Killing Time for Cancer Stem Cells (CSC): Discovery and Development of Selective CSC Inhibitors

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Abstract: Can cancer be cured or will it have to be controlled as a chronic disease? Despite a better understanding of the biology of tumour cells, the treatment of most cancers has not significantly changed for the past three decades. Are current cancer drugs targeted at the wrong kind of cells? Accumulating evidence has implicated that cancer is a disease of stem cells. In this context, a small fraction of cancer cells adopt the properties of stem cells. In some cases, the cancer stem cells (CSC) could be the close derivative of normal tissue stem cells. In either situation, the net result will be the same, in that CSC are the cells to be used as targets in the development of molecular and pharmaceutical therapies to treat and prevent human cancer. This could be a paradigm shift in the treatment of cancer, away from targeting the blast cells and towards the targeting of the CSC. A challenge to this approach will be to find a way to specifically target CSC without toxicity to normal cells. In this article, we propose how CSC can be used in therapy programs (target identification, drug discovery, etc.). Therefore, in the future, it might be possible to rid a patient of all his/her cancer cells, including the cancer stem cells.

Key words: Cancer, cancer stem cells (CSC), stem cells, mouse models, CSC inhibitors, drug discovery.

INTRODUCTION

An axiom in the treatment of tumours is that the remission is, in general, more difficult to achieve with each relapse. Despite a better understanding of the biology of tumour cells, the treatment of most cancers has not significantly changed for the past three decades and the decreasing mortality has been the result of early detection and prevention, rather than the consequence of effective therapeutics [1].

For decades, tumour initiation and development have been regarded as a multistep process, reflected by the multiple genetic alterations found in the tumour mass cells that drive the transformation of normal human cells into highly malignant derivatives [2], and by the absence of animal models able to reproduce the human cancer with only one genetic mutation. Currently, increasing evidences indicate that the events leading to tumour initiation are orchestrated by cancer stem cells (CSC) [3-6].

CANCER STEM CELL HYPOTHESIS

Although many tumours contain cells that display stem cell like-features, the identity of the stem cell that acquires the initial genetic mutation leading to tumour formation has remained elusive [7]. A normal stem cell is defined by its dual properties of self-renewal and multilineage differentiation potential, repopulating the mature cells of the organ system that it serves [3,8]. A cancer stem cell would function in a similar way to sustain the growth and spread of tumours, however, a cancer stem cell would not be subject to the same intrinsic and extrinsic controls as normal stem cells.

The "cancer stem cell hypothesis" about the origin of cancer represents a modern day interpretation of that suggested by the pathologists Rudolph Virchow and Julius Cohnheim 150 years ago, based on the histological similarities between the developing foetus and certain types of cancers, such as teratocarcinomas [9,10]. Evidence for cancer stem cells was first documented in the haematological malignancies, acute and chronic myeloid leukaemias, where only a small subset of cancer cells, $CD34^{+}CD38^{-}$ (stem cells immunophenotype), was able to form new tumours [11,12].

Recent studies in solid tumours indicate that the concept of cancer as a hierarchy initiated and maintained by a rare population of stem cells may have larger implications beyond haematopoiesis field. Identification in the previous years of breast cancer stem cells [13] and characterisation of central nervous system stem cells responsible for the maintenance of some brain tumours [6,14], have increased the evidence of the veracity of rare cancer stem cells that drive the formation of a number of different tumours types, raising the question of whether all cancers originate from and are maintained by cancer stem cells [8,15].

ARE CURRENT CANCER DRUGS TARGETED AT THE WRONG KIND OF CELLS?

If cancer is maintained by cancer stem cells (CSC), and characterised by low rates of division and proliferation, it is clear that present therapies such as chemotherapy or radiation, which depend on high division and proliferation rates, would not be effective at targeting CSC [16,17]. Certain antigens, indeed, currently targeted by biological treatments may not be expressed on CSC surface due to their in immature state moreover, if CSC are the driving force of

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tumour formation and current therapies are orientated to reduce the main tumour mass, the initial tumour reduction can be a fact, sparing the cancer stem cells and causing the relapse to be inevitable Fig. (**1**).

Tumours of many, if not all organs contain a cancer stem cell population responsible for tumour growth. The identification and characterisation of this group of cells, the common and different properties between these and their normal stem cells counterparts will provide novel targets for future cancer therapies.

MOUSE MODELS MIMICKING HUMAN CANCER AS A SOURCE OF CSC

A recurring theme in the molecular pathogenesis of human cancer is the activation/inactivation of cellular genes. Understanding these genetic pathways is the prerequisite for the development of molecular and pharmacological therapies to treat and prevent cancer. However, cancer is a disease of the organism, and not simply of the abnormal cells, although cell lines have led to remarkable advances in our understanding of the molecular and biochemical changes in cancer cells, their use in the identification of effective cancer therapies is limited [16,18]. The fact that biological properties of cancer cell lines differ from the cancer cells from which they were derived can explain, at least in part, why cell lines are often poor predictors of drug efficacy in a clinical setting [16].

In order to accurately identify the genetic pathways responsible for cancer development, it is necessary to perform experiments in an *in vivo* setting in which neoplasm emerges in the appropriate microenvironment [19,20]. Research in mice has integrated the complexity of an organ and its different cell types with the dynamic physiological status of the animal [21]. Therefore, the generation of mouse models mimicking human cancer pathology must be a prerequisite not only for understanding of the genesis and maintenance of human cancer, but also for the development of molecular and pharmacological therapies to treat and prevent human cancer.

Most of our current conceptualisation of how tumourigenesis occurs in humans is strongly influenced by mouse models of cancer development [22]. Several different mouse models have been generated in the last decades. Murine models created by i) constitutive transgenic expression [23,24], ii) germline-knock-out [25], and iii) inducible oncogene expression [26-29] (tissue-specific expression of oncogenes is induced by small molecules such as doxycycline or tamoxifen) have led to major advances in our understanding of the molecular and genetic pathways involved in tumourigenesis and malignant transformation. Conversely, in these models, the mutation occurs in every cell of the organism or tissue, unlike the genetic lesions in human cancer that occur sporadically in single cells during prenatal or postnatal development.

In contrast to the germline-transgenic or knock-out mice, models now exist in which the tumour-initiating genetic event occurs at the level of the individual cell by conditional gene targeting [30,31]. These are more accurate models of the clonal origin of naturally occurring spontaneous human tumours, and these approaches enable tumour growth to occur in a field of genetically wild-type neighbouring cells.

The recent discoveries that critical genetic events take place within somatic primitive cells in some human cancers have led to enthusiasm within the scientific community for generating cancer mouse models, targeting specific

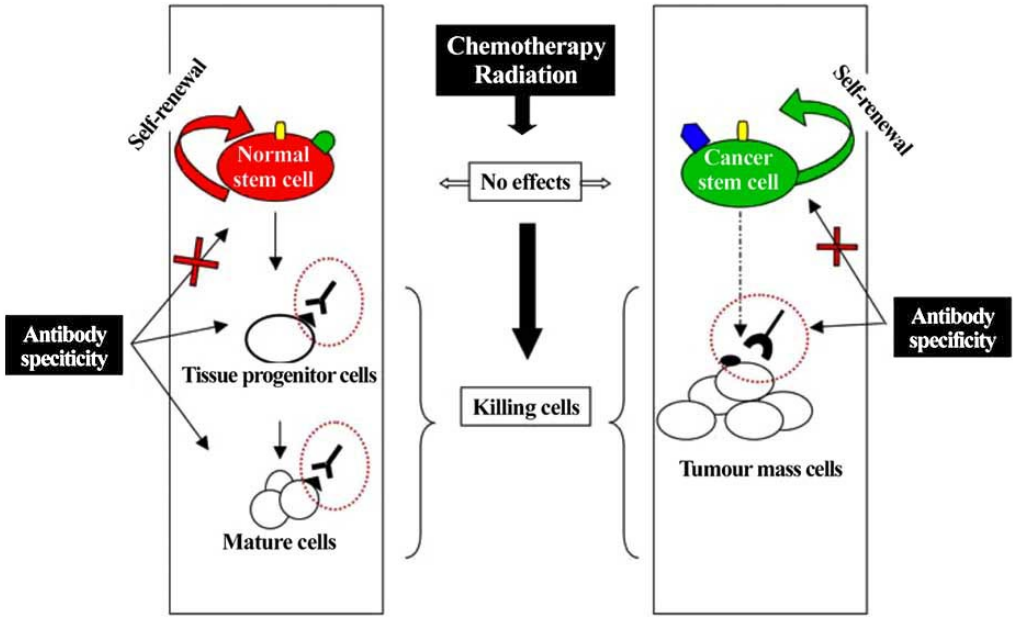

Fig. (1). Current anticancer therapies in the stem cell/cancer stem cell and mature cell populations. The increasing idea of the cancer stem cells being the source of origin of cancer has swayed the recent therapeutic intervention directed to the cancer cell mass. If cancer results from cancer stem cells, characterised by very low rates of proliferation and division, it has become clear that therapies such as chemotherapy or radiation, were dependent on high division and proliferation rates, and new antibodies designed against mature cell antigens, would not be effective at targeting CSC.

mutations to stem cells using specific promoters. Subsequently, studies in mice in which alteration has not been directed to the specific cells of origin (stem cells), as in current mouse models, must be interpreted cautiously.

HOW TO MANAGE CSC MOUSE MODELS EFFECTIVELY FOR DRUG DISCOVERY SUCCESS

The era of the chemotherapy began in the 1940s with the first uses of nitrogen mustards and antifolate drugs. Since then, cancer drug development has been transformed in a multi-billion industry. Cytotoxic chemotherapy or radiotherapy of cancer is limited by serious side effects that arise from toxicities to sensitive normal cells. The conventional chemotherapy is mostly based on the evidence that proliferating cells are more sensitive to anticancer agents than non-dividing cells. This is the main reason why these compounds are not tumour specific and their selectivity is generally in favour of rapidly growing cells (haematopoietic or intestine. i.e.), rather than discriminating against any fundamental biological difference between normal and tumour cells. In addition, the toxicity invariably associated with drug treatment limits the dose of the drug that can be used and the low therapeutic index prevents the clinical development of potentially effective agents. A further obstacle to the success of treatments is that after a first line of successful treatment, the majority of tumours have a chemotherapy sensitivity relapse in developing resistance mechanisms not only towards the same class of drugs, but also against structurally different compounds ("multi drug resistance" or MDR) [32,33].

A significant fraction of current research in this area is now moving away from relatively non selective cytotoxic drugs towards the new generation of molecular therapeutic agents that target the key molecular abnormalities that drive malignant transformation and progression and which, as a result, have a fundamental impact on cancer cell survival. Indeed, the current view of anticancer agent drug discovery and development is based on the concept that more selective and effective therapies (and thus lesser harmful to normal cells than traditional cytotoxic agents) will emerge by identifying the genetic defects that create and drive the malignant phenotype. These attempts are also supported by the recent advent of new technologies (such as "microarrays" and "protein-arrays") and the progresses made in the field of molecular cell biology [34].

This transition represents an important advance, but the basic principles for cancer treatment and drug resistance, as developed in the period from 1950 to 1980, remain the same. All therapies, targeted or non-targeted, play a significant role in reducing the proliferation of cancer cells. In addition, targeted therapies showed a clinical benefit in only small subpopulations of patients, where as in extended clinical trials, patients treated with these target therapies encountered a relapse. The challenge therefore remains in how to design and develop novel cancer treatments to select effective targeted anticancer drugs? In other words, can cancer be cured?

A significant and successful effort by our laboratory in the last years has led to the development of an *in vivo* genetic cancer stem cell mouse model system, based upon alterations in cancer stem cells. These models, in contrast to other mouse cancer models, accurately recapitulate the human cancer pathology and its response (and lack of response) to commonly used therapeutic agents, representing an important resource which, if used effectively, can be the basis for the design of novel, broadly used and commercially successful therapeutic agents Fig. (**2A-2B**). Recent unpublished data about cancer stem cells has already shown that i) *in vivo* ablation can eliminate cancer, ii) CSC differs from the normal stem cells, allowing the opportunity to select and remove these cells specifically without causing

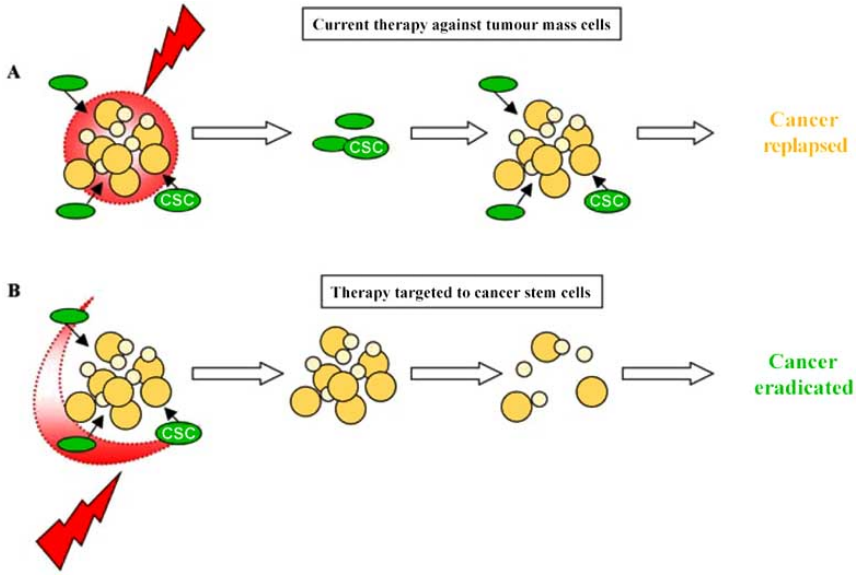

Fig. (2). Cellular target guides therapeutic result. Nowadays, human solid tumours treated with current therapies often shrink. 2A) The elimination of the bulk of cancer cells, in these cases, permits not only the initial tumour reduction but also spares the cancer stem cells and results in an inevitable relapse. 2B) Design of new therapies targeted to cancer stem cells will eliminate cells responsible of tumour regeneration and, consequently, patient relapses.

unacceptable side effects in the normal population, and the most important iii,) CSC from different cancer types are similar, implying that a similar therapeutic approach can have a broad range of applications in many different cancers. The major challenge faced is to find a way to specifically target these cancer stem cells, but as an initial step it is necessary achieve some major tasks such as the generation of new and improved animal models and the identification of novel CSC targets.

GENERATION OF NEW AND IMPROVED ANIMAL MODELS: MOLECULAR IMAGING.

Although we have effectinely developed available models for CSC, for the discovery and testing of CSC-related targets in haematopoietic and lung cancers, data recovered in the last years have revealed the use of these possible targets for a wide range of additional cancers. Nevertheless, it will be of high value to develop new mouse models for common solid cancers such as colon, breast and prostate, able to reproduce the human disease by directing the typical genetic lesion to the stem cells population. While new approaches to mouse modelling have taken place over the past decade, parallel advances have occurred in molecular imaging that permit the characterisation and measurement of molecular events in living animals with high sensitivity and spatial resolution [35,36]. The development of dedicated small animal imaging equipment for micro-computer tomography and optical imaging has facilitated an accelerated progress of this technique in recent years. The creation of a new generation of mouse models, designed in a suitable way for advanced molecular imaging techniques, will allow a similar cancer approach to those currently being used in the clinic, providing a potent powerful tool to study different protein activities during tumour initiation and progression in a living animals model. This imagining assessment, in combination with innovative target therapies, would allow assessment of therapeutic effectiveness at a molecular level, long before phenotypic changes occur [35]. One of the key elements, however, necessary for sampling molecular information is the use of special imaging probes with high specificity. It is this reason that application of gene expression profile might be used to select innovative imaging approaches for cancer with the combination of functional and anatomical imaging Fig. (**3**).

IDENTIFICATION AND VALIDATION OF NOVEL CSC TARGETS.

The feasibility of using gene expression profiling to identify molecular targets for imaging different cancers, such as lung, recently published [36], has shown the advantages of these two new techniques in the cancer research field. Insights into the genes and gene pathways that regulate stem cell function will advance not only our basic understanding of stem cells, but also the entire regenerative medicine and cancer fields.

Although stem cell functions might have a stochastic component, increasing evidence indicates that extrinsic signals from the stem cell microenvironment, or niche, can converge intrinsic cellular signals to regulate stem cell proliferation and cell fate functions [37]. The specific signals involved, however, are poorly understood. Indeed, several

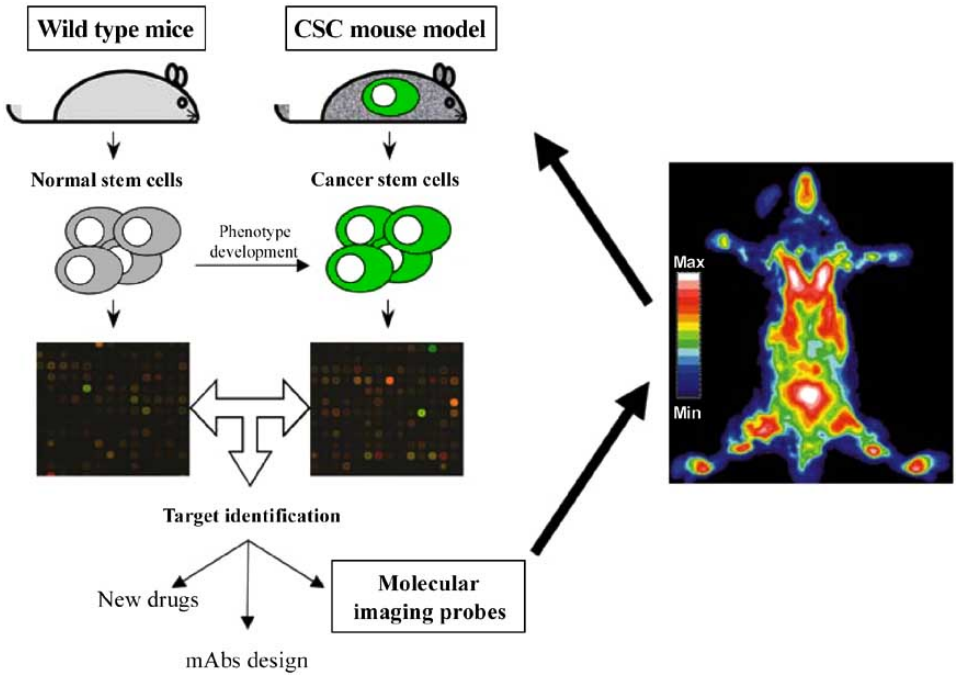

Fig. (3). Gene expression profile applications in the selection of innovative imaging approaches for cancer. Gene expression profile analysis of CSC mouse models at different stages of cancer development and in different cancer types, versus normal stem cells, has provided enough information about target genes specific for these CSC, offering a large range of potential drug therapeutic targets and *in vivo* models analysis. Designing specific probes through the CSC signature will provide a powerful tool to study different genes activities during tumour initiation and progression.

studies in the last years have tried to define a specific gene expression pattern of both, embryonic and adult stem cell populations [38,39] and, even if these analysis have provided some insights into the genetic mechanisms responsible for the stem cell phenotype, there are clear inconsistencies in the resultant lists of stemness genes [40]. Gene expression profile analysis of CSC mouse models at different stages of cancer development and in different cancer types has provided relevant information about certain specific target genes for these CSC and for CSC maintenance in respect of the normal stem cell population, offering a large range of potential drug therapy targets. Some of these targets could be of relevance for drug discovery, because of their characteristics (kinases, cell-surface receptors, ion channels, etc) or could be analysed in *in vivo* models for their ability to detect cancers at an early stage, as well as because of their ability to predict early therapeutic outcomes Fig. (**3**).

DISCOVERY AND DEVELOPMENT OF SELECTIVE CSC INHIBITORS

To treat cancer effectively, novel therapies must be developed to eliminate CSC. Targets obtained and validated from CSC mouse models as described above, provide the basic tools to design such drugs. These targets can be used to identify either small molecule or antibody-based drug candidates, due to the fact that not all these candidate targets will be suitable to generate antibody drugs.

1. Use of CSC and CSC Targets for Small Molecule Drug Screening

To identify agents that interfere specifically with CSC resulting in cell death, it will be necessary to develop CSC-based and CSC-target assays able to detect specific inhibitors of cancer cells in cell cultures. Although cell lines have led to important advances in the understanding of the molecular biology of cancer as we have previously mentioned, cell lines are imperfect predictors of drug efficacy in human tumours, mainly because of the different biological properties between cancer cell lines and *in vivo* human cancer cells. The use of CSC originating from CSC mouse models, able to reproduce the human disease associated to a specific genetic defect, will allow us to eliminate differences in biological properties found in cancer cell lines, there by representing a better assay to detect CSC inhibitors independently of the molecular mechanism of the particular compound.

2. Use of Targets to Generate Antibody Therapeutics

A century ago, Paul Ehrlich hypothesised that a "magic bullet" could be developed to selectively target the disease. This vision became practical with the development of hybridoma technology. Effective monoclonal antibody (mAb)-based therapies for the treatment of cancer have proved, however, more elusive than originally envisioned. Owing to their murine origins, the first generation of mAbs evaluated in the clinic were limited by their immunogenicity and poor ability to recruit immune effector mechanism [41,42]. In the last years, modifications to create mAbs with more human characteristics have attenuated the foreign nature of these proteins and allowed them to interact efficiently with the receptors expressed on immune effector cells. The next generation of mAbs currently under development, incorporated additional beneficial modifications like the increased ability, to penetrate in solid tumours [43]. Antibody affinity will need tuning and customisation to inhibit binding to normal tissues, to improve tumour penetration and retention, and to optimise anti tumour effects. The identification, using gene expression profiles of new functional targets and epitopes on cancer stem cells

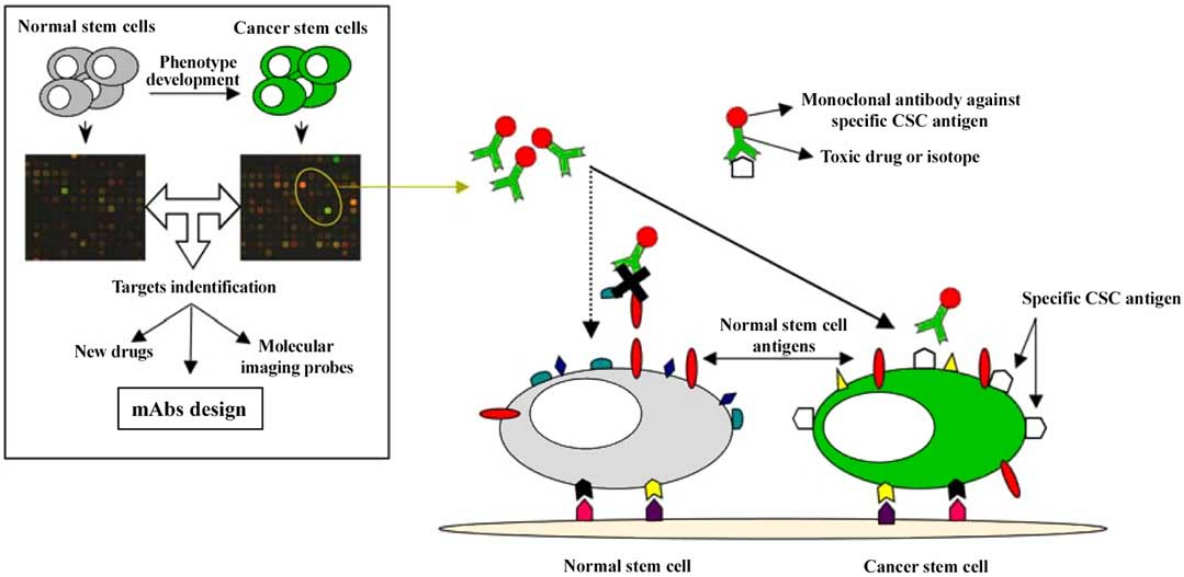

Fig. (4). Using CSC gene expression profile in the generation of therapeutics mAbs. Creation of modified mAbs with more human characteristics in the last years has allowed the efficient binding of these with the receptors expressed on immune effector cells. The identification, using gene expression profiles of new functional targets and epitopes on cancer stem cells from our CSC mouse models, would allow us to generate improved specific inhibitory antibodies capable to recognise and eliminate cancer stem cells responsible for the maintenance of the cancer cell population.

from our CSC mouse models, would allow us to generate new specific inhibitory antibodies capable of marking, following and destroying stem cells responsible for the maintenance of the tumour cell population Fig. (**4**).

3. Identification of Biomarkers

Individual molecular markers and patterns of markers are sought after by oncologists and pharmaceutical companies, in order to successfully identify and predict patient survival and/or selection of the most satisfactory therapy. In this sense, CSC mouse models are also a unique source to identify biomarkers able to monitor the stages of cancer and guide therapeutic application in humans. Detailed analysis of CSC from CSC mouse models by gene expression has already provided a large inventory of candidate biomarkers with projected value of risk assessment, screening, diagnosis, prognosis, and selection of monitoring of therapy. Moreover, CSC strategy derived from biomarkers could be important not only after diagnosis of a particular disease (in monitoring therapy, selecting additional therapy and detecting recurrence) but also before disease diagnosis, discerning between local and/or disseminated cancer, as CSC mice are programmed to develop cancer.

Currently, we have identified the first biomarker that serves as i) an early marker of dissemination in a broad spectrum of human carcinomas (breast, ovarian, colon) and ii) has helped to monitor treatment benefit and relapse [44]. Biomarkers validation and development require, alternatively, a number of stages illustrated in Fig. (**5**), before they can be considered for clinical practice.

The identification of CSC similarities between different CSC populations from different types of cancer would expand the range of cancers that can be effectively treated from this technology.

CONCLUSION

Current results have pointed out the existence of a subset of cells in a tumour, capable of self-renewal and very much effective in regenerating the tumour when serially grafted into recipient animals. These cells with tumour regenerating capacity have been termed as “cancer stem cells”. The term “cancer stem cell” relates to a broad group of cells that share some common properties, such as self-renewal and the ability to maintain a tumour. Considering the fact that these CSC are the driving force of tumour formation, the principal challenge will be to identify and characterise this group of cells. It has been suggested that the self-renewal and multilineage differentiation characteristics of stem cells is the result of a genetic programme that is common to stem cells of all origins and, therefore, stem cells may conserve a universal molecular signature. The inability, however, to identify a consensus stem cell expression signature nowadays by different groups, is probably due to differences

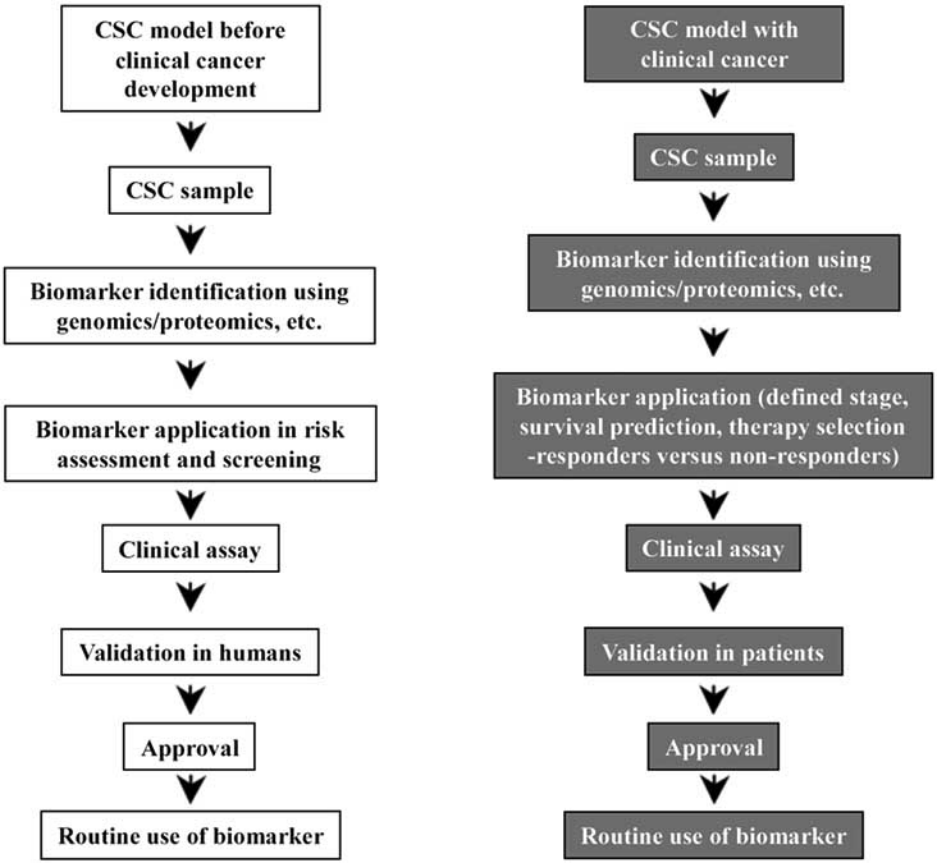

Fig. (5). Identification and validation of biomarkers before and after cancer development. Illustration of the different stages recommended for a fine validation and development of improved biomarkers in the clinical practice. Detailed analysis of CSC from CSC mouse models gene expression has already provided a large inventory of candidate biomarkers and it maybe important not only after diagnosis of a determined disease but also before disease diagnosis.

in the technical resources, stem cells population isolation, and even phenotypic definition of the interesting population, which has led some researchers to question this common signature idea.

In this chaos of a defined idea, the existence of a cancer stem cell, and the absence of proofs that demonstrate the gene expression similarities in the common properties between stem cells, in the last years we have been led to the development of an *in vivo* genetic cancer stem cell mouse model system, based upon alterations in cancer stem cells able to recapitulate the human cancer pathology. Our group has shown that CSCs from different cancer types are similar, implying that a similar therapeutic approach could be used in many different cancers. The challenge is now to find a way to specifically target CSC without causing toxicity to normal cells.

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