, this model takes advantage of the increased susceptibility of CD-1 mice to ischemic insults. They have used this model to show that neurogenesis is reduced in the hippocampal dentate gyrus after stroke (Kadam et al. 2008), in part due to a decrease in cell proliferation in the subgranular zone, which significantly correlated with the severity of the injury (Kadam et al. 2009). Moreover, although the percentage of newly formed neurons was not significantly changed, the percentage of newborn glial cells was slightly increased in the hippocampus after stroke (Kadam et al. 2008). An interestingly finding was that neurogenesis impairments were not restricted to the ischemic hemisphere, but could also be observed in the contralateral hemisphere (Kadam et al. 2008), suggesting that this effect could be at least partially mediated by soluble factors in the cerebrospinal fluid and/or in the plasma.

In sharp contrast to the effects in hippocampal neurogenesis, the proliferation of cells in the SVZ was transiently increased in the first week after stroke in CD-1 mice (Kadam et al. 2009). However, one study has demonstrated that SVZ do not contribute to the generation of new neurons after transient middle cerebral artery occlusion (MCAO) in postnatal day 7 rats. In this study, intraventricular injections of a green fluorescent protein (GFP)-expressing lentivirus were used to track the fate of NSPC of the SVZ. The injury favored the generation of astrocytes, decreasing the percentage of newly formed oligodendroglial cells, despite the overall reduction in the number of both SVZ-derived astrocytes and oligodendrocytes in the striatum. In addition, SVZ-derived neurons were not observed in the striatum or in the cerebral cortex and the number of SVZ-derived neurons reaching the olfactory bulb decreased after stroke (Spadafora et al. 2010).

In summary, this observations suggest that unlike HIE, acute arterial ischemic stroke might compromise hippocampal neurogenesis in the neonatal brain. It also indicates that neonatal stroke does not induce a robust regenerative response in the SVZ. Therefore, future studies investigating the cellular and molecular mechanisms involved in the regulation of neurogenesis after stroke in the developing brain are absolutely needed.

Germinal Matrix Hemorrhage and Intraventricular Hemorrhage (IVH)

IVH is a common cause of mortality and long-term neurological impairment in very low birth weight preterm neonates (<1,500 g of body weight). IVH initiates as a germinal matrix hemorrhage, i.e., an injury in the highly proliferative periventricular neurogenic zone localized adjacent to the head of the caudate nucleus (IVH grade I). Then, in more severe cases, the ventricles can be filled with blood (grade II), the ventricular system can be distended (grade III) and secondary venous infarcts and intraparenchymal lesions may occur (grade IV). Strikingly, IVH grade III–IV is significantly associated with mental retardation and up to three quarters of these infants will develop cerebral palsy (McCrea and Ment 2008).

A recent study has analyzed the brain of preterm neonates with germinal matrix hemorrhage, demonstrating that multiple alterations take place in the human ganglionic eminence, a region that contributes to the generation of neocortical GABAergic interneurons and of thalamic neurons. It was observed a decrease in cell proliferation

in the ventral SVZ within 24 h after the injury, persisting for up to 4 weeks. Furthermore, other alterations were reported in the SVZ, including the appearance of activated microglial cells 2–63 days after the hemorrhage, the presence of cystic degenerative changes in 40% of the infants who survived 24–98 days and a decrease in the expression of the oligodendroglial lineage markers Olig2 and platelet-derived growth factor receptor alpha (PDGFRα). All these alterations have the potential to disrupt the development of important brain regions, possibly contributing to the pathogenesis of neurological deficits in these infants (Del Bigio 2011).

Periventricular Leukomalacia

Approximately 50% of the very low birth weight preterm neonates develop cerebral white matter injury, particularly in the form of noncystic periventricular leukomalacia (PVL), which occurs when focal necrotic lesions in the white matter are microscopic in size, progressing to the formation of a glial scar. In addition, in a small number of patients, larger necrotic lesions can occur, which can evolve to the formation of cysts in the white matter (cystic PVL). In both cases, PVL is also characterized by the presence of a more diffuse injury of premyelinating oligodendrocytes (pre-OLs), the predominant cell type of the oligodendroglial lineage in the last half of gestation. The increased vulnerability of pre-OLs to free radical attack and to excitotoxicity seems to be the underlying cause of the diffuse component of PVL, which in turn leads to myelination deficits in the brain of the affected infants (Volpe et al. 2011). Moreover, it has been demonstrated that despite the proliferation and accumulation of OPC and pre-OLs in the damaged brain after hypoxia-ischemia in the preterm equivalent postnatal day 3 rat, this robust regenerative response is followed by an arrest of the differentiation of pre-OLs into mature oligodendrocytes, resulting in a failure to initiate myelination (Segovia et al. 2008). Although this effect is still not completely understood, it is probably caused by alterations in the microenvironment, such as the presence of hyaluronic acid in the glial scar and might involve toll-like receptor 2 (TLR-2) signaling on OPC (Volpe et al. 2011).

Recent evidence has also indicated that neuronal damage (including residual subplate neurons and late-migrating GABAergic neurons in the white matter) is also an important component of PVL. Therefore, it is possible to speculate that the regenerative response to PVL could include the compensatory generation of new neurons. Indeed, an interestingly study has observed an increased number of doublecortin-positive immature neurons in the SVZ, in the subcortical white matter, as well as within and immediately adjacent to the foci of necrosis in the white matter in the brain of human PVL cases, compared to age-matched controls (Haynes et al. 2011).

Chronic Hypoxia in Low Birth Weight Neonates

Chronic hypoxia due to respiratory distress syndrome or to chronic lung disease is an important cause of brain injury in extremely low birth weight preterm neonates (<1.000 g of birth weight). To investigate the endogenous regenerative response of the developing brain subjected to chronic hypoxia, Vaccarino and colleagues have used a model of chronic subletal hypoxia in the early postnatal period that consists of rearing postnatal day 3 mice for 7 days in a hypoxic environment (9.5–10.5% O2). This model results in a 30% decrease in the number of cortical neurons and in a 24% decrease in cortical volume, despite the absence of a robust reactive astrocytosis (Fagel et al. 2009; Bi et al. 2011). One of the most interesting characteristics of this model is that the number of cortical excitatory neurons returns to normal levels one month after the injury, while the number of parvalbumin$^+$ and calretinin$^+$ GABAergic interneurons remains altered, indicating a robust, but selective, regenerative capacity of the newborn brain (Fagel et al. 2009).

This regenerative response involves NSPC from the SVZ, as well as cortical progenitors, and is regulated by fibroblast growth factor (FGF) signaling (Ganat et al. 2002; Fagel et al. 2009;

Bi et al. 2011). In this regard, chronic hypoxia induces the upregulation of the FGF receptor-1 (FGFR-1) in ependymal cells and RGC. Furthermore, genetic ablation of FGFR-1 in GFAP$^+$ glial cells (which includes RGC) results in an incomplete cortical neurogenic response and in a decreased proliferation of SVZ cells, preventing the recovery of cortical excitatory neurons after chronic hypoxia (Fagel et al. 2009).

Given that chronic hypoxia increases the number of radial glia-like cells in the ependymal and subependymal layers (Ganat et al. 2002), one interesting question that arises from this model is whether the hypoxic insult is preventing the differentiation of RGC into astrocytes (maintaining them as NSPC) or inducing other precursors to acquire a RGC-like phenotype.

Additional evidence for the important role of FGF signaling on the regulation of brain regeneration came from one study that used a model of cerebral ischemia in the preterm-like brain (bilateral occlusion of the common carotid artery in postnatal day 3 rats). In this study, it was demonstrated that the ischemic brain damage is followed by an increased generation of new neurons, oligodendrocytes and astrocytes and that the intraventricular administration of FGF2 further enhances this regenerative response (Jinqiao et al. 2009).

Finally, the most recent study from Vaccarino and colleagues has shown that hypoxia affects the fate of GFAP$^+$ progenitors localized in the cerebral cortex. Using genetic fate mapping, they observed that the generation of neurons by these cortical progenitors occurs in mice subjected to chronic hypoxia, but not in normoxic controls. Moreover, cortical GFAP$^+$ progenitors from hypoxic animals generate pluripotent, long-term self-renewing neurospheres, in contrast to GFAP$^+$ cortical cells from control mice (Bi et al. 2011).

Future Directions

Taken together, current evidence indicates that neurogenesis in the newborn brain can be either stimulated or compromised, depending on the type and on the timing of the insult. Furthermore, even when the generation of new neurons is induced, as occurs in animal models of HIE, this process appears to be not efficient enough to compensate the full spectrum of neuronal damage. Moreover, the need to replace a large variety of neurons in several brain regions represents a major obstacle for regeneration. For instance, although the newly formed neurons migrate to the damaged areas after HIE, they have a limited potential to differentiate into the appropriate neuronal subtypes (Yang et al. 2008). Similar observations were made in the model of subletal chronic hypoxia, in which the replacement of a subset of interneurons does not occur, despite the robust regeneration of excitatory neurons (Fagel et al. 2009). In addition, the survival of these newly generated neurons is still very low and their functional integration has not yet been demonstrated. Thus, it will be important to investigate if newborn neurons form new connections and extend long axons, restoring function. Taking advantage of this self-repair mechanism, increasing the formation of new neurons, their migration, survival, differentiation into the appropriate phenotypes and integration into the neural circuitry, is a promising new therapeutic strategy. In this regard, preclinical studies screening molecular targets and potential drugs that could modulate the function of NSPC and their progeny are urgently needed.

One promising drug that promotes neural regeneration in the developing brain is erythropoietin (EPO), which is already used for the treatment of anemia in premature infants. EPO administration induces both neurogenesis and oligodendrogenesis in the animal model of HIE, increasing cell proliferation in the SVZ and expanding the number of newly generated neurons that migrate to the striatum and to the cerebral cortex. Furthermore, EPO improves neurological outcome after HIE through several mechanisms, including neuroprotection, anti-inflammatory effects, induction of revascularization and reduction of brain edema. Importantly, the results of several clinical trials have shown that the administration of an early high dose of EPO is safe and feasible in preterm newborns and have indicated that EPO treatment might improve the neurodevelopmental outcome in these infants

(including those with IVH). Similar results were obtained in two clinical trials in term infants with HIE (Xiong et al. 2011).

Currently, several studies are testing the efficacy of erythropoietin administration in very preterm infants (http://clinicaltrials.gov; Identifier: NCT00413946), extremely preterm infants born at less than 28 weeks of gestation (http://clinicaltrials.gov; Identifier: NCT01378273) and in very low birth weight preterm infants (http://clinicaltrials.gov; Identifier: NCT01207778). One phase I/II clinical trial is also evaluating the safety and pharmacokinetic profile of darbepoetin alfa (darbe), a recombinant human erythropoietin-derived molecule, for the treatment of HIE (http://clinicaltrials.gov; Identifier NCT01471015).

Other possible therapeutic candidates are the modulators of the endocannabinoid system, such as WIN55212-2, a synthetic cannabinoid receptor agonist, which has a strong neuroprotective effect in the hypoxic-ischemic brain, through activation of the cannabinoid receptors CB1 and CB2. WIN55212-2 increases the generation of OPC in the injured white matter and the number of mature oligodendrocytes in the striatum, resulting in the remyelination of the external capsule after HIE in rats. Moreover, WIN55212-2 treatment further increases cell proliferation in the SVZ and the number of immature neurons in the striatum (Férnandez-López et al. 2011). However, given that the clinical use of this compound is limited by the production of psychoactive effects, it is necessary to identify nonpsychoactive cannabinoid compounds that could promote a similar therapeutic effect in the developing brain.

The lack of appropriate trophic support to the newly formed neurons is possibly an important obstacle for brain regeneration. In this regard, a recent study has observed that a 2-week intraventricular infusion of brain-derived neurotrophic factor (BDNF) and EGF, 5 weeks after the injury, increases the functional recovery in neonatal hypoxic-ischemic rats. This effect was partially mediated by the stimulation of NSPC proliferation in the SVZ and by the increased survival of newly generated striatal neurons (Im et al. 2010).

Finally, stimulation of hippocampal neurogenesis could potentially prevent some of the cognitive deficits that are frequently observed after almost all types of perinatal brain damage. It was shown that besides decreasing brain injury, lithium treatment increases both the proliferation and the survival of NSPC in the hippocampal dentate gyrus, for up to 7 weeks after HIE (Li et al. 2011). However, the possible side effects of this drug in the developing newborn brain are still unknown, despite the current use of lithium for the treatment of bipolar disorder in children.

In conclusion, a growing body of evidence has shown a robust regenerative potential of NSPC in the animal model of HIE. In contrast, neonatal stroke impairs hippocampal neurogenesis and PVL is followed by an unsuccessful attempt to replace the lost pre-oligodendrocytes. It will be important to investigate the mechanisms underlying these effects and how they can be manipulated in each condition. The developing brain has an enormous pool of NSPC and these cells represent a promising target for the development of new therapies that could improve the neurological outcome of extremely and very low birthweight neonates, as well as to reduce the neurological sequels of infants with HIE or perinatal stroke.

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