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Alvaro Mordente
Bruno Giardina *Editors*

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Editors
Roberto Scatena
Department of Laboratory Medicine
Catholic University
00168 Rome, Italy
r.scatena@rm.unicatt.it

Bruno Giardina
Department of Laboratory Medicine
Catholic University
00168 Rome, Italy
bgiardina@rm.unicatt.it

Alvaro Mordente
Institute of Biochemistry
and Clinical Biochemistry
Catholic University
School of Medicine
00168 Rome, Italy
alvaro.mordente@rm.unicatt.it

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Preface

The classic hallmarks of cancer are a poorly differentiated phenotype, and a cellular and genetic heterogeneity. In the past, the cellular diversity of cancer has mostly been attributed to the genetic instability of its cells. As the tumor cell population expands, individual cells pick up random mutations, and their molecular identity starts to diverge. By the time the cancer is detected, the millions of cells that make up the tumor have become as different from each other.

Cancer stem cells (CSCs) or, as defined by other authors, tumor-maintaining cells or cancer stem-like cells are a subpopulation of cancer cells that acquired some of the characteristics of stem cells to survive and adapt to ever-changing environments. These include the ability to self-renew and the capacity to produce progenitors that differentiate into other cell types.

It has been originally hypothesized that CSCs could potentially arise from normal stem or early progenitors. Now, the longstanding notion that fully committed and specialized cells might de-differentiate over the course of tumor initiation and progression to originate CSCs has been reevaluated. At present, data emerge to indicate that cancer cells that resemble stem cells need not be part of the original tumor but rather may emerge during later stages of tumor development. The observed tumor heterogeneity is probably a combination of growing genomic instability and epigenetic instability associated with the acquisition of a stem cell-like phenotype. These instability promote a new a fundamental peculiarity of CSCs, i.e., genetic plasticity.

CSCs represent the ideal justification for a lot of intriguing and obscure aspects of cancer pathogenesis (i.e., cancer cell dormancy, chemoresistance, local and distant relapses). The complex pathophysiology of CSCs and its important direct and indirect implications in molecular and cellular biology of cancer, at present, render this topic particularly interesting for Chemists, Biochemists, Pharmacologists, Biologists, Geneticists who are studying different aspect of experimental oncology. Moreover, considering the enormity of the clinical implications related to CSCs and/or to "cancer cells like stem cell," a growing number of researchers should modify and/or adapt its field of study in consideration of this relatively new topic.

At last, the identification of a molecular phenotype for these modified stem cells, associated to an accurate definition of their typical derangement in cell differentiation and metabolism, can represent a fundamental advance in terms of early diagnosis and selective therapy of cancer. At last but not least, the knowledge of pathogenetic mechanisms at the basis of CSCs can enlarge and ameliorate the therapeutic applications of the normal adult stem cells (i.e., regenerative medicine, tissue engineering, biotechnology applications) by reducing the risk of a deranged, uncontrolled, and thereby potentially tumorigenic stem cell differentiation.

A critical and continuous updating to the different pathophysiological aspects of this CSC may certainly help the development of a research, not only limited to cancer but also really useful and harmless for patients, by stimulating potential clinical applications in terms of diagnosis and above all of therapy.

Rome, Italy

Roberto Scatena
Alvaro Mordente
Bruno Giardina

Contents

Contributors

Carlos Gamallo Amat Pathology Department, Medical School, Autónoma University, Madrid, Spain

Cinzia Bagalà Division of Medical Oncology, Department of Internal Medicine, Catholic University, Largo A. Gemelli 8, 00168 Rome, Italy
cinziabagala@libero.it

Carlo Barone Division of Medical Oncology, Department of Internal Medicine, Catholic University, Largo A. Gemelli 8, 00168 Rome, Italy
carlobarone@rm.unicatt.it

Patrizia Bottoni Department of Laboratory Medicine, Catholic University, Largo A. Gemelli 8, 00168 Rome, Italy
patrizia.bottoni@rm.unicatt.it

Benedetta Bussolati Renal and Vascular Physiopathology Laboratory, Department of Internal Medicine, Molecular Biotechnology Centre and Research Centre for Molecular Medicine, University of Torino, Cso Dogliotti 14, 10126 Torino, Italy
benedetta.bussolati@unito.it

Giovanni Camussi Renal and Vascular Physiopathology Laboratory, Department of Internal Medicine, Molecular Biotechnology Centre and Research Centre for Molecular Medicine, University of Torino, Cso Dogliotti 14, 10126 Torino, Italy

Alfonso Colombatti Division of Experimental Oncology 2, National Cancer Institute IRCCS-CRO, Via Franco Gallini 2, 33081 Aviano, Italy
acolombatti@cro.it

Carla Danussi Division of Experimental Oncology 2, National Cancer Institute IRCCS-CRO, Via Franco Gallini 2, 33081 Aviano, Italy

Jun Dou Department of Pathogenic Biology and Immunology, Medical School, Southeast University, Nanjing 210009, China
njdoujun@yahoo.com.cn

Markus H. Frank Transplantation Research Center, Children's Hospital Boston and Brigham and Women's Hospital, Harvard Medical School, 300 Longwood Avenue, Boston, MA 02115, USA

Department of Dermatology, Brigham and Women's Hospital, Harvard Medical School, 77 Avenue Louis Pasteur, Boston, MA 02115, USA

Bruno Giardina Department of Laboratory Medicine, Catholic University, Largo A. Gemelli 8, 00168 Rome, Italy
bgiardina@rm.unicatt.it

Enrique González González Surgery Department, Hospital del Henares, Madrid, Spain

Ning Gu School of Biological Science and Medical Engineering, Southeast University, Nanjing 210096, China
guning@seu.edu.cn

Xin-Yuan Guan Department of Clinical Oncology, Li Ka Shing Faculty of Medicine, Queen Mary Hospital, The University of Hong Kong, Pok Fu Lam, Hong Kong
xyguan@hkucc.hku.hk

Toru Kondo Laboratory for Cell Lineage Modulation, RIKEN Center for Developmental Biology, 2-2-3 Minatojima-Minamimachi, Chuo-ku, Kobe, Hyogo 650-0047, Japan

Department of Stem Cell Biology, Ehime University
Proteo-Medicine Research Center, Shitsukawa, To-on, Ehime 791-0295, Japan
tkondo@m.ehime-u.ac.j

Serdar Korur Laboratory of Molecular Neuro-Oncology, University Hospital Basel, Buchserstrasse 26, Bern CH-3006, Basel, Switzerland
sedar.korur@unibas.ch

Steven W. Lane Division of Immonology, Queenland Institute of Medical Research, Brisbane, Australia
steven.lane@gimr.edu.au

Jennifer Y. Lin Department of Dermatology, Brigham and Women's Hospital, Harvard Medical School, 77 Avenue Louis Pasteur, Boston, MA 02115, USA

Maria Maddalena Lino Laboratory of Molecular Neuro-Oncology, University Hospital Basel, Buchserstrasse 26, Bern CH-3006, Basel, Switzerland

Stephanie Ma Department of Pathology, Li Ka Shing Faculty of Medicine, Queen Mary Hospital, The University of Hong Kong, Pok Fu Lam, Hong Kong
stephanie.ma@gmail.com

Giuseppe Ettore Martorana Institute of Biochemistry and Clinical Biochemistry, Catholic University, School of Medicine, Largo F. Vito, 1, 00168 Rome, Italy

Adrian Merlo Laboratory of Molecular Neuro-Oncology, University Hospital Basel, Buchserstrasse 26, Bern CH-3006, Basel, Switzerland adrian.merlo@gmx.ch

Elisabetta Meucci Institute of Biochemistry and Clinical Biochemistry, Catholic University, School of Medicine, Largo F. Vito, 1, 00168 Rome, Italy

Alvaro Mordente Institute of Biochemistry and Clinical Biochemistry, Catholic University, School of Medicine, Largo F. Vito 1, 00168 Rome, Italy alvaro.mordente@rm.unicatt.it

Oswaldo Keith Okamoto Centro de Estudos do Genoma Humano, Departamento de Genética e Biologia Evolutiva, Instituto de Biociências, Universidade de São Paulo, CEP 05508-090, São Paulo – SP, Brazil keith.okamoto@usp.br

Deric M. Park Department of Neurological Surgery, University of Virginia, 800212 Charlottesville, VA 22908, USA dmp3j@virginia.edu

Eliana Pivetta Division of Experimental Oncology 2, National Cancer Institute IRCCS-CRO, Via Franco Gallini 2, 33081 Aviano, Italy

Alessandro Pontoglio Department of Laboratory Medicine, Catholic University, Largo A. Gemelli 8, 00168 Rome, Italy espeedy@libero.it

Erica N. Proctor Departments of Surgery and Molecular and Integrative Physiology, University of Michigan Medical Center, 1500 E Medical Center Drive, TC 2210B, Ann Arbor, MI 48109, USA eproctor@med.umich.edu

Nuria Rodríguez Salas Medical Oncology Unit, Hospital Universitario Infanta Leonor, C/ Gran Vía del Este nº 80, 28031 Madrid, Spain nuria.rodriguez@salud.madrid.org

Salvatore Scarà Department of Laboratory Medicine, Catholic University, Largo A. Gemelli 8, 00168 Rome, Italy

Roberto Scatena Department of Laboratory Medicine, Catholic University, Largo A. Gemelli 8, 00168 Rome, Italy r.scatena@rm.unicatt.it

Tobias Schatton Transplantation Research Center, Children's Hospital Boston and Brigham and Women's Hospital, Harvard Medical School, 300 Longwood Avenue, Boston, MA 02115, USA tobias.schatton@tch.harvard.edu

Mark E. Shaffrey Department of Neurological Surgery, University of Virginia, 800212 Charlottesville, VA 22908, USA

Andrea Silvestrini Institute of Biochemistry and Clinical Biochemistry, Catholic University, School of Medicine, Largo F. Vito, 1, 00168 Rome, Italy

Diane M. Simeone Departments of Surgery and Molecular and Integrative Physiology, University of Michigan Medical Center, 1500 E Medical Center Drive, TC 2210B, Ann Arbor, MI 48109, USA
simeone@med.umich.edu

Akio Soeda Department of Neurological Surgery, University of Virginia, 800212 Charlottesville, VA 22908, USA

Paola Spessotto Division of Experimental Oncology 2, National Cancer Institute IRCCS-CRO, Via Franco Gallini 2, 33081 Aviano, Italy

Kwan Ho Tang Department of Pathology, Li Ka Shing Faculty of Medicine, Queen Mary Hospital, The University of Hong Kong, Pok Fu Lam, Hong Kong

Ugo Testa Department of Hematology, Oncology and Molecular Medicine, Istituto Superiore di Sanità, Viale Regina Elena 299, 00161 Rome, Italy
ugo.testa@iss.it

David A. Williams Division of Hematology/Oncology, Children's Hospital Boston, Department of Pediatrics, Harvard Medical School, Boston, MA, USA

Department of Pediatrics Oncology, Dana-Farber Cancer Institute, Harvard Stem Cell Institute, Boston, MA, USA

Chapter 1
Cancer Stem Cells: A Revisitation of the "Anaplasia" Concept

Roberto Scatena

Introduction

Cancer stem cells (CSCs) or, as defined by other authors, tumor-maintaining cells or cancer stem-like cells represent one of the most interesting topics of cancer pathophysiology studied in the last decade.

The American Association for Cancer Research Stem Cell Workshop defined a cancer stem cell as a cell within the tumor that possesses the capacity to self-renew and, in doing so, gives rise to the heterogeneous lineages that comprise the tumor (Clarke et al. 2006). This intriguing subpopulation of cancer cells should permit to justify some lethal clinical aspects of cancer, above all recidivism and radio/chemoresistance. Moreover, CSCs may represent a real new and more selective approach in cancer treatment. These innovative clinical potentials originate from an important revision of cancer molecular biology, with the clonal model of tumor evolution passing to a hierarchical model.

Specifically, the term "CSCs" describes a representative subpopulation of cancer cells with peculiar molecular aspects that resemble some of those typical of normal stem cells. In fact, these cells are capable of self-renewal (i.e., replenishing the repertoire of identical cancer cells), differentiation (i.e., creating heterogeneous progeny that differentiate into more mature cells), and show extraordinary proliferative potential (Stricker and Kumar 2010). Importantly, this particular subpopulation of tumor cells seems to show other similarities to normal stem cell physiology, including the following:

- They appear to be primarily in a more quiescent or dormant cell-cycle state
- Long-lived cells typically give rise to short-lived, more differentiated cells

R. Scatena (✉)
Department of Laboratory Medicine, Catholic University,
Largo A. Gemelli 8, 00168 Rome, Italy
e-mail: r.scatena@rm.unicatt.it

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- They are highly influenced by signals from their microenvironment
- They are characterized by specific surface markers and/or signal transduction pathways that are also important in stem cell biology
- They express high levels of ABC transporters and DNA repair mechanisms, which, together with their low proliferation index, give these cells a particular resistance to classical radiotherapeutic and chemotherapeutic protocols (Visvader 2011; Wang and Dick 2008).

On the whole, these peculiar biological properties contribute to the possibility that CSCs are responsible for cancer recurrence, metastatic dissemination, and chemoresistance (Zhou et al. 2009). As a result, these intriguing cells seem to play an important role in the pathophysiology of cancer, with dramatic clinical implications in terms of prognosis and therapy. In this sense, it may be more appropriate to call them "tumor-maintaining," "tumor-sustaining," or "tumor-propagating" cells.

Interestingly, in the same way, cancer stem-like cells seem to introduce a modification to the definition of the term "anaplasia," which derives from the term Greek ἀνα-πλάσσω, meaning to mold, to shape, or to model again. In fact, classically, it implies a dedifferentiation or, better, a loss of the structural and functional peculiarities of normal cells, which lose the morphological and functional features of the original cell, either partially or totally (Stricker and Kumar 2010). This peculiar reversion and/or lack of differentiation are considered hallmarks of malignant neoplasms (tumors). This term also includes a derangement of the normal cytoarchitecture of the tissue/organ from which the tumor originates and sometimes, an increased proliferative potential.

Generally, to define anaplasia, a number of morphologic changes are required (Stricker and Kumar 2010), including the following:

(a) *Pleomorphism.* The cells and the nuclei characteristically display considerable variations in size and shape.
(b) *Abnormal nuclear morphology.* Nuclei are typically hyperchromatic and disproportionately large; nuclear shape is extremely variable, and chromatin is often coarsely clumped and distributed along the nuclear membrane. Large nucleoli are usually present.
(c) *Mitoses.* Anaplastic cells usually show large numbers of mitoses; moreover, unusual mitotic figures (tripolar, quadripolar, or multipolar spindles) are often present. These gross perturbations of the mitotic apparatus could also be responsible for the formation of tumor giant cells, some possessing only a single huge polymorphic nucleus and others having two or more nuclei.
(d) *Loss of polarity.* In addition to cytological abnormalities, the orientation of anaplastic cells is markedly deranged, indicating a complex derangement of cell–cell and cell–matrix interactions. These alterations cause sheets or large masses of tumor cells to grow in an anarchic, disorganized fashion.
(e) *Stromal alterations*. The structural and functional perturbations of cancer cells derange cell–cell and cell–matrix interactions that could have a causative role in the classic tumor cell–matrix disorganization. This occurrence, in turn, from a morphological point of view, may cause large areas of ischemic necrosis in

tumor masses, while, from a functional standpoint, this architectural disorder could have a role on the induction of the Warburg effect.

For many years, these morphological features have been associated with a simple dedifferentiation process, fitting well with the classic, clonal evolution of cancer.

It is now known, however, that cancer may arise from stem cells or progenitor cells in different tissues. In these tumors, failure of differentiation and/or abnormal differentiation, rather than dedifferentiation of specialized cells, accounts for the undifferentiated neoplastic cell. In fact, according to hierarchical model, instead of a neoplastic cell, it would be more appropriate to discuss about heterogeneous populations of tumor cells, which consist of both essentially differentiated cells with no or poor mitotic potential and rarer cells that function as a tumor reservoir by sustaining malignant growth. This mixed pathogenesis has important biological, pathological, and thereby clinical implications that push to reevaluate the morphological criteria of anaplasia.

Background

Are CSCs really progenitor/stem cells and the actual cells of origin in cancer? The answer is becoming more complex as more data is accumulating.

Virchow first suggested that some tumors could arise from embryonic cells (1855). Generally, however, the modern concept of cancer cells as cells that show or acquire some of the fundamental characteristics of normal stem cells (i.e., self-renewal, multipotency, and proliferation potential) was initially postulated by Pierce and Speers (1988) and more recently confirmed by Bonnet and Dick (1997), who, by adopting some clusters of differentiation as CSC biomarkers, elegantly showed that a single leukemic cell was able to transmit systemic disease when transplanted into a mouse. A decade following the initial prospective isolation of leukemic stem cells, Al-Hajj et al. (2003) showed that human breast cancers also seem to adhere to the hierarchical or CSC model.

Thereafter, similar stem cell-like cells were discovered in various solid tumors, including melanoma (Quintana et al. 2008), colon (O'Brien et al. 2007), prostate (Collins et al. 2005), liver (Sell and Leffert 2008), pancreas (Li et al. 2007), and brain tumors (Singh et al. 2003).

Specifically, Bonnet and Dick (1997), in acute myeloid leukemia, showed that only a small subset of CD34+CD38− cells harbored serial leukemic transplantation potential, whereas the bulk of leukemic cells did not show this capability. Thereby, just a defined subset of leukemic cells is responsible for maintaining the disease. Other evidence seems to confirm that few, but not all, cancers are organized in a hierarchical manner (Bonnet and Dick 1997; Reya et al. 2001). Moreover, a number of caveats of the CSC model were evident early that limited a general acceptance of the CSC concept and the hierarchical organization of cancer (Visvader and Lindeman 2008).

It is essential to appreciate that the field of CSC research is a work in progress. Specifically, as recently reviewed by Clevers (2011), the meaning of CSC is undergoing a profound re-evaluation in its main postulates. In reality, additional data give a more mature vision of the CSC concept and, at the same time, mitigate an overly enthusiastic approach to its potential clinical applications. These new data, moreover, reply efficaciously to criticism about the existence of CSCs and their pathophysiological role in cancer (Gupta et al. 2009; Maenhaut et al. 2010).

CSC Peculiarities

CSC Plasticity

Importantly, the most intriguing and new evidence about this subpopulation of CSCs seems to be their plasticity (Rapp et al. 2008; Leder et al. 2010). This term introduces a dynamic concept in the CSC definition, with fundamental biological and clinical implications.

For example, Roesch et al. (2010) isolated different melanoma subpopulations of tumor cells, using the H3K4 demethylase JARID1B as a biomarker. Using this technique, the authors characterized a small subpopulation of slow-cycling melanoma cells that cycle with doubling times of >4 weeks within the rapidly proliferating main population. These isolated JARID1B-positive melanoma cells give rise to highly proliferative progeny. Moreover, knockdown of JARID1B leads to an initial acceleration of tumor growth followed by exhaustion, which seems to suggest that the JARID1B-positive subpopulation is essential for maintaining tumor growth. Importantly, the expression of JARID1B was dynamically regulated and did not follow the typical hierarchical CSC model because JARID1B-negative cells can become positive, and even single melanoma cells, irrespective of selection, are tumorigenic. These results seem to suggest a new understanding of melanoma heterogeneity, with tumor maintenance as a dynamic process mediated by a temporarily and dynamically distinct subpopulation of cancer stem-like cells. These data pushed the authors to conclude that at least some stem-like cells from solid tumors may actually not be static entities, but rather tumor cells that may transiently acquire some stemness properties, depending on the tumor context (microenvironment, physical and chemical milieu). Moreover, analogous to normal stem cells, the epithelial–mesenchymal transition (EMT) seems to be a key developmental program that can induce not only the acquisition of mesenchymal traits but also the expression of functional and phenotypic stem cell markers, confirming previous studies (Mani et al. 2008).

At this point, it is already evident that the morphological concept of anaplasia is not static, but now extends to an extremely complex and dynamic, molecular pathophysiological disorder.

Strong evidence about this new complexity originates from the research of Anderson et al. (2011) and Notta et al. (2011). These authors, by adopting a Ph^+ acute lymphoblastic leukemia (ALL) xenograft model, carried out a combined genetic

and functional study of the genomic diversity of functionally defined tumor-initiating cells derived from a diagnostic patient sample. The results clearly showed that multiple tumor clones coexist in the diagnostic patient sample and that these clones undergo divergent evolution from the diagnostic clone, supporting a branching model of tumor progression. Specifically, these genetically diverse subclones seem to be related through a complex evolutionary process and vary in their xenograft growth properties and leukemia-initiating cell frequency. Importantly, this intratumoral heterogeneity seems to promote clonal evolution by increasing the number of selectable traits under any given stress. This selective pressure could contribute to the genetic diversification that is probably important for tumor survival and evolution, which also affects outcomes in terms of clinical aggressiveness.

In practical terms, the evidence that, at diagnosis, genetically distinct subclones already possess variably aggressive growth properties points to the need to develop effective therapies to eradicate all intratumoral genetic subclones, to prevent further evolution and recurrence. In this sense, the ability to segregate even minor subclones in xenografts could be a useful tool for the preclinical development of new therapeutic strategies, but in reality, this ability will likely significantly complicate a true radical therapeutic approach.

From a pathophysiological point of view, the isolation of individual genetic subclones in xenografts could provide an opportunity to study the functional genetic evolution of subclones present in diagnostic samples. Moreover, because gene silencing and other epigenetic events may contribute to tumor progression, a genome-wide methylation analysis of individual subclones would be an interesting undertaking. In fact, this evolution by branching, stressing subclonal complexity, underscores the importance of gaining a better molecular understanding of each subclone. Most important, this research has shown that outgrowth of subclones in serial xenografts can only be sustained by leukemia-initiating cells, establishing that genetic diversity occurs in this functionally important cell type, as well. Moreover, in the opinion of the authors, the discoveries that specific genetic events influence leukemia-initiating cell frequency, and genetically distinct leukemia-initiating cells evolve through a complex evolutionary process, indicate that a close connection must exist between genetic and functional heterogeneity.

All that brings together the classical clonal evolution and the hierarchical model related to CSC. This unifying vision allows consideration of the leukemia stem cell not as a static entity, but as a cell able to evolve genetically in response to the selective pressure of tumor microenvironments. As tumors evolve, the frequency of leukemia stem cells can increase and eventually progress at different grades of differentiation until they lose the characteristics of a CSC.

From a clinical point of view, the isolation of CSCs should be interpreted with considerable care in tumors composed of genetically diverse subclones, as fractionation of CSC and non-CSC populations could segregate genetically distinct subclones with variable tumor-initiating cell capacity, different epigenetic/developmental programs, and possibly different phenotypic peculiarities. Finally, these findings indicate that more commonalities may exist between clonal evolution and CSC

models of cancer than previously thought, and in the future, a unification of these concepts will likely be realized.

Moreover, data from the Anderson and Dick groups confirm a re-evaluation of a Darwinian model for cancer propagating cells and resultant clonal architecture. According to this revisitation, cells with self-renewing properties have varying genotypes that provide the units of selection in the evolutionary diversification and progression of cancer. Moreover, data have shown that sequential and concurrent genotypic variation in propagating cells occur in ALL and are likely to do so in other cancers, providing a rich substrate for disease progression.

Importantly, it is likely that genetic diversity of these new cancer stem-like cells may be associated with both frequency variation and diversity of functional properties, for example, differentiation status, niche occupancy, quiescence and drug or irradiation sensitivity. This picture may help to explain some of the criticisms related to the CSC hypothesis (Visvader and Lindeman 2008; Rosen and Jordan 2009; Greaves 2010).

In summary, plasticity and related genomic diversity in cancer varies in extent with stage of disease (Park et al. 2010; Anderson et al. 2011) and probably with time, but this diversity also varies according to space, depending on the local microenvironments, chemical and physical conditions of each cell, effects of intraclonal competition, and intrinsic genetic instability. In fact, in metastasis or recurrences, for example, data seem to indicate a continued diversification of propagating cells with a prevalence of dominant or therapy-resistant subclones (Scatena et al. 2008; Liu et al. 2009).

In this situation, a CSC-targeted therapy, directed at mutant molecules, may have limited efficacy if the targets themselves are not initiating lesions, but secondary mutations segregated into subclones. In other terms, this genetic and functional variation of cancer-propagating cells may represent a significant roadblock to effective, specific therapy (Scatena et al. 2011). CSCs plasticity thereby seems to reconcile clonal and hierarchical models, but it significantly complicates the pathophysiology of CSCs.

An additional new aspect of CSCs that also indirectly confirms to the concept of plasticity comes from the observation of Visvader (2011), who stressed that the cell of origin of cancer, i.e., the normal cell that acquires the first cancer-promoting mutation, is not necessarily related to the CSCs, the cellular subset that uniquely sustains malignant growth. In other words, the cell-of-origin and CSC concepts refer to cancer-initiating cells and cancer-propagating cells, which should be considered distinct.

Thereby, a stem cell might sustain the first oncogenic hit, but subsequent alterations required for the genesis of a real CSC can occur in descendent cells. For example, in chronic myeloid leukemia (CML), the hematopoietic stem cell (HMS) is the cell of origin in the more indolent phase of the disease, but in patients with CML blast crisis, granulocyte–macrophage progenitors acquire self-renewal capacity through a β-catenin mutation and emerge as the probable CSCs (Jamieson et al. 2004).

Interestingly, the stemness of CSC was indirectly validated by Janic et al. (2010) who showed that a number of genes typically involved in germline programming in

fruit flies were also involved in the formation of glioblastoma. The authors found that inactivation of these germ cell genes can suppress tumor growth. Importantly, some of these genes have a related human counterpart known to be abnormally expressed in certain cancers and not only in glioblastoma.

Further strong evidence of CSC plasticity and/or stemness comes from the observations of Wang et al. (2010) and Ricci-Vitiani et al. (2010), which show that, in addition to recruiting vessels from the outside, glioblastomas may induce vessel formation by differentiating its tumor cells into cancer endothelial-like cells. Specifically, some cancer cells in the immediate environment of the nascent vessel are co-opted for this purpose. The co-opted cells are thought to retain most of their tumor-cell characteristics, while acquiring a limited number of endothelial-cell features. In fact, both authors showed independently that a subset of endothelial cells lining tumor vessels carry genetic abnormalities (i.e., monosomy of Cep 10 or polysomy of Tel19 and LSI22) found in the tumor cells themselves. Moreover, a comparable proportion of a cell population expressing endothelial cell markers and a population of neighboring tumor cells harbored three or more copies of either the *EGFR* gene or other parts of chromosome 7. Such cell populations also shared a mutated version of the oncogene *p53*. Another indicator of the tumor origin of some tumor vessel endothelial cells is that, as well as expressing characteristic endothelial cell markers, such as von Willebrand factor and VE-cadherin, they expressed the nonendothelial tumor marker GFAP. Moreover, the glioblastoma cell population that could differentiate into endothelial cells and form blood vessels in vitro was enriched in cells expressing the tumor stem cell marker CD133. Further, Wang et al. (2010) showed that a clone of cells derived from a single tumor cell, which expressed CD133 but not VE-cadherin, was multipotent in vitro, and these cells may differentiate into both neural cells and endothelial cells.

Interestingly, Ricci-Vitiani et al. (2010), on the basis of this evidence, hypothesized some clinical applications. In fact, studies examining exposure to the clinical antiangiogenesis agent bevacizumab (Calabrese et al. 2007) or to a γ-secretase inhibitor (Gilbertson and Rich 2007) utilizing knockdown shRNA have demonstrated that blocking VEGF or silencing *VEGFR2* inhibits the maturation of tumor endothelial progenitors into endothelium, but not the differentiation of CD133+ cells into endothelial progenitors, whereas γ-secretase inhibition or *NOTCH1* silencing blocks the transition into endothelial progenitors. These data may provide new perspectives on the mechanisms of failure of antiangiogenesis inhibitors currently in use.

In conclusion, such data demonstrate that lineage plasticity and the capacity to generate tumor vasculature of putative CSCs within glioblastoma are strong findings that provide new insight into the biology of gliomas and, above all, into the definition of cancer stemness.

These findings further confirm the pathophysiological role of CSCs in cancer. In fact, the expression of these multipotency factors, normally limited to early developmental stages, may inappropriately contribute "to specify and characterize" CSCs that can divide and differentiate into heterogeneous cell types. Importantly, from a therapeutic point of view, the direct and/or indirect drug-induced loss or inhibition of these stem cell program genes might prevent the formation of CSCs

or lead to their death, thereby facilitating the prevention or cure of cancer. These observations clearly established that cancer cells, during their evolution, might acquire some stem cell peculiarities that are fundamental for the resultant course of disease.

If, on the one hand, this cancer stemness stresses the difference between stem cells and CSCs, which may better be defined as cancer "stem-like" cells, on the other hand, it induces the study of the physiology and pathophysiology of stem cells, allowing a better understanding of the molecular mechanisms that extend these new functional conditions of cancer cell, with dramatic clinical implications. Again, intriguingly, the term "anaplasia" does not seem to contain this new armament of powerful functional capabilities that, until now, have not been characterized by a peculiar morphological picture.

CSC Biomarkers

The revival of CSCs originated from the possibility to isolate these cells by adopting hypothetical, somewhat specific markers (i.e., the original research of Dick (2008) on the CD34+/CD34− fraction from AML). Afterward, other various biomarkers have been discovered that show the peculiarity to be more present in cancer cells with stemness properties, such as the following:

(a) Other cell surface proteins (CD 133, IL-3r, EpCAM, CXC chemokine receptor type 4 (CXCR-4) also known as fusin or CD184)
(b) Peculiar signaling pathways generally related to self-renewal mechanisms (Hedgehog, Notch, Wnt/β-catenin, BM1, BMI, Pten)
(c) Structural and/or functional components of the stem cell niche
(d) Various detoxifying mechanisms (ABC transporters, aldehyde dehydrogenase ALDH)
(e) Telomerase and pathways related to cellular senescence
(f) Oncogenes and oncosuppressors (p16INK4 – Rb)
(g) Cell differentiation-inducing pathways
(h) Various microRNAs

Each marker and its pathophysiological implications in cancer will be discussed in other sections; now it is important to outline that these structural molecules and/or functional pathways are not specific for CSCs but are present both in normal differentiated cells and stem cells. This could partially hamper potential clinical implications of these markers in diagnosis and, above all, therapy of cancer. In fact, recently developed drugs capable of modulating some of these functions and utilized in the preclinical phase have provided interesting results in terms of response to therapy but showed significant side effects (Von Hoff et al. 2009; Yauch et al. 2009).

As already cited, Dick (2008) adopted CD34+CD38− fractions to identify leukemic stem cells. Similarly, Al-Hajj et al. (2003) used the marker combination

CD24–/CD44+ in breast cancer. CD133 has been a widely used CSC marker, despite criticisms that it is also present in normal cells of different organs. From this original research, different authors have adopted several differentiation-clustering panels to characterize CSCs of various origins. However, as previously cited, CSCs may present other structural and functional characteristics that, at least partially, should permit identification (ALDH, Hedgehog, Notch, Wnt/β-catenin, BM1, BMI, telomerase and so on). It is important to reiterate, however, that these characteristics, although they prevail in CSCs, are not unique to this subpopulation of cancer cells. This difficulty to identify and isolate CSCs can impair research on the molecular pathophysiology of CSCs, in particular, and cancer, in general, with significant implications on the therapeutic index of drugs that could selectively target these tumor-maintaining cells. In fact, the possibility to recognize and selectively kill such cells could represent a real revolution in cancer treatment, with beneficial effects on the frequency of recidivism and metastasis. This therapeutic potential has caused an upsurge in research on different molecular aspects of this topic, and some new and old drugs have been rapidly produced to destroy these cells. Some of these molecules are already in the clinical phase, with conflicting results. In reality, actual CSC biomarkers are not specific and are present in normal stem cells, as well as in normal cells from different organs and tissues, a fact that is too often disregarded.

To further complicate the matter, the molecular mechanisms at the basis of CSC are really complex and above all, not static, but highly dynamic. This means that phenotypic and functional characteristics of these cells can vary by minimal influences of microenvironments, rendering their identification and analysis problematic. Just as an example to better understand the serious, but disregarded, aspect of the dynamic plasticity of CSCs, the dissimilar biomarker profiles of human colon CSCs from two different European and American biotechnology companies that produce various CSCs for research purposes are presented below:

- USA company positive markers of human colon CSCs : Vimentin, Variable S100, CEA, Galactosyl Transferase II, CK-7, CK-20, Smooth Muscle Actin (polyp), Bcl2, Ki-67, P504S, Mucin (MUC-1 and MUC-3)
- European company positive markers of human colon CSCs: CD133, CD44, CD34, CD 10SSEA3/4, Oct4, Tumorigenicity (<1,000 cells), Alkaline Phosphatase, Aldehyde Dehydrogenase, Telomerase, Sox2, cKit, Lin28

It is evident that genetic, proteomic, cellular and functional studies of these two groups of CSCs could give different results, with serious consequences in terms of translational research and thereby on pharmacotoxicological implications.

All these facts stress that the present fundamental task, not only from a pharmacological point of view, is the accurate identification/targeting of these CSCs. Such attempts must consider that these cells are tumor stem-like cells with only some aspects typical of physiological stem cells. This sharing of certain structural and functional characteristics not only should permit more selective therapeutic targeting but also may expose normal stem cells to iatrogenic insult, with potentially dangerous side effects.

Identification and Isolation of CSCs by Xenograft Assay

Another debated aspect of CSC validation is related to xenograft assay validity. Xenotransplantation of sorted cancer cells into immunodeficient mice is the choice method to identify CSCs. The transplanted cancer cell should be able to regenerate the original neoplasia. The frequency of cells able to regenerate tumor in the host depends also on the level of mouse immunocompromise, belittling in such a way the concept of CSCs is rare subpopulation of cancer cells (Quintana et al. 2008). Moreover, the frequency of CSCs can be dramatically improved if the species barrier is avoided. On the other hand, in some mouse leukemia models, CSC isolation by tumor cell transplantation has not been obtained. These data are ambiguous, as these models significantly limit the pathogenic role of the microenvironment that, in other different cancer experimental models, seems to have a fundamental importance in driving tumor progression (LaBarge 2010; Allen and Louise Jones 2011). Further, isolation by xenotransplantation could significantly impair morph-functional studies on CSCs because all fundamental niche functions are abruptly modified, with unavoidable alteration of proteome and genome expression of the original CSCs. Importantly, xenotransplantation attests that these cancer stem-like cells may survive and proliferate independently by otherwise fundamental interactions with adhesion molecule and growth factors. This seems to indicate that potential anticancer drugs targeting niche interactions would be, or would become, easily ineffective. Thereby, the interesting experimental results reported by Liu et al. (2011), which show that enforced expression of miR-34a in bulk or purified CD44(+) prostate cancer stem-like cells may inhibit clonogenic expansion, tumor regeneration, and metastasis by directly repressing CD44, should all be validated by in vivo studies. Similarly, the attractive data of Sodir et al. (2011), which showed that short-term systemic Myc inhibition in the (SV40)-driven pancreatic islet mouse tumor model is sufficient to trigger tumor regression by collapse of the tumor microenvironment, with concomitant death of endothelial cells, attenuation of inflammatory cells, vascular collapse, and hypoxia, need to be confirmed with more prolonged studies. In fact, it is fundamental to verify if such plastic and highly adaptable cancer stem-like cells can overcome this molecular stress signaling.

Existence of Distinctive CSCs Biomarkers?

The defined, so-called peculiarities of CSC, in terms of cluster of differentiations, signal transduction pathways, ATP-binding cassette transporters (ABC transporters), and so on, are not specific. It is evident that only one intriguing aspect is truly distinctive, i.e., its genetic, functional and phenotypic plasticity (Woodward and Sulman 2008; Scatena et al. 2011). The extreme adaptability of these cells to minimal variations of the environment recalls, in the opinion of some authors, Darwin's evolutionary theory, with its classic branching pattern of evolution that is based on natural selection. However, considering the high rate of this cellular evolution/

adaptation, it is probably in some ways more complex because it contains a further important factor, i.e., the mutator phenotype of cancer cells (Bristow and Hill 2008; Brégeon and Doetsch 2011).

This definition originates from the well-known observation that malignancies are characterized by a high rate of mutations. Normal human cells replicate their DNA with exceptional accuracy. It has been estimated that approximately one error occurs during DNA replication for each 10^9–10^{10} nucleotides polymerized. Typically, malignant cells exhibit genetic instability, which causes multiple chromosomal abnormalities and thousands of alterations in the nucleotide sequence of nuclear DNA that tend to progressively accumulate. Pathogenic mechanisms, which accelerate this process, may be favored carcinogenic pathways. Mutator mutations are, in fact, mutations in genetic stability genes that increase the mutation rate, speeding up the accumulation of oncogenic mutations. The mutator hypothesis states that mutator mutations play a critical role in carcinogenesis (Beckman 2010).

Importantly, this mutator phenotype can be not only the starting point for tumor development but also might promote the emergence of a more aggressively growing tumor, frequently characterized by the appearance of poorly differentiated cells with some typical properties of a more embryonic phenotype. Moreover, during the tumor course, considerable biochemical heterogeneity becomes manifest in the growing tumor and its metastases.

Thereby, the loss of genetic stability is expected to increase the rate of growth-promoting or survival-promoting mutations that could drive tumor growth. Importantly, genetic instability may also increase the rate of deleterious mutations that could kill cells before they develop into tumors. Understanding how these factors balance out will ultimately be the key to understanding tumor development via genome destabilization. Moreover, understanding this balance may also have clinical implications for cancer diagnosis, prognosis, and therapy. If deleterious, genome-destabilizing mutations with their phenotypic counterparts are found in the population of developing cancer cells, these targets may provide opportunities for more efficacious diagnostic and therapeutic procedures (Barbie et al. 2008).

Specifically, the unscheduled alterations caused by genetic instability may be either temporary or permanent within the genome. These genetic changes are generally categorized into two major sites of instability, at the chromosomal level and at the nucleotide level (Perera and Bapat 2007).

At the chromosomal level, for example, telomere attrition has been correlated with genome instability. The shortest telomeres, in fact, can cause telomere fusions and genomic rearrangement. Thus, telomere-related carcinogenesis may involve induction of senescence by shortened telomeres, followed by primary genomic instability, leading to acquisition of mutations in cells. Some mutations may provide a proliferative advantage. The induced cell proliferation may induce further telomere shortening. Telomeres that are shortened below their stability threshold can induce breakage-fusion-bridge (BFB) cycles, formation of dicentric or ring chromosomes, and so on (Raynaud et al. 2008).

Instability at the nucleotide level occurs because of faulty DNA repair pathways, such as base excision repair and nucleotide excision repair, and includes instability

of microsatellite repeat sequences (MSI) caused by defects in the mismatch repair pathway. The second form of instability, chromosomal instability (CIN), defines the existence of an accelerated rate of chromosomal alterations, which result in gains or losses of whole chromosomes, as well as inversions, deletions, duplications, and translocations of large chromosomal segments. Aneuploidy, which refers to an abnormal karyotype, is a hallmark of many cancer cells and is thought to develop as a result of CIN. To date, several pathways and processes have been implicated in CIN including the following: (1) pathways involved in telomere and centromere stability, (2) cell cycle checkpoint pathways and kinases, (3) pathways regulating diverse proteins via posttranslational modifications, (4) sister chromatid cohesion and chromosome segregation, and v. centrosome duplication.

Valeri et al. (2010) and Tili et al. (2011) have proposed a new, fascinating cause of genetic instability. These authors showed that miR-155 might significantly down-regulate the core MMR proteins hMSH2, hMSH6, and hMLH1, inducing a mutator phenotype and MSI. Moreover, Tili et al. (2011) showed that miR-155 enhances the mutation rate by simultaneously targeting different genes that suppress mutations and even can reduce the efficiency of DNA safeguard mechanisms by targeting cell-cycle regulators, such as WEE1. In conclusion, by simultaneously targeting tumor suppressor genes and inducing a mutator phenotype, miR-155 could allow the selection of gene alterations required for tumor development and progression.

Genetic instability in cancer may also depend on abnormal protein synthesis because of the following: (1) lapses in RNA polymerase (RNAP) fidelity, generating aberrant transcripts that are translated into erroneous proteins; (2) lapses in ribosome fidelity, caused by exposure to a genotoxic agent; and (3) modification of RNA molecules that could induce the production of erroneous proteins during translation because of their potentially altered codon–anticodon pairing during tRNA selection. This transcriptional mutagenesis, which alters proteins and possibly changes the physiology of the cell, could be crucial for cancer stem origin because, as opposed to the DNA replication-dependent production of erroneous proteins, the lapses hit quiescent or slowly replicating cells (Brégeon and Doetsch 2011).

For completeness, it could be useful to stress that derangement of cellular metabolism could also have a role in genetic instability of cancer cells, in general, and CSC, in particular. In fact, in an experimental model of radiation-induced genomic instability (Dayal et al. 2009), mitochondrial dysfunction of complex II caused increased steady-state levels of hydrogen peroxide, which increased mutation frequency and induced gene amplification. These results seem to indicate that mitochondrial ROS could have a role in inducing genetic instability. These data, when applied to the intriguing metabolism of CSC, open an interesting field of research.

Finally, it is useful to cite the work of Conway et al. (2009), who showed that CSC generation is associated with the acquisition of nonclonal genomic rearrangements not found in the original population. This study was carried out in a transplantation model of testicular germ cell tumor, created by transplanting murine embryonic germ cells into the testis of the adult severe combined immunodeficient mouse model. Interestingly, pretreatment of EGCs with a potent inhibitor of self-renewal, retinoic acid, prevented tumor formation and the emergence of genetically

unstable CSCs. Moreover, microarray analysis revealed that EGCs and first- and second-generation CSCs were highly similar. Further, approximately 1,000 differentially expressed transcripts could be identified that corresponded to alterations in oncogenes and genes associated with motility and development. In the opinion of the authors, these data suggest that activation of oncogenic pathways in a cellular background of genetic instability, coupled with an inherent ability to self-renew, is involved in the acquisition of metastatic behavior in the CSC population of tumors derived from pluripotent cells.

Conclusions

The evidence that cancer cells may assume some functional characteristics of stem cells is substantially modifying cancer research. In fact, CSCs not only have led to the consideration that cancer is caused by a morphologically heterogeneous population of malignant cells but also have focused the attention of researchers on a particular subpopulation of cancer cells with intriguing, yet too often disregarded, functional, and consequently clinical, implications.

The pathophysiology of these particular malignant cells is progressively becoming more complex with the advances in the understanding of their various biological properties, from a simple vision of stem cells that acquire a malignant phenotype, maintaining some of their typical characteristics (i.e., self-renewal, differentiation, proliferation potential), to a model of a neoplastic cell with extraordinary genomic plasticity that permits adaptation, also by assuming some functions of stem cells, to the minimal modifications of the microenvironment to satisfy their primordial need, i.e., proliferation.

The original definition of CSC pointed to the research on stem cells, which, across their long lives, may easily undergo and accumulate mutations that cause neoplasia. This pathogenesis may perfectly adhere to the hierarchical model of cancer proliferation. Moreover, this definition of CSC permits the adoption of some typical biomarkers of stem cells, to selectively target "transformed" stem cells.

This targeting of various subpopulations of isolated CSCs has produced innovative, but debated, results. Most important, the possibility of targeting the cells responsible for recidivism and/or metastasis has induced a series of pharmacological studies on potential anti-CSCs drugs. Considering the peculiarity of these biomarkers (clusters of differentiation, signal transduction pathways including Hedgehog, WNT, TK), some preclinical and clinical studies have shown interesting results, but the real therapeutic index should be evaluated, considering, above all, the partial selectivity of these biomarkers for CSCs.

It is probably time to update the definition of CSCs. It could be sufficient to stress, as already adopted from some authors concerning "cancer-maintaining cells" or "cancer stem-like cells," the differences between these cells may be not only phenotypic and functional but also origin. Moreover, the frequently adopted definition of "tumor-initiating cell," as recently reviewed by Visvader (2011), should be limited

to that stem, progenitor, or terminally differentiated cell that presents the first mutative hit leading to cancer. This cell could be different from, or subsequently become, the cancer "stem-like" cell.

Finally, it is beyond doubt that CSCs have stimulated attention toward some aspects of the pathophysiology of cancer, which, until recently, have been neglected, specifically:

- It is clear that metabolism of these cells, which can be considered dormant or with a low proliferation index, should be different from that of classical highly proliferating cancer cells, justifying a re-evaluation of the Warburg effect (Scatena et al. 2010), which could mean that all cancer cell metabolism should be revisited according to this functional heterogeneity of cancer cells. Could metabolic drugs, capable of inhibiting the cancer "stem-like cell" and inducing cell differentiation toward more specialized and less multipotent cancer cells, be developed?
- What is the role of the epigenome in maintaining the genetic program induced by selection pressure and/or genetic instability? Could epigenetic drugs, capable of deranging the mechanisms that permit the cancer cell to acquire its high and dramatic genetic plasticity, be developed?
- Could it be possible to pharmacologically target the molecular mechanisms at the basis of this pathogenetically relevant genetic plasticity of cancer "like-stem" cells?

In conclusion, the advances in knowledge on CSCs are confirming that the clonal and hierarchical models of cancer growth coexist, at least at some points, during neoplastic evolution.

Moreover, the complex molecular pathophysiology of the cancer cell, in general, and the CSC, in particular, should be considered when discussing the anaplastic cell.

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Chapter 2
Stem Cells and Cancer Stem Cells: New Insights

Toru Kondo

Prologue to Cancer Stem Cell Research

Although the concept of the cancer stem cell (CSC) was advocated more than several decades ahead, it was not accepted widely due to the lack of a direct proof method. However, recent progresses in the stem cell biology and developmental biology revealed that cancers contain the hierarchy similar to normal tissues and that only CSCs in tumors have a strong self-renewal capability and are malignant (Fig. 2.1) (Reya et al. 2001). It is thought that the existence ratio of CSCs is several percent or less in tumors and cancer cell lines and the other cells (non-CSCs) are either cancer precursor cells, which have limited proliferation ability, or nondividing cancer cells. Together these findings suggest that characterization of CSCs is essential for the curable cancer therapy.

Definition of CSCs

CSCs were initially defined by their extensive self-renewal capacity, tumorigenicity, and multipotentiality. As a number of oncogenes, including *inhibitor of differentiation* (*Id*), *hairy and enhancer of splits* (*Hes*) and *Notch*, are expressed in CSCs as well as tissue-specific stem cells (TSCs) and block cell differentiation, it remains uncertain as to whether CSCs actually give rise to multilineage cells. Further evidence also exists suggesting that cancer cells co-express a number of lineage-specific

T. Kondo (✉)
Laboratory for Cell Lineage Modulation, RIKEN Center for Developmental Biology,
2-2-3 Minatojima-Minamimachi, Chuo-ku, Kobe, Hyogo 650-0047, Japan

Department of Stem Cell Biology, Ehime University Proteo-Medicine Research Center,
Shitsukawa, To-on, Ehime 791-0295, Japan
e-mail: tkondo@m.ehime-u.ac.jp

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