



CENTRE DE RECERCA MATEMÀTICA

This is a preprint of: *Metabostemness: a new cancer hallmark*
Journal Information: *Frontiers in Oncology*,
Author(s): J.A. Menendez, T. Alarcon.
Volume, pages: 262 1-21, DOI:[doi.org/10.3389/fonc.2014.00262]



Metabostemness: a new cancer hallmark

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The acquisition of and departure from stemness in cancer tissues might not only be hardwired by genetic controllers, but also by the pivotal regulatory role of the cellular metabolotype, which may act as a “starter dough” for cancer stemness traits. We have coined the term metabostemness to refer to the metabolic parameters causally controlling or functionally substituting the epitranscriptional orchestration of the genetic reprogramming that redirects normal and tumor cells toward less-differentiated cancer stem cell (CSC) cellular states. Certain metabolotypic alterations might operate as pivotal molecular events rendering a cell of origin susceptible to epigenetic rewiring required for the acquisition of aberrant stemness and, concurrently, of refractoriness to differentiation. The metabostemness attribute can remove, diminish, or modify the nature of molecular barriers present in Waddington’s epigenetic landscapes, thus allowing differentiated cells to more easily (re)-enter into CSC cellular macrostates. Activation of the metabostemness trait can poise cells with chromatin states competent for rapid dedifferentiation while concomitantly setting the idoneous metabolic stage for later reprogramming stimuli to finish the journey from non-cancerous into tumor-initiating cells. Because only a few permitted metabolotypes will be compatible with the operational properties owned by CSC cellular states, the metabostemness property provides a new framework through which to pharmacologically resolve the apparently impossible problem of discovering drugs aimed to target the molecular biology of the cancer stemness itself. The metabostemness cancer hallmark generates a shifting oncology theory that should guide a new era of metabolo-epigenetic cancer precision medicine.

Keywords: stemness, metabolism, reprogramming, cancer stem cells, oncometabolites, Waddington, epigenetic landscapes

INTRODUCTION

We are accumulating ever-growing evidence that metabolism and stemness are highly intertwined processes in tumor tissues. Cancer is beginning to be understood as a disease of reprogramming that appears to involve the progressive resetting of the metabolic infrastructure and metabolite levels concomitantly with changes in cellular differentiation (1–9). The modulation of metabolism and associated signaling is being increasingly implicated in the determination of cell identity during nuclear reprogramming and oncogenesis. The transformation of cellular metabolism precedes changes in stemness and, therefore, metabolic reprogramming appears to reflect the molecular dynamics fundamental for the rearranging and redirection of cell-fate (10–30). Moreover, we have recently learned that certain metabolites can be oncogenic themselves and, crucially, the malignant activity of these oncometabolites, i.e., small-molecule components (or enantiomers) of normal metabolism whose accumulation causes signaling dysregulation to establish a milieu that initiates and drives carcinogenesis (31–45) likely relies on their ability to epigenetically block the acquisition of differentiation markers while inducing the expression of stem cell maintenance genes. A key question, however, remains unanswered: how can metabolism and metabolites

exert influence over the transcriptional factors, the chromatin structure, and the epigenetic circuits that establish and maintain the self-renewal and differentiation capacities owned by cancer stem cells (CSCs), which are suggested to drive tumor-initiation and metastatic progression?

Herein, we propose that the molecular logic behind the conversion of non-CSCs into CSCs can be better understood in terms of cellular metabolotypes that operate as pathways or roadblocks by facilitating or impeding, respectively, the epitranscriptional orchestration of the genetic reprogramming that drives the intrinsic and microenvironmental paths to CSC cellular states. We therefore postulate that a bona fide metabolo-epigenetic reprogramming of stemness exists in pre-malignant and cancer tissues, a new cancer trait to which we have coined the name “metabostemness.”

METABOLISM: EMERGING AS A CANCER HALLMARK

When Hanahan and Weinberg revisited the hallmarks of cancer in 2011 (46), they raised the question of whether the deregulation of cellular metabolism in tumor tissues might be viewed as a core hallmark capability of cancer cells that is as fundamental as the six well-established core hallmarks formerly proposed in 2000 (47). Alternatively, the reprogramming of cancer metabolism might be

merely viewed as an evolutionary conserved target that is upstream programmed by oncogenic gain-of-function events and the loss of tumor-suppressors (48). The latter view implies that a stereotyped pattern of cancer-associated metabolic changes including accelerated glucose transport, reduced mitochondrial oxidative phosphorylation (OXPHOS) accompanied by aerobic glycolysis and lactate production (i.e., the Warburg effect), and augmented *de novo* fatty acid biogenesis (i.e., the lipogenic phenotype), can be all induced by most common genetic alterations in the oncogenic PI3K/AKT/mTOR/HIF axis and in the tumor-suppressor p53 system (49–53). Not surprisingly, the metabolic signatures of cancer cells have been frequently perceived by traditional biochemists as indirect, secondary phenomena that are merely required to support oncogene-directed anabolic proliferation and survival. Instead of adopting the challenging notion that tumor cells might essentially exhibit increased autonomy in maintaining an anabolic phenotype because proto-oncogenes and tumor-suppressors originated through evolution as components of metabolic regulation, Hanahan and Weinberg rather considered cluster analyses showing that several cancer-driving mutations converge on metabolic pathways. Subsequently, they designed cancer metabolic reprogramming as an “emerging hallmark” to highlight the unresolved issues surrounding its functional independence from the bona fide cancer hallmarks (46, 47).

STEMNESS: A FORGOTTEN CORE CANCER CAPABILITY

Several researchers have advocated incorporating the two key properties of stem cells, i.e., the ability to proliferate without lineage commitment (i.e., self-renewal), and the capacity to differentiate into one or more specialized cell types (i.e., pluripotency), as a new-dimensional hallmark of cancer (54–58). The role of stemness as a cancer attribute was originally identified from the analysis of the outcomes of high-throughput gene expression datasets revealing that biologically aggressive, poorly differentiated tumors display transcriptional profiles characterized by the overrepresentation of gene signatures usually enriched in embryonic stem cells (ESCs) (59–63). Some carcinomas appear to hijack the stemness transcriptional factors’ machinery to support tumor-initiation, aberrant proliferation, and metastasis; accordingly, the activation of reprogramming-like dedifferentiation mechanisms driven by master regulators of self-renewal and pluripotency (e.g., Sox2, Oct4, and Lin28) has been repeatedly shown to generate cell populations enriched with CSC-like cells that possess tumor-initiation and colonization capacities (64–72). However, the pioneer suggestion by Bond et al. (73) almost 20 years ago that the apparent dedifferentiation accompanying malignant progression can play a causal rather than passive role in the critical tumors-behavior-switch from well-differentiated to highly aggressive forms has been commonly forgotten. Most cancer researchers have adopted an alternative view, in which tumors adhere to essentially irreversible top-down hierarchies of CSC-driven cellular differentiation that caricature those occurring in normal tissues. As for metabolic reprogramming, the stemness-related loss of differentiation, one fundamental characteristic of most tumor tissues, was not considered a distinct hallmark in the framework provided by Hanahan and Weinberg in 2011.

STEMNESS IN CANCER TISSUES: WHAT IS THE ORIGIN OF CANCER STEM CELLS?

Carcinogenesis involves the accumulation of numerous mutational events over long periods of time. In tumors that originate from tissues with high cellular turnover, only adult stem cells (ASCs), with their innate self-renewal capacity, can remain in the tissue long enough to accumulate the number of oncogenic alterations that are necessary to support a complete malignant transformation. This has led to the hypothesis that tumor-initiation and progression are driven by CSCs, commonly defined as the fraction of tumor cells specifically endowed with self-renewal and tumor-seeding potential and the ability to spawn non-CSC progeny (74–76). Not surprisingly, ASCs have been commonly hypothesized to represent the cells of origin in most tumors because they can be directly targeted with primary transforming events; more committed progenitors can also similarly gain the ability of self-renewal and function as CSCs through oncogenic transformation. In this hierarchical organization with rare, self-renewing CSCs residing at the top, the disorganized tumoral mass should be merely viewed as an aberrant version of the ASC-driven mechanisms that govern the corresponding tissue’s normal development (77–79). However, while this is the case for cancers with a stem cell origin such as hematopoietic malignancies, for those with non-stem cell origin including liver, breast, lung, pancreatic, and prostate cancers, although CSCs obviously exhibit stem cell properties, they do not necessarily originate from the direct transformation of normal tissue stem cells or progenitor cells.

We now know that non-cancerous and differentiated cancerous cells possess enough plasticity to aberrantly reprogram and acquire bona fide CSC properties. Indeed, the inherent aggressiveness of carcinomas appears to derive not from the pre-existing content of CSCs, but rather from the intrinsic proclivity of a given tumor tissue to generate new CSC from non-CSC cell populations (80–90). Such plasticity potential of non-CSCs to acquire a CSC cellular state depending on their epigenetic/transcriptional signature and in their interpretation of multiple microenvironmental signals (e.g., hypoxia, starvation, inflammation, and therapeutics) is fully absent in the conventional depiction of the one-way stem/progenitor cell hierarchy and is revolutionary changing our current perception of the CSCs’ biology. By understanding cancer as a disease of differentiation, CSCs can be generated at any time during cancer progression so long as appropriate oncogenic lesions that can enable epigenetic reprogramming to a stem-like cellular state are present or, alternatively, tumor-suppressors are hindered. Because CSCs are emergent, dynamic cellular states, i.e., CSCs are also made and not just born, multiple independently derived and molecularly distinct CSC cell populations may evolve depending on the likelihood of reprogramming phenomena within tumors. The resulting heterogeneity manifests as diverse clones of CSC that vary in terms of their dormancy, proliferative, biomarkers, metastatic, and/or chemo-sensitivity profiles. Indeed, the molecular heterogeneity and stochasticity of gene expression in cancer tissues can drive a continuum of cancer cell states to rapidly shape cancer’s evolution through a greater probability of entering CSC cellular states.

CANCER STEM CELLS REPROGRAMING: A NEW PARADIGM FOR UNDERSTANDING TUMORS' SOCIAL STRUCTURE

Cancer stem cell reprogramming is a molecular process able to establish the bidirectional control of tumors' epigenetic hierarchy. On the one hand, cancer genetic alterations can reset the epigenetic and transcriptional status of an initially healthy cell to establish a newly acquired, pathological differentiation program of aberrant stemness (i.e., a CSC-like cellular state) that ultimately leads to cancer development. On the other hand, differentiated tumor cells can dynamically alter their transcriptional and epigenetic circuits to acquire a less-differentiated CSC cellular state. Differentiated (normal and tumor) cells and CSCs are therefore distinct cellular states that could convert each other to achieve a balanced equilibrium within heterogeneous cancer cell populations. Crucially, the reprogramming-differentiation cancer model easily explains many of the apparently paradoxical aspects of the CSC-related tumor biology: (1) the apparently contradictory reconciliation of the rarity (of CSC number) with robustness (of CSC properties) in some tumors; (2) the challenging application of hierarchical models to some tumors such as metastatic melanoma [i.e., an extreme example of stem cell reprogramming in which certain epigenetic makeups endow almost the entire tumor cell population with the easiest ability to acquire CSC qualities; (91)]; (3) the lack of bona fide CSC markers enabling the general identification of stemness across different cancer types and even during the natural history of a given tumor type (61, 92–94); (4) the occurrence of unique stem-like states due to the continuous evolution and adaptation to new constraints [e.g., the conversion of tumor cells into functional vascular endothelial cells that resist antiangiogenic therapy or the transient assumption by individual cells of a reversible drug-tolerant state to protect the cancer cell population from eradication due to potentially lethal exposures; (95, 96)]; and (5) the accumulation of CSCs following treatment with therapeutics [i.e., cancer therapies do not necessarily enrich cancer tissues with pre-existing, treatment-refractory CSCs, as an accelerated production of *de novo* CSC cellular states from the residual cancer cells may easily repopulate their ranks while the older CSCs die; (97)]. The reprogramming-dedifferentiation cancer model illuminates the fact the “hierarchy” within tumors' social structure is not rigid because self-renewal and differentiation are acquired traits. Importantly, CSC reprogramming does not exclude the pivotal role of non-strictly genetic factors (e.g., metabolites and microenvironment), which can significantly impact the conversion probability between non-CSCs' and CSCs' cell stages.

CANCER METABOSTEMNESS: A CONCEPTUAL DESCRIPTION

A key peculiarity of the abovementioned model of stem cell reprogramming is that the dysregulation of specific signaling pathways, rather than the type of the cell of origin, dictates the emergence and phenotype of CSCs in a given tissue. If any differentiated cell can be reprogrammed to an induced pluripotent state through the right combination of transcription factors, then, following the same line of reasoning and in theory, non-CSC cells could similarly dedifferentiate to a CSC cellular state given that an appropriate stemness transcription factor is strongly activated. Moreover, by balancing counteracting differentiation forces (98), reprogramming to a CSC functional state might be also achieved through the establishment

of a fine-tuned equilibrium that might not require of the traditionally considered master regulators of stemness. However, by following the numerous parallels between the process of reprogramming differentiated cells-to-induced pluripotent stem cells (iPSCs) and differentiated cells-to-CSCs, when considering the slow kinetics and efficiencies of iPSCs generation (99) it might tempting to suggest that not all of the normal or cancerous cells would possess the equivalent ability *ab initio* to permit their successful reprogramming to CSC cellular states. Whereas certain populations are seemingly refractory to reprogramming, we now know that the ability to generate iPSCs is intrinsic to any cell given sufficient time and the appropriate reprogramming push. Thus, additional expression of the so-called Yamanaka factors, the use of additional stemness transcription factors, the use of chemicals, or the direct modification of critical epigenetic components can greatly enhance (even to efficiencies nearing 100%) and accelerate reprogramming to iPSCs (100–104). Ever-growing iPSCs-based findings showing that metabolic reprogramming phenomena might be essential for transcription factor-induced stemness have further confirmed that the elaborate regulation of key master metabolic switches seems to contribute to the metabolic changes that take place in the transition between a differentiated cell and a stem cell, to the maintenance of the stemness properties in stem cell cellular states, and to the exit from the pluripotent state to become primed for differentiation (10–30, 105). In this scenario, we recently reasoned that the dedifferentiation of somatic cells into iPSCs as well as the *de novo* generation of CSC cellular states from non-CSCs may represent mechanistically related, metabolically dependent reprogramming phenomena in which epigenetic remodeling, the activation of genes related to the establishment and maintenance of stemness, and/or the scavenging of fate determinants related to cell differentiation might be co-opted in the absence of functional tumor-suppressing mechanisms. In other words, the metabolic infrastructure and functioning might operate as the key molecular constraint controlling the kinetics of stemness reprogramming for the optimal routing of non-CSC to CSC-like cellular states during cancer genesis and progression.

We propose that the acquisition of and departure from stemness in pre-malignant and cancer tissues might not be governed exclusively by genetic and epigenetic controllers, but also by the pivotal regulatory role of the cellular metabolotype, which may act as “starter dough” for cancer stemness traits. When viewing cancer stemness as a flexible quality that might be gained and lost in a metabolic-dependent manner, the cellular metabolotype then operates as a supra-genetic dimension guiding the ability of epigenetic and transcriptional circuitries to redirect normal and non-CSC tumor cells toward a CSC-like cellular state. Certain metabolotypic shifts might function as very early molecular events that render a (normal or cancerous) differentiated cell more susceptible to transcriptional and epigenetic rewiring required for the acquisition of aberrant stemness and, concurrently, of refractoriness not only to apoptosis, but also to differentiation. Subsequent hits, occurring in variable orders, combinations, and/or intensities can then confer the definitive quality of cancer stemness and dictate the dynamic cellular hierarchy within a tumor tissue. We have therefore coined the term “metabostemness” to refer to the metabolic parameters causally controlling or functionally

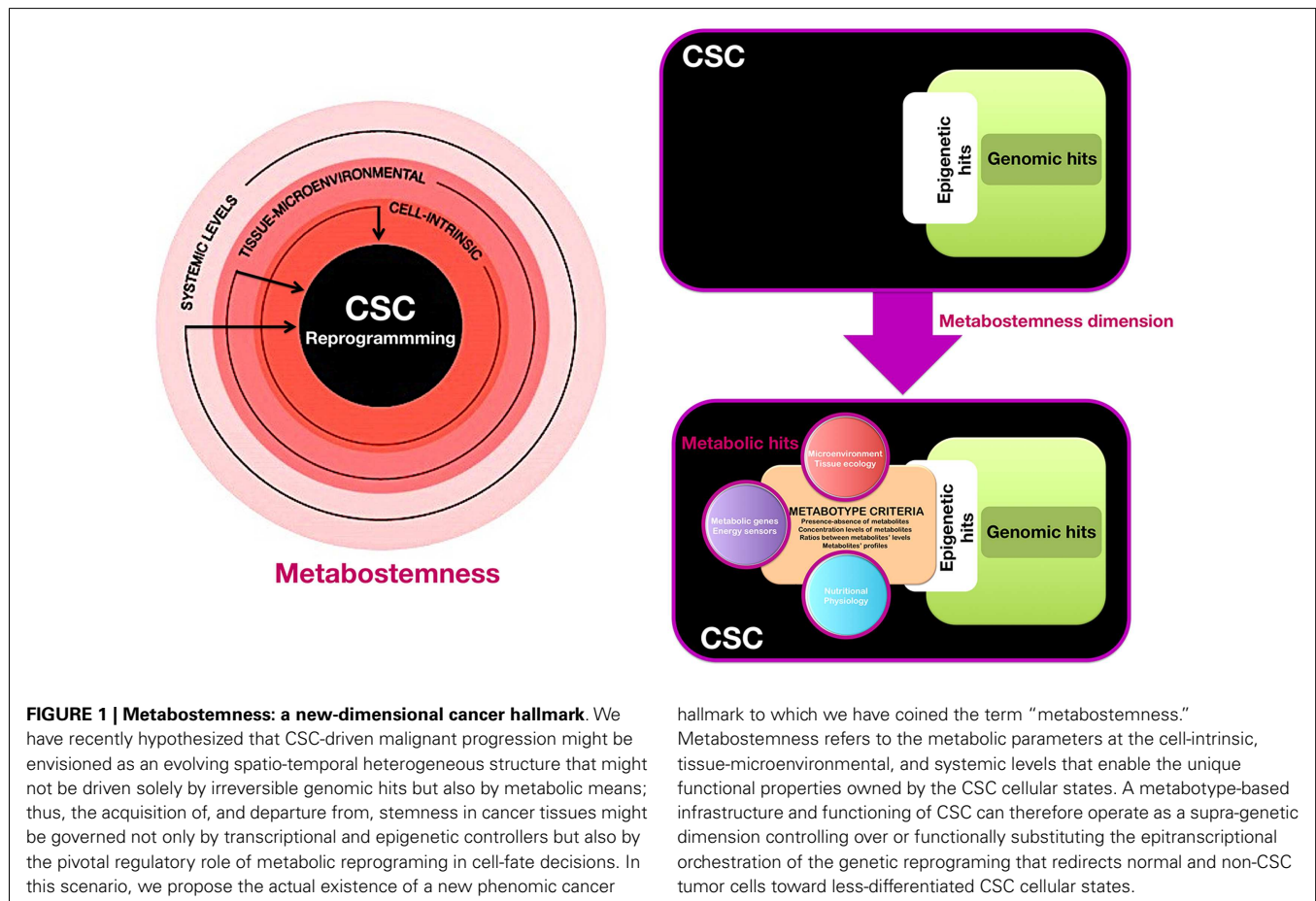
substituting the epitranscriptional orchestration of the genetic reprogramming that redirects normal and non-CSC tumor cells toward less-differentiated CSC cellular states (Figure 1). As such, the metastemness trait can be understood as the physiological glue that metabolically connects all the omic layers with a self-autonomous CSC cellular quality; operatively, metastemness is a systeomic function of observable metabolic phenotypes (i.e., the CSC metabolophenome) that predate systems biology and its sub-disciplines (i.e., genomics, transcriptomics, proteomics, and metabolomics) at the level of CSC cellular states. From a holistic perspective, the metastemness hallmark comprises the intrinsically, microenvironmental, and physiologically determined metabolic parameters that enable the self-renewal and differentiation capacities owned by the CSC cellular states in tumor tissues (Figure 1). Four main features can conceptually define the metastemness process in cancer tissues:

- (1) the cellular metabolotype determines the global success of the epitranscriptional reprogramming that redirects normal and non-CSC tumor cells toward less-differentiated CSC cellular states;
- (2) the cellular metabolotype imposes the “permitted” and “protected” cellular modes that allow or prevent, respectively, the completion of the molecular journey from non-CSC to CSC cellular states;

- (3) the closer a cellular metabolotype is to that of a CSC, the higher its reprogramming capacity for acquiring a CSC cellular state; and
- (4) metabolic interventions can reprogram cellular metabolotypes in a manner that successfully impedes the aberrant acquisition and functioning of stemness in cancer tissues.

CANCER METABOSTEMNESS: AN OPERATIONAL DELINEATION

Transcription factors are commonly viewed as the key intrinsic regulators of cell-fate, i.e., the cell-fate choice exclusively involves modulating networks of transcription factors. It is also generally accepted that a given cellular type including that of stem cells must not be excessively sensitive to random, or unpredictable, small fluctuations in the levels of specific signals. This indispensable requirement to withstand modulating factors that may perturb the gene regulatory network (GRN) architecture – i.e., the gene–gene relationships, their directionality (“who controls whom”), their interaction modalities (inhibition versus stimulation), and the modes of cooperation – defines a cell type in a timely manner and strongly limits the number of solutions or “cellular states” for a given genetic system including those of cancer tissues (106–108). The evolving dynamics of molecular connections between the genes and gene products that can interact with each other within a cell, including the underlying regulatory logic that govern



these interactions, must therefore provide robustness with respect to varying extrinsic signals and intrinsic factors, i.e., the noise. Such stability of the cell state is obviously required in stem cells to support the self-renewal and maintenance of an uncommitted state, but must also afford certain flexibility in the choice of cell-fate to permit the diversification and differentiation of cell types in response to intrinsic cues or extrinsic signals.

During normal development, cells make very precise transitions between network states of gene expression patterns, which must be stable and irreversible in terminally differentiated cells, at least under homeostatic or physiological conditions. Conversely, the potential for reverse differentiated-to-stem cell state conversions has been revolutionarily exemplified by the nuclear reprogramming of somatic cells to a pluripotent stem cell state driven by a small number of stemness transcription factors. Crucially, a very similar consideration of cell states transitions apply to pathological states such as cancer, where the misexpression of transcriptional regulators can reset the status of an initially healthy cell to establish a newly acquired, pathological differentiation program of aberrant stemness that ultimately leads to cancer development. Considering the above-depicted scenario, it is not surprising that the major challenge that is being faced by regulatory biology in the post-genomic era of understanding cancer diseases is to map the core transcription factors' networks associated with different cancer cell types, especially the underlying regulatory logic that governs their behavior as differentiated versus CSC cellular states. The accurate delineation of such a map will provide crucial insights into the rules that define cancer cellular states (i.e., cancer heterogeneity) and how transitions between cancer cellular states are achieved, thus providing unforeseen therapeutic solutions to the apparently irresolvable problem of targeting cancer dynamical models such as the model of reversibility, or cancer cell reprogramming, in which heterogeneous populations of CSC can arise by reversion of more differentiated cancer cells. Indeed, multiple different CSC populations have been described for a given cancer type, despite presenting different gene and protein expression signatures, thus leading to the currently accepted view that the characterization of CSCs can no longer be based on marker expression, but instead at their functional level, strongly supporting the model of reversibility, or CSC reprogramming, in which the populations of CSCs arise by reversion of more differentiated cells (85, 109–113).

As for bona fide pluripotency, CSCs should be viewed as functional states rather than discrete cellular entities characterized by well-defined and static gene networks, thus highlighting cancer stemness as a statistical property resembling a macrostate in statistical physics (108, 114, 115). If these macrostate entities of functional CSCs are correct, the gene expression signature for a CSC in a given tumor tissue may be dynamic or non-unique, which can create a challenge when trying to unambiguously establish mathematical frameworks describing CSC reprogramming phenomena. We propose that incorporating metabolism and metabolites into the intrinsic variability of the CSCs' epigenetic and genetic signatures can significantly reduce the high-dimensional problem of understanding reprogramming dynamics both at the single-cell level and the population level. But how can we model the incorporation of the metabolism and metabolites into the epigenetic and genetic signatures accounting for CSC variability during the

generation, maintenance, and evolution of CSC cellular states in cancer tissues? To definitely consider the process of reprogramming to CSC into a rigorous, quantifiable theory, the establishment of a mathematical framework able to accurately mapping the landscape pertaining the transition between non-CSC and CSC cell states needs to incorporate a never before considered metabolic dimension.

DYNAMIC PERSPECTIVE BY DEVELOPING PROBABILISTIC DESCRIPTIONS OF CELL STATES

Cancer cell states (e.g., non-CSC versus CSC) can be parameterized as vectors of molecular characteristics, \hat{S} , which are generally considered a set of gene expression levels ($\hat{S} = [g_1, g_2, g_3, \dots, g_N]$) (Figure 2). A cancer cell at any time t can exist in a point of this state space, and its state can change with time in response to particular cell-autonomous and non-cell-autonomous conditions, i.e., in a CSC reprogramming scenario, \hat{S} is a function of time $\hat{S}(t)$ as a result of noise, reprogramming, or differentiation. By plotting one trajectory during a state change from t_0 to t_1 , we can fully describe the transition between cancer cell states during this time $S(t) = [g_1(t), g_2(t), g_3(t), \dots, g_N(t)]$. For simplicity, we show a cancer cell state space generated by the level of N different genes, g_1 to g_N , where each arrow represents an axis corresponding to the expression level of that particular gene transcript. Such a cancer cell state space provides an accurate means to quantitatively organize and visualize different states of a cancer cell with a fixed genome. Indeed, often cancer cells will cluster in particular regions of the cancer cell state space, which can be viewed as stable types of cancer cells that express particular markers. Those regions where no cancer cells are found correspond to cell states that are somehow unstable for the given genome of the cancer cell in the microenvironmental conditions considered. Thus, N is generally reduced to two to three more manageable dimensions through statistical techniques [e.g., principal component analysis (PCA)]. Figure 2 shows a two-dimensional representation of the cancer cell state space, where each axis (g_a and g_b from PCA) is a linear combination of genes g_1 to g_N and, therefore, stable types of cancer cells exist at particular points of this graph. By quantitatively mapping the gene expression levels of a large sample of single stable cancer cells in the same space, the probability of occupying each point in this space can be plotted in a continuous fashion, and regions with high densities of spots can define observable cancer cell types. The continuous probabilistic description of cancer cell types staying at a particular point in state space can be represented as a landscape, by calculating $-\ln[P(\hat{S})]$ at each point. In this landscape, $V(\hat{S})$ represents “energy barriers” between transitions involving any two cellular states and thus may provide a more thorough description of non-CSC-to-CSC transitions. However, we should acknowledge that while the nature of the cancer cell substates can be elucidated in terms of a landscape picture in which stable differentiated, non-CSC, and CSC cellular states are mathematically defined as “attractors” – i.e., observable cancer cell types – the architecture of these apparently stable “network states” is described exclusively in terms of genes, or at the most epigenetic, expression patterns. Indeed, when selecting a particular set of characteristics as being informative for the transition between two cell states of interest, functional cell types are almost

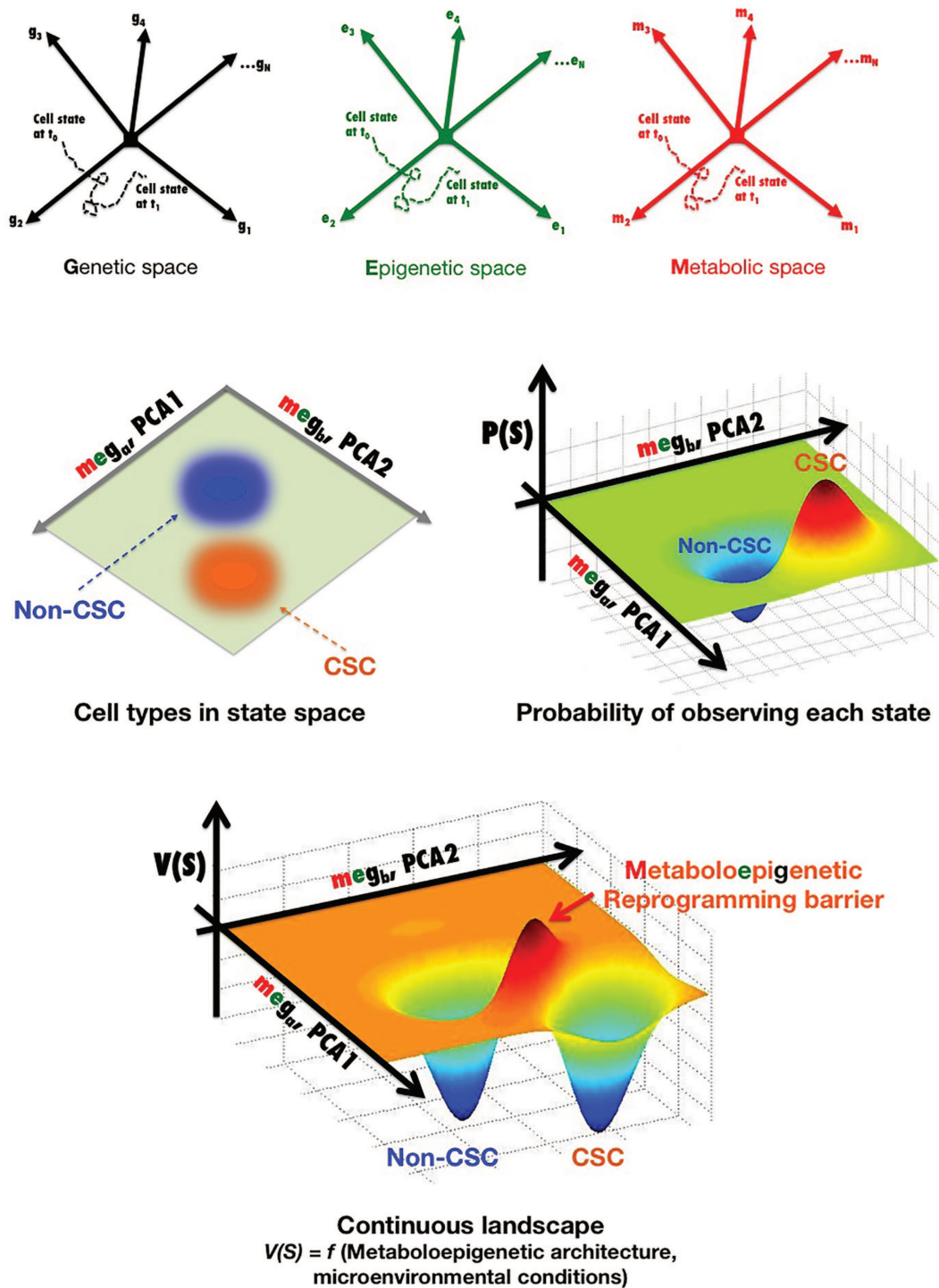


FIGURE 2 | Metabostemness: developing probabilistic descriptions of non-CSC and CSC cell states. (See text for a more detailed explanation).

exclusively defined based on levels of gene expression changes on the timescale of days to weeks. In this probabilistic framework, the genetic background defines the abovementioned architecture of the cell state space, meaning that particular gene–gene relationships, interaction modalities, and integrating transfer functions

(i.e., the GRN) are largely “hardwired” by the genome. A more detailed characterization not only of gene expression, but also of biochemical noise in signaling processes (e.g., binding of epigenetic modifiers to particular genome loci, inherent stochastic nature of molecular binding events, secondary messengers, and

variable cell–cell contact) and of the epigenetic state (e.g., levels of epigenetic methylation or acetylation marks on DNA or chromatin) is expected to provide more insight into the missing key parameters and required transcriptional changes controlling the kinetics of CSC reprogramming. In this regard, we propose that the regulatory network architecture of a cancer landscape comprises not only the genes and proteins that can interact within a cancer cell, but also the cellular metabolome, which can necessarily and sufficiently characterize different functional cell states within heterogeneous cancer cell populations through different types and/or levels of certain metabolites.

But how do metabolism and metabolites hierarchically integrate with genetic expression programs to coordinately regulate CSC function and fate? Because DNA transcription is regulated by chromatin organization, the occurrence of metabolic inputs into epigenetic modifications of chromatin and transcription should be viewed as the molecular bridge that links metabolism to epigenetics and gene expression during non-CSC to CSC transitions. We are accumulating ever-growing evidence suggesting that, beyond the classically delineated transcriptional output of growth factor/hormonal signaling pathways, a variety of metabolic signals can play also critical roles in determining chromatin structure, thus directly linking metabolic perturbations to the dysregulation of cellular differentiation (116–118) (**Figure 3**). Indeed, CSC metabolism is linked to epigenetics and gene expression in a multifaceted and bidirectional manner:

- Metabolic fluxes can be controlled by metabolic enzymes that are directly regulated by stemness transcription factors such as c-Myc, which can act both locally and globally on chromatin to exert wide-ranging effects on the biology of stem and tumor cells (119–122).
- Epigenetic alterations (e.g., demethylation or methylation of gene promoter regions, protein acetylation) can contribute to the deregulated expression of key enzymes involved in cell metabolism. Thus, the reversible acetylation of histones and non-histone proteins has been shown to affect cell metabolism, and glycolytic versus OXPHOS pathway gene expression and DNA methylation patterns change during reprogramming to stemness. The rate-limiting glycolytic enzyme Hexokinase II (HK2) and the key enzyme of gluconeogenesis fructose-1,6-biphosphatase (FBP1), with opposing roles in glycolysis, have recently been identified as epigenetically regulated by promoter demethylation and methylation, respectively (123–126). PKM2, which catalyzes the conversion of phosphoenol pyruvate to pyruvate, is targeted for degradation in a glucose-dependent manner by acetylation at lysine K305; the epigenetic silencing of FBP1, which catalyzes the energy-consuming conversion of fructose-1,6-biphosphate to fructose-6-phosphate, is employed by CSC as a mechanism of glucose flux maintenance via glycolysis and other associated biosynthetic pathways (127). The tumor tissues' extra requirements of glucose, an essential nutrient for CSC that, when present in the culture environment, significantly increases the percentage of CSC-like cells in the overall cancer cell population (128), can be achieved via the epigenetic silencing of DERL3, the gene responsible for degrading the glucose transporter SLC2A1 [glucose transporter 1 (GLUT1)] (129). Moreover, posttranscriptional modifications of p53, the key connector of reprogramming to pluripotency and tumorigenesis (130–136), may affect cell metabolism through downstream targets such as TIGAR (TP53-induced glycolysis and apoptosis regulator) and interaction with PGC-1 α [peroxisomal proliferator-activated receptor (PPAR) gamma coactivator 1] (137–142).
- Epigenetic modifications of DNA and histones by methylation and acetylation reactions require cofactors that are derived from various metabolic pathways including glycolysis, fatty acid oxidation, tricarboxylic acid (TCA) cycle, and OXPHOS (**Figure 3**). Among these cofactors, we can mention the following: (a) S-adenosyl-L-methionine (SAM), which is a cofactor for methylation reactions by DNA- and histone-methyltransferases (DNMTs and HMTs) (143–145); (b) Flavin adenine dinucleotide (FAD), which is a cofactor for lysine specific demethylase 1 (LSD1) (144, 146); (c) α -ketoglutarate, which is an electron donor and cofactor for the α -KG/Fe²⁺-dependent dioxygenases Jumonji-C domain (JmJC) histone demethylases (HDMs) and ten–eleven translocation (TET) proteins, respectively (143, 144); (d) Nicotinamide adenine dinucleotide (NAD), which is a cofactor for the sirtuins family of histone deacetylases (HDACs) and poly(ADP-ribose)polymerase (PARP) (143, 147–151); and (e) Acetyl Coenzyme A, which is not only an important precursor for the *de novo* biogenesis of fatty acids, but also an essential cofactor for histone acetyl transferases (HATs) and the acetylation of non-histone proteins involved in cell metabolism (145, 152–155). Because alterations in the supply of these cofactors may affect DNA methylation, alter chromatin structure, and change posttranslational modifications of non-histone proteins that influence the regulation of gene expression reprogramming to stemness, and because cofactors and modifying enzymes are always present at some level, the current challenge is to understand how localized fluctuations in levels of metabolites control chromatin modifiers in space and time to operate as mechanisms of specificity that prevent all genes from being regulated synchronously. Enzyme recruitment with DNA-binding factors, local depletion or excess cofactors, or the modification of spatial and temporal sublocalization of enzymes within the nucleus might likely account for a hierarchy of target chromatin regions and associated genes (116, 156), thus translating CSC metabolomes into CSC methylation maps.
- Beyond the more general effects on epigenetics brought about by changes in the availability of substrates or cofactors for enzymes that regulate chromatin structure and gene expression, mutations in metabolic enzymes have been linked to the generation of oncometabolites, which directly drives epigenetic reprogramming in cancer cells. Ever-growing *in vitro* and *in vivo* studies have provided strong biochemical, cell biological, and genetic evidence that oncometabolites can drive tumorigenesis and tumor maintenance by regulating histone and DNA modifications and engaging a metabolic block in cellular differentiation. This is the case of the currently recognized oncometabolites R(-)-2-hydroxyglutarate (2-HG), fumarate, and succinate, which accumulate due to defects in the TCA cycle enzymes isocitrate dehydrogenases (IDHs), fumarate hydratase (FH), and succinate dehydrogenase (SDH), respectively. Defects in these TCA cycle

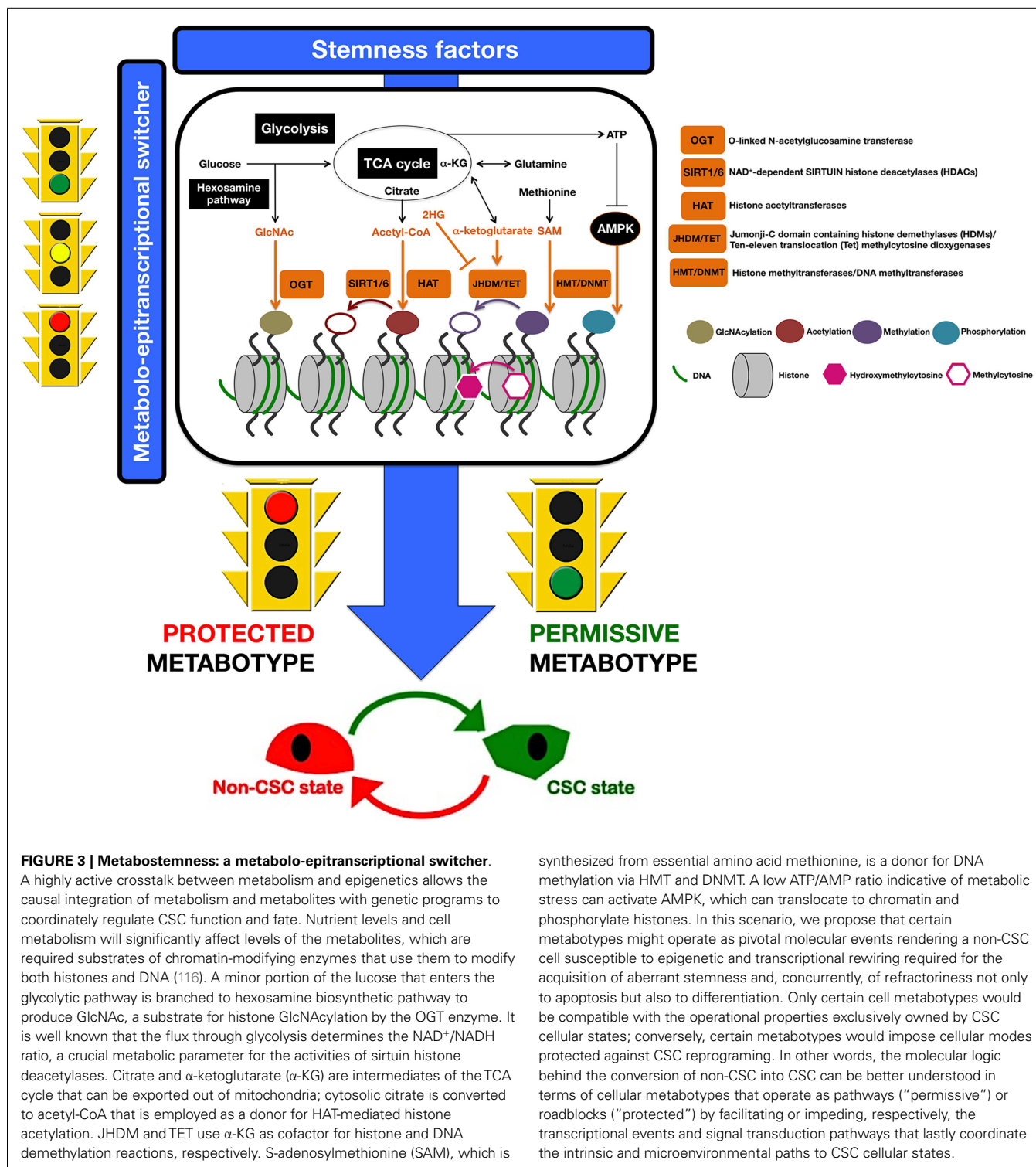


FIGURE 3 | Metabostemness: a metabolo-epitranscriptional switcher.

A highly active crosstalk between metabolism and epigenetics allows the causal integration of metabolism and metabolites with genetic programs to coordinately regulate CSC function and fate. Nutrient levels and cell metabolism will significantly affect levels of the metabolites, which are required substrates of chromatin-modifying enzymes that use them to modify both histones and DNA (116). A minor portion of the glucose that enters the glycolytic pathway is branched to hexosamine biosynthetic pathway to produce GlcNAc, a substrate for histone GlcNAcylation by the OGT enzyme. It is well known that the flux through glycolysis determines the NAD⁺/NADH ratio, a crucial metabolic parameter for the activities of sirtuin histone deacetylases. Citrate and α -ketoglutarate (α -KG) are intermediates of the TCA cycle that can be exported out of mitochondria; cytosolic citrate is converted to acetyl-CoA that is employed as a donor for HAT-mediated histone acetylation. JHDM and TET use α -KG as cofactor for histone and DNA demethylation reactions, respectively. S-adenosylmethionine (SAM), which is

synthesized from essential amino acid methionine, is a donor for DNA methylation via HMT and DNMT. A low ATP/AMP ratio indicative of metabolic stress can activate AMPK, which can translocate to chromatin and phosphorylate histones. In this scenario, we propose that certain metabolotypes might operate as pivotal molecular events rendering a non-CSC cell susceptible to epigenetic and transcriptional rewiring required for the acquisition of aberrant stemness and, concurrently, of refractoriness not only to apoptosis but also to differentiation. Only certain cell metabolotypes would be compatible with the operational properties exclusively owned by CSC cellular states; conversely, certain metabolotypes would impose cellular modes protected against CSC reprogramming. In other words, the molecular logic behind the conversion of non-CSC into CSC can be better understood in terms of cellular metabolotypes that operate as pathways ("permissive") or roadblocks ("protected") by facilitating or impeding, respectively, the transcriptional events and signal transduction pathways that lastly coordinate the intrinsic and microenvironmental paths to CSC cellular states.

enzymes caused inherited benign or malignant tumors by altering DNA and histone modifications to cause widespread transcriptional dysregulation (31–45). Indeed, the identification of cancer-associated mutations in metabolic enzymes has provided the strongest support ever for the argument that the metabolism rewiring observed in cancer plays a significant role in cancer

development. Crucially, by highlighting the similarities in the nuclear reprogramming pathways that are involved in the generation of iPSCs and of the tumor-initiating action of CSC-like cells, it has recently been suggested that a stemness-related hallmark of cancers may be mutations or expression changes in metabolic genes that are implicated in the regulation of DNA

methylation plasticity such as IDHs (7). Lu and Thompson (116) originally suggested that, during progenitor cell differentiation, the inhibition of the HDM JHDM by the oncometabolite 2-HG, which is aberrantly synthesized by the neomorphic mutations of IDH enzymes, causes defective histone demethylation and blocks the accessibility of differentiation-related genes. The 2-HG-driven inhibition of JHDM as well as that of the TET family of DNA demethylases, which operate as failsafe mechanisms to protect promoters from aberrant DNA methylation, should lead to progressive DNA hypermethylation and permanently “lock” differentiation-related genes in a silent state; the resulting differentiation arrest might facilitate cancer development through the accumulation of undifferentiated cells capable of self-renewal. Goding et al. (7) now propose that the cell of origin dictates the metabolo-epigenetic relationship between reprogramming and cancer. As originally proposed by Lu and Thompson (116), gain-of-functions IDH mutations can lead to global hypermethylation, preventing the demethylation of genes that are implicated in differentiation, and consequently promoting an increase in the number of stem cells that may be targetable by oncogenic mutations in cancers with a stem cell of origin, such as hematopoietic malignancies. For cancers with a non-stem cell origin including liver, breast, lung, pancreatic, and prostate cancers, in which mutations on metabolic genes are not widespread, Goding et al. (7) propose that an intact metabolic function of IDH would be necessary to maintain DNA methylation plasticity and a flexible epigenetic landscape, as seen during the generation of iPSCs.

Nevertheless, as metabolic abnormalities in cancer continue to be uncovered, it can be expected that new metabolites or metabolic perturbations that affect chromatin structure will be identified (53, 157). Moreover, during reprogramming to a CSC cellular state, a positive feedback loop might be established between bioenergetics reprogramming and the activity of stemness transcriptional factors; accordingly, there is a progressive resetting of metabolite levels that parallels the progressive changes seen in global epigenetic modifications and gene expression over prolonged times of induced pluripotency maintenance. We propose that the probabilistic description of cell states in cancer tissues can be better described by incorporating the connections between metabolism and chromatin dynamics; therefore, in order to determine how metabolism and metabolites exert influence over epigenetic and genetic reprogramming circuits that establish and maintain CSCs’ self-renewal and differentiation abilities, the transition between cancer cell states should incorporate not only a set of gene expression levels, but also the metabolo-epigenetic state, i.e., $S(t) = [\text{meg}_1(t), \text{meg}_2(t), \text{meg}_3(t), \dots, \text{meg}_N(t)]$ (Figure 2).

DYNAMIC PERSPECTIVE USING WADDINGTONIAN LANDSCAPES

It is well-recognized that the Waddington landscape (158), a metaphoric representation of cell differentiation in which pluripotent stem cells are positioned at the top of a hill progressively losing differentiation potential while going downhill into different valleys representing irreversible differentiated cellular states, allows for the modeling of a complex network of molecular barriers governing cell-fate transitions. The complexity of the enormous number of

possible intracellular factors that can mitigate or impede cellular reprogramming is notably reduced into an effective energy landscape in the Waddington model, in which cell-fates are defined by stable states, known as “attractors,” and cell transitions are represented as flows from one energy state to another. In this quasi-potential Waddingtonian landscape that captures the global dynamics of high-dimensional attractor states (i.e., distinct entities and of regulatory networks representing the equilibrium solutions of how the concentrations of interacting transcripts, proteins, and other variables in a GRN evolve over time to exhibit the natural properties of cell types), the cancer cell types within a tumor tissue are therefore self-stabilizing states basically defined by their “energetic” minimum (i.e., the commitment is defined as the progression toward the minimum energy) and the shape of the potential cleft. While they are robust to small perturbations, they still allow “all-or-nothing” transitions to other attractors including those of CSCs given sufficiently high perturbations, as they are separated by “hills” that correspond to “unstable” states. The latter truly represent the “epigenetic barriers” originally proposed in the 1940s to explain cell-fate determination in contraposition to the commonly, but erroneously, usually claimed “epigenetic landscape” in which unique chromatin marks such as DNA methylation and histone acetylation/methylation control the activity of specific genes, thereby acting as the crucial modulators of cell-fate. Thus, according to a bona fide Waddingtonian model based on a rather reversed role of chromatin modification as the *prima causa* of lineage-specific gene expression patterns by “upstream” controlling the access of transcription factors to DNA target sites (i.e., the stemness transcription factors themselves, endowed with sequence-recognition capability, are in charge of initiating the opening of chromatin at specific sites, followed by a directional cooperation with the chromatin-modifying enzymes that they recruit to their target loci), we argue that the metabolo-epigenetic nature of the potential barriers between non-CSC and CSC cell states actually determines how metabolism and metabolites exert influence over or functionally substitute the genetic and epigenetic circuits that establish and maintain stem cell reprogramming in cancer tissues. In this new scenario, the underlying regulatory logic that governs the interactions between genes and proteins should include the cellular metabolite to better define the actual “metabolo-epitranscriptional regulatory network (MGRN)” architecture of cancer tissues.

A glossary of terms might facilitate the understanding of the metabolo-epigenetic landscapes in cancer tissues. The term “metabotype” refers to a cellular phenotype characterized by different metabolites’ levels that can be described by means of four variability criteria: (a) the presence-absence of metabolites; (b) the concentration levels of metabolites; (c) the relative levels or ratios between metabolites; and (d) metabolic profiles. The term “MGRN” comprises the epigenetic state, genes transcripts, proteins, and metabolites that can interact with each other within a cell, including the underlying regulatory logics that govern the interactions between chromatin dynamics, transcripts, proteins, and metabolites. The delineation of “metabolo-epitranscriptional rate equations” refers to mathematical representations of how the concentrations of interacting cellular methylome, gene transcripts, proteins, and metabolites in an MGRN evolve over time in a cell. A

“metabolo-epitranscriptional attractor” describes the equilibrium solutions of the metabolo-epitranscriptional rate equations that represent observable cellular states and can be visualized as wells, or depressions, in a metabolo-epigenetic landscape. A “metabolo-epitranscriptional basin of attraction” includes the set of initial conditions of the metabolo-epitranscriptional rate equations that describe how a particular cell moves to particular metabolo-epitranscriptional attractors. It therefore represents a fate-primed subset of a given population, i.e., a particular cellular state.

The concepts of attractors and the associated landscape pictures provide a very useful conceptual framework in considering the metabolo-epigenetic nature of the non-CSC and CSC substates. Although it might be argued that the power of a dynamic perspective of cancer metastemness using Waddingtonian metabolo-epigenetic landscapes does not extend beyond intuition, we should acknowledge that, in some examples, it has been possible to mathematically describe the relevant attractors and to make predictions about the paths that differentiating cells follow from the undifferentiated, undetermined stem cell state, and vice versa (106, 115, 159). Moreover, the importance of pursuing mathematical models for stemness and differentiation issues should be emphasized when considering that a CSC cellular state should represent a “metabolo-epitranscriptional attractor” in which the maintenance of stemness might not only involve an active process of maintaining this “ground-state,” but also prevent cells from leaving it (160). In this scenario, where CSC cellular states are expected to have an innate program for self-replication that might not require extrinsic instruction, which may certainly account for their latent tumorigenicity, the development of the methods and procedures to mathematically model MGRNs involved in determining self-renewal and pluripotency will not only provide new insights into switch and reprogramming properties in cancer tissues, but will also reveal specific, metabolically driven fluctuating behaviors; in particular, the delineation of minimal metabolo-epitranscriptional requirements for cancer self-renewal can pinpoint missing metabolic components and interactions from CSC functionality that can be confirmed in the laboratory, thus generating an iterative procedure in which the occurrence of “metabostemness factors” and “metabolic rules of attraction” is modeled.

In a first attempt to provide a defined platform for the precise description and dissection of the CSC state in minimal metabolo-epitranscriptional terms, we recently developed the methods and procedures to mathematically model the MGRNs that might be involved in determining pluripotency and self-renewal in mammary epithelial cells; similarly to previous studies, our model formulation considered that the establishment of a balance between Oct4 and Sox2 – the core regulators of pluripotency – and/or counteracting lineage specifiers facilitate reprogramming to stemness (98). Our model formulation, however, introduced for the first time ever the epigenetic regulation of the lineage-specific genes, which is an essential element that allows the effects of metabolic regulation in the nuclear reprogramming process to be taken into account (Menendez et al., unpublished observations). In particular, we added nucleosome modification by HDM as an essential reprogramming element regulated by oncometabolites such as 2-HG. When studying the impact of 2-HG-induced reduction of HDM activity on both the epigenetic landscape of pluripotency

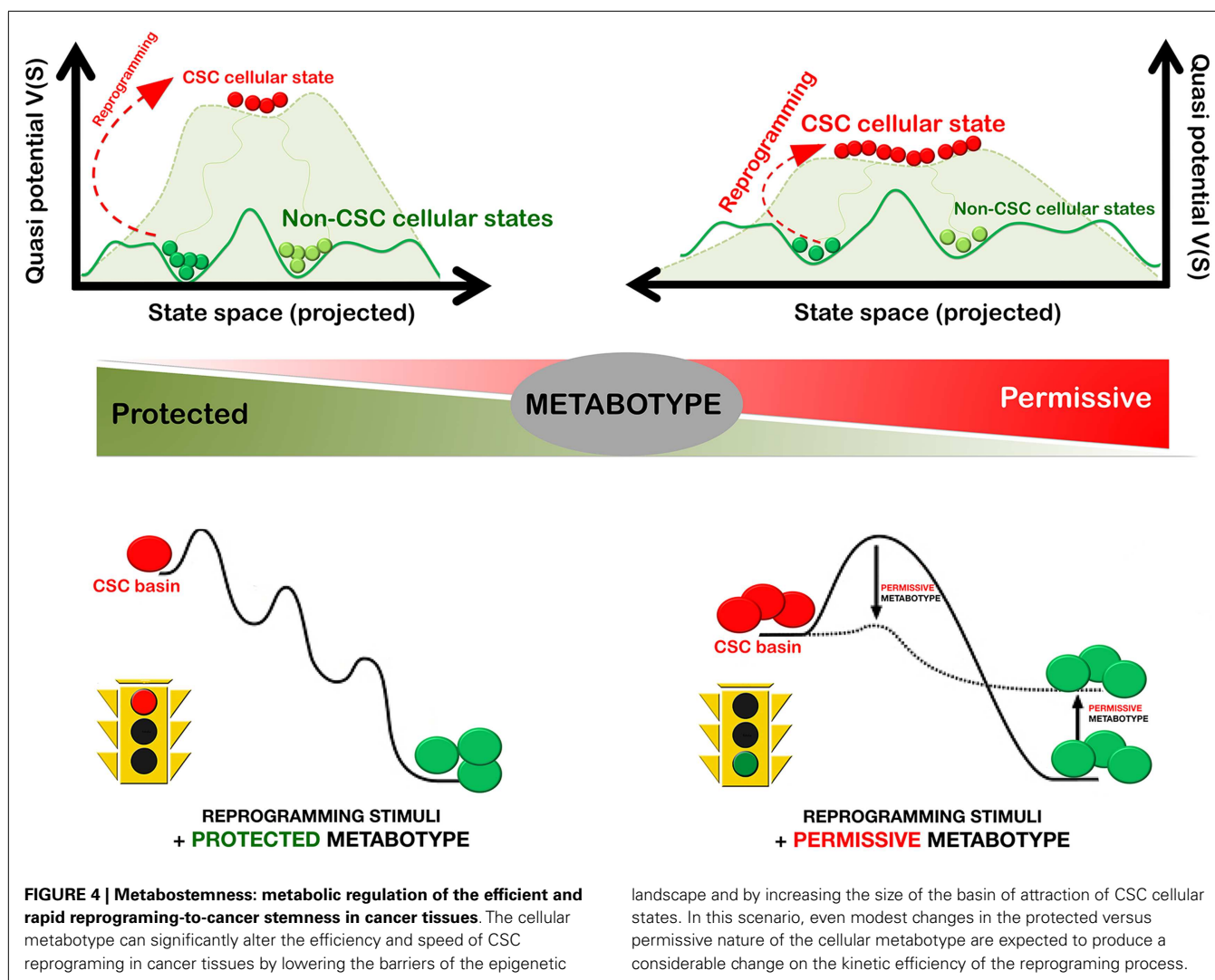
and the reprogramming kinetics (Figure 4), the following was predicted:

- (a) a metabolically driven small reduction of HDM activity should be sufficient to notably lower the barriers of the epigenetic landscape, thus allowing differentiated cells to more easily enter into stem cell macrostates;
- (b) the reduction in average reprogramming time should vary exponentially with the metabolically imposed reduction of HDM activity, suggesting that even modest metabolic decreases of histone demethylation would produce a considerable increase in reprogramming efficiency; and
- (c) the metabolic-induced reduction of HDM activity should transfer a portion of the basin of attraction of the differentiated to the pluripotent state thus increasing the size of the basin of attraction of the macrostate occupied by stem cells.

Second, the creation of non-transformed mammary epithelial cells with clinically relevant heterozygous knock-in of the 2-HG-producing IDH1 R132H mutation allowed us to generate proof-of-concept data supporting the actual reprogramming activity of oncometabolites predicted in our mathematical model. The experimental validation of functional predictions confirmed the following:

- (a) the endogenous accumulation of oncometabolites such as 2-HG is likewise sufficient to significantly enhance and accelerate the efficient generation of induced CSC-like-cells when using the four transcription factors Oct4, Sox2, Klf4, and c-Myc;
- (b) even transient exposure to oncometabolites can operate as a microenvironment-driven dedifferentiation event that facilitate “pathological reprogramming” because several hours of exposure with a cell-permeable form of 2-HG can fully recapitulate the promoting effects of IDH1 mutations on switching differentiated mammary epithelial cells into induced CSC-like cells; and, more importantly
- (c) 2-HG can substitute for Klf4 and c-Myc in reprogramming-factor combinations leading to the enhancement and acceleration of reprogramming non-CSC into induced CSC-like cells when solely using Oct4 and Sox2.

By impacting the epigenetic remodeling that facilitates the transition between non-CSC and CSC functional states, oncometabolites such as 2-HG likewise appear to operate as bona fide “metabostemness reprogramming factors” that determine the cancer tissue proclivity of generating a given number of CSCs in a given time. Because we employed a non-tumorigenic, non-transformed genetic background that, upon introduction of defined reprogramming factors, has been found to generate tumorigenic cells with CSC properties (161), our findings demonstrating how small-molecule components of metabolism such as 2-HG efficiently drive significant enhancement and acceleration of CSC reprogramming strongly support the notion that metabostemness factors could poise cells with chromatin states competent for rapid dedifferentiation, creating a persistent pre-neoplastic state suitably primed for later transcriptional alterations to finish the reprogramming of non-CSC into CSC cellular states.



Given that metabolism represents a junction system receiving cumulative signals from upstream (genome, transcriptome, and proteome) and downstream (microenvironment) systems, pre-malignant, and cancer cells can adapt, resist, or react to multi-omic effects through different types of metabolism and, therefore, based on differential regulation, synthesis, and availability of cellular metabolites. Indeed, all the -omic characteristics that drive cancer plasticity concurrently provide to metabolism a flexibility that can be described by means of, at least, four variability criteria, i.e., the occurrence of certain “elite” metabolites, their concentration levels, their relative levels or ratios between metabolites, and metabolic profiles. The occurrence of particular metabolites should be viewed as a qualitative criterion concerning a strong metabolomic parameter specifically stimulated by particular cancer cell states or that locks cancer cells in a given state by merely being present (e.g., oncometabolites). Although apparently simplistic, this binary aspect of associating unique metabolomic parameters with radically different non-CSC versus CSC cellular “species” is strongly supported by the abovementioned findings with the oncometabolite 2-HG. In this scenario,

metabotypes based on the presence-absence of some particular metabolites can have high taxonomic value at specifically differentiating non-CSC versus CSC cellular states in cancer tissues. The increase in the concentration levels of some metabolites could also operate as intrinsic factors governing the proclivity of non-CSC-to-CSC transitions. For instance, the upregulation of the middle glycolysis intermediate fructose-1,6-bisphosphate upon epigenetic silencing of the gluconeogenic enzyme fructose-1,6-bisphosphatase is differentially employed by CSCs as a mechanism of glucose flux maintenance and the lowering of reactive oxygen species (ROS) via glycolysis and other associated biosynthetic pathways (127). Forthcoming studies should elucidate whether metabolic ratios between the concentration levels of structurally close metabolites can also provide biochemical functional discrimination between non-CSC and CSC cellular states in heterogeneous cancer populations. In the same regard, should CSC cellular states possess specific changes in the capacities and kinetics of certain metabolic modes, the discovery of unique, CSC-associated metabolic flux imprints will remain a challenge for the field (see below).

TISSUE-DEPENDENT CANCER METABOLIC PROGRAMS: THE “METABOLIC MEMORY” OF REPROGRAMED CSC

A dynamic perspective of the metastemness trait using Waddingtonian metabolo-epitranscriptional landscapes can explain why we are accumulating evidence that tumor cells' metabolism, often considered a single entity differing from normal cell metabolism, rather exhibits a wide diversity of metabolic phenotypes (162). The heterogeneous expression of metabolic genes is observed across tissue types and, therefore, the metabolotypic expression pattern appears to reflect cancer cells' propensity to adapt the pre-existing metabolic network to successfully support the cancer tissue's altered needs. Where it has been studied, the metabolotype of tumors is a function of both the genetic lesions driving tumorigenesis and the tissue from which the cancer arose (163). Although it has been suggested that this heterogeneous metabolic pattern across tissue types can reflect cancer cells' propensity to adapt the pre-existing metabolic network to support the neoplastic tissue's altered metabolic needs (162), the metastemness trait can provide a radically different view of the occurrence of tissue-dependent cancer metabolic phenotypes.

A shift in the balance between mitochondrial OXPHOS and glycolysis that reconfigures the cellular anabolic requirements (i.e., high glycolytic carbon flux and the increased decoupling from ATP production in mitochondria) precedes the appropriate acquisition of stemness traits in iPSCs (10–30, 105). Similar to well-recognized genetic and epigenetic factors, bioenergetics reprogramming crucially operates as an enabling regulator of cellular reprogramming to stemness because the self-renewal and pluripotency attributes cannot be efficiently acquired in the presence of an inadequate bioenergetic metabolotype. Thus, the efficiency of reprogramming is higher the closer the glycolytic/OXPHOS energy metabolism profiles of the starting somatic cells are to the pattern observed in ESCs. Moreover, bioenergetics resetting has an early, active role during reprogramming because manipulations that inhibit glycolysis reduce, whereas augmenting glycolysis enhances reprogramming efficiency, respectively. Therefore, only when a crucial, very early step of engagement to the stemness' bioenergetics metabolotype is correctly initiated can transcriptional regulators of self-renewal and pluripotency then induce additional endogenous factors to acquire a bona fide stem cell cellular state while maintaining the reprogramming cells' bioenergetics' competence. Accordingly, induced pluripotency can be achieved with a combination of only one stemness transcription factor and small molecules able to facilitate the metabolic transition from mitochondrial OXPHOS to glycolysis. Moreover, there is a progressive resetting of metabolite levels that parallels the progressive changes seen in global epigenetic modifications and gene expression with late passage iPSCs significantly closer to the metabolo-epigenetic profiles of ESCs (19, 164).

The abovementioned findings strongly suggest that, similar to the “epigenetic memory,” a “metabolic memory” also exists that can be partially retained through the reprogramming process and might significantly influence the functioning and differentiation potential of iPSCs from cells of different tissues. Although little is known about the bioenergetics resetting of CSCs, it appears that, similarly to iPSCs, a direct link might exist between the occurrence of a metabolic switch from OXPHOS to aerobic glycolysis and the

occurrence and maintenance of CSC cellular states. Compared to their more differentiated progeny, the acquisition of a stem cell cellular state might necessarily promote changes in the bioenergetics metabolotype because CSCs appear to preferentially perform glycolysis over OXPHOS, at least in some cancer types. Accordingly, recent studies have confirmed that breast CSCs shift from mitochondrial OXPHOS toward fermentative glycolysis and are sensitive to treatment with 2-deoxyglucose, a well-known inhibitor of glycolysis (165). A direct link between glucose metabolism and cancer stem/initiating cells has similarly been established in glioblastoma cancer tissues (166). Once again, by following the numerous parallels between the processes of reprogramming differentiated cells-to-iPSCs and differentiated cells-to-CSC, it seems reasonable to suggest that certain bioenergetics features such as the Warburg effect can no longer be viewed as the single metabolic entity shared by all the cancer tissues, but rather as the archetypical aspect of the undifferentiated state owned by CSCs. As recently pointed out by Pacini and Borziani (167), the specific metabolic phenotype known as the Warburg effect might not be considered a metabolic signature that is acquired during the oncogenesis process; conversely, the Warburg effect might represent an aberrant expression of a metabolic layout that is distinctive from the undifferentiated state owned by CSCs. Pacini and Borziani (167) further consider that there is a gradual and irreversible establishment of an undifferentiated state, with a gradual or complete loss of OXPHOS, rather than respiration in itself, which is often present in neoplasms, as originally described by Otto Warburg almost a century ago. Thus, these authors view the permanent shift toward a Warburgian energetic and metabolic state as an essential contributory cause of cancer, as it might represent the central link between genetic/epigenetic instability and the CSC theory considering that a characteristic and essential feature of each neoplasm is the lack of differentiation.

Our metastemness proposal provides an alternative explanation to the apparently contradictory fact that certain bioenergetic metabolotypes such as the OXPHOS-to-glycolysis bioenergetics resetting can be commonly adopted by CSC cellular states from different cancer tissues, whereas, the metabolic networks of individual tumors more closely resemble those of the normal tissue from which the tumor arose than other tumors that develop in different organ sites (168). From a Waddingtonian perspective, establishing a CSC cellular state is an “uphill battle” against the global slope in the landscape, which accounts for the arrow of time of cancer development. Certain metabolic events (e.g., the Warburg effect) might lower the barriers of the epigenetic landscape to provide “smoothly ascending slopes” while concomitantly enlarging the CSC attractor basin and thus promoting the ground-state character of the CSC cell state that is self-maintaining (Figure 4). However, the Warburgian metabolic state of CSC will be nevertheless globally situated at a “high altitude,” which affords the state a strong urge to “differentiate away” and populate all other cancer states attractors situated at a lower “altitude”; upon certain perturbations the metabolo-network state will “flow down” the valleys to the much lower attractors of differentiated cancer cells, which, because of their “metabolic memory” will convey their tissue-specific metabolic patterns without needing any “instructive” signal.

CANCER METABOSTEMNESS: THE CHALLENGES AHEAD

DESCRIPTION OF THE CSC-METABOLIC PHENOME: THE METABOLIC IMPORTANCE OF THE HOST

Could we determine whether the cellular states of CSCs specifically engage structural or functional changes in metabolic enzymes to produce either specific or differentially enriched CSC-metabolites? We should acknowledge that there are currently no studies on the intracellular metabolic fluxes of CSCs. The elucidation of CSCs' shift between OXPHOS and aerobic glycolysis based solely on transcriptomic data would imply there are no specific changes in CSC metabolism, as most metabolic changes are not accompanied by significant changes in the transcript levels of metabolic genes. The inclusion of proteomics data would shed further light on CSC-associated metabolism; for example, increased protein expression throughout the glycolytic pathway could suggest that this pathway's activity is increased in CSC cellular states. However, proteomics data will not be conclusive regarding changes in the activity of certain metabolic pathways. Indeed, detailed knowledge of cell physiology, in particular at the level of cellular metabolism, is entirely lacking for CSCs due to the difficulty of measuring the *in vivo* metabolic fluxes of mammalian cells. A better understanding of these fluxes will be critical in order to exploit the metastemness trait to eliminate tumor tissues in patients, especially when considering that the tumor cell microenvironment can profoundly affect metabolism and influence how nutrients are metabolized (169, 170). Moreover, tumor tissues are composed of a heterogeneous mixture of cancer cells and normal cells, and symbiotic metabolic relationships have been described in both normal and malignant-tissues contexts (171–175). Because whole body metabolic regulation can also affect tumor tissue metabolism (176), incorporating the complex interplay between genetics, microenvironment, tissue heterogeneity, and whole body metabolism at the CSC-metabolic level remains an enormous challenge for the field. In this regard, we recently reasoned that certain extracellular nutrients might be the major determinant of the maintenance and/or expansion of the metabolic states characteristic of CSC cellular states. Using a standardized high-throughput metabolic phenotyping platform [i.e., the Phenotype MicroArrays for Mammalian Cells (PMM) technology] based on a single-assay metabolic profile of several hundred nutrient sources, we performed a comprehensive evaluation of the phenotypic variations among non-CSC and CSC-like isogenic cells as a starting point for uncovering CSC cells' microecological nutritional niches (5). Our study revealed that the acquisition of stemness traits by breast epithelial cells is sufficient to cell-autonomously enable the vectorial transfer of energy-rich nutrients from the extracellular microenvironment to energy-producing catabolic pathways in the CSC-like cells. We presented the first evidence for the existence of a cell-autonomous “reverse Warburg effect” where CSC reprogramming appears to pre-activate energy-producing pathways that can efficiently use bona fide ketone bodies (e.g., β -hydroxy-butyric acid) and other high-energy metabolites such as lactate and pyruvate from the extracellular milieu to feed mitochondrial energy production, especially upon starvation. Because cancer cells' utilization of the high-energy metabolites pyruvate, lactate, and ketones has been shown to increase the transcriptional expression of gene profiles normally associated with stemness, including genes commonly upregulated

in ESCs (177, 178), our results strongly suggest that global nutrient utilization analyses should be viewed as crucial complements of the metabolo-phenotypic characterization of CSC cellular states.

But how should a definitive elucidation of the metabolo-phenomic maps in CSC be approached? Metabolic flux analysis (fluxomics) is known to represent the physiological counterpart of its sibling's transcriptomics, proteomics, and metabolomics. Fluxomics integrates *in vivo* measurements of metabolic fluxes with stoichiometric network models to allow the determination of absolute flux through large networks of the central carbon metabolism. Thus, fluxomics, by comprising all metabolic conversion rates in a cell, is being increasingly used in fundamental and applied sciences to unravel metabolic infrastructures and activities of metabolic networks and their regulation (Figure 5). Accordingly, forthcoming studies should afford the systematic determination of “CSC fluxomes” by directly inferring the immeasurable *in vivo* central metabolic reaction rates through rigorous mathematical modeling. By performing metabolomic flux analyses using isotopic tracers and mass spectrometry (MS), it might be possible to determine whether CSC cellular states possess highly specific structural or functional changes in metabolic enzymes and nodes that allow them to produce specific or differentially enriched CSC-associated metabolites. CSC fluxomic experiments are currently underway in our laboratory, which consist of feeding the culture of EMT-induced CSC-like cells and isogenic parental cells with a defined ^{13}C -labeled substrate, and measuring, through nuclear magnetic resonance (NMR) or MS, the isotopic enrichment in intracellular metabolites. This information is expected to be “stored” in terms of isotopomers (i.e., each of the possible labeling states in which a particular metabolite can be found). The resultant ^{13}C -labeling in the intracellular metabolites is expected to impose important constraints on how the labeled carbon substrate is distributed throughout the metabolic network and, hence, on the identity of the non-CSC- and CSC-associated metabolic fluxes.

GERONCOGENESIS AND METABOSTEMNESS

The recently proposed “geronco genesis” hypothesis states that metabolic changes during aging (i.e., the normal decline in oxidative metabolism and the development of a Warburgian glycolytic metabolism in normal tissues) constitute an early and important “hit” that pushes cells toward complete cellular transformation. Wu et al. (179) propose that “is not simply the time taken to accumulate genomic hits that accounts for the increased rate of cancer with age, but the decline in metabolic homeostasis and gene regulation that occurs normally as we age.” The authors place the sirtuins, a family of NAD^+ -dependent deacetylases (180) that have evolved as coordinators of physiological responses to nutrient intake and energetic demand, as central to this aging-induced dysregulation of mitochondrial metabolism. In the geroncogenic scenario, aging induces a gradual reprogramming of metabolism toward a “cancer-like” state. This pre-metabolic transformation certainly correlates well with our current proposal of metastemness, which calls for the need to broaden our current perception on how metabolism and metabolites exert influence over the transcriptional factors, the chromatin structure, and the epigenetic circuits that establish and maintain the self-renewal and

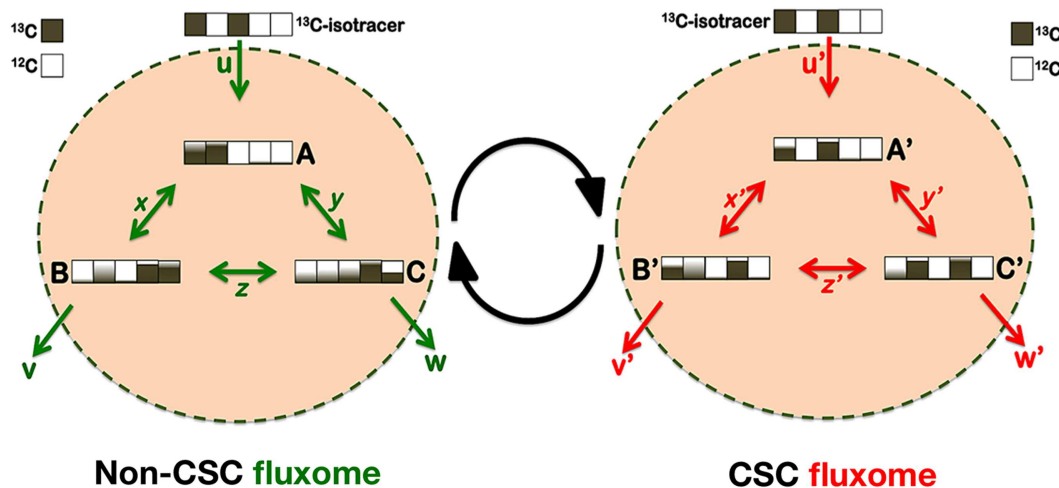


FIGURE 5 | Metabostemness: a fluxomic perspective. CSC cellular states should include changes in the capacities (enzyme abundance) and kinetics (enzyme activity) of certain metabolic nodes that might then generate CSC-associated metabolites or metabolomic flux imprints. Metabolic flux analysis (MFA) has become a standard tool to probe cellular metabolism and elucidate *in vivo* metabolic fluxes, which are estimated from isotopic labeling measurements combined with extracellular uptake and excretion rates. Because ^{13}C -MFA has been shown to provide an inherently more realistic representation of *in vivo* cellular metabolism, the application of the isotopic ^{13}C -MFA methodology might allow obtaining an integrated picture of the metabolic fluxes specifically or differentially occurring in CSC cellular states. In brief, a ^{13}C -labeled substrate can be incorporated into the carbon backbone of a wide range of CSC-metabolites and the CSC metabolome, either through exchange or synthesis. The dynamic distribution of labeled carbon traversing along CSC-metabolic pathways is expected to generate a characteristic

imprint of labeling patterns whose mass signature will be observed by MS. All fluxes can be determined on an absolute scale because the physiological fluxes in and out of non-CSC and CSC cells will be available under experimental conditions. Mass isotopomers can be analyzed at different times to follow the label incorporation immediately after incubating cells with a labeled substrate. To translate the time-series labeling data into metabolic fluxes, MS-related mathematical models could combine the balances of the total metabolite pools and of individual isotopomers to obtain complete information about the transitions of the labeled carbons within metabolites. The model inputs will be the isotopomer ^{13}C time-courses and some metabolite pools determined experimentally, in addition to the consumption or production rates of metabolites measured in cell supernatants. Because the isotopic transient ^{13}C -MFA methodology does not rely on uncertain cofactor balances it allows the estimation of fluxes through both parallel and cyclic pathways as well as through bidirectional reactions.

differentiation capacities of CSCs. Certain aging-related metabolic alterations (e.g., a shift toward a predominantly glycolytic, Warburg-like metabolism) might operate as pivotal molecular events rendering any type of cell of origin susceptible to epigenetic rewiring required for the acquisition of aberrant stemness and, concurrently, of refractoriness not only to apoptosis, but also to differentiation. From a geronogenic perspective (179), certain “gerometabolites” (43) can operate as bona fide metabostemness reprogramming factors that poise cells with chromatin states competent for rapid dedifferentiation while concomitantly setting the idoneous metabolic stage for later genetic alterations to finish the reprogramming of non-cancerous into tumor-initiating cells. From a dynamic perspective using Waddingtonian landscapes, metabolic changes during aging could causally control or functionally substitute the epitranscriptional orchestration of the genetic reprogramming redirecting normal and non-CSC tumor cells toward less-differentiated CSC cellular states by accelerating the number of “stochastic transitions” (i.e., a case in which the metabolo-epitranscriptional rate equations occurring in a given MGRN cannot specify how a cellular state will move from non-CSC attractors to CSC attractors) and increasing the probability of “deterministic transitions” (i.e., a case in which the metabolo-epitranscriptional rate equations occurring in a given MGRN specify how a cellular state will move from non-CSC attractors to CSC attractors once appropriate initial conditions such as genomic

hits are defined). By impacting the epitranscriptional remodeling that facilitates the transition between non-CSC and CSC functional states, the metabostemness trait determines the cancer tissue proclivity of generating a given number of CSCs in a given time-frame, thus providing a functional extension of the geronogenesis hypothesis at the stem cell level. The gero-metabostemness scenario is consistent with the strong association between cancer prevalence and type 2 diabetes, obesity, and sedentary lifestyle and might be counteracted by exercise, calorie restriction (CR), and CR mimetics that may prevent aging tissues from undergoing the metabolic switch in the first place. Forthcoming studies should mechanistically evaluate how organismal diet, stem cell function, and cancer initiation are interconnected (181) and the implications for cancer prevention using metabolic drugs (e.g., biguanides) or natural dietary products including polyphenolic xenohormetins (8, 182, 183).

METABOSTEMNESS AND THERAPEUTICS: FROM METABO-STEMOTOXIC DRUGS TO ANTI-CSC SMART FOODS

The fact that CSC can be generated *de novo* from more differentiated cells adds a crucial concern to the therapeutic elimination of CSC cellular states: how can we resolve the apparently impossible problem of suppressing the molecular biology of the stemness itself as the sole credible target against CSC? The proposed metabostemness hallmark critically contributes to the gaining and

retaining of the stem cell-like fate in pre- and malignant-tissues because, as mentioned above, it operates as the physiological glue that connects all the omic layers with a self-autonomous CSC-metabolic quality, thus establishing a therapeutically targetable metabolic continuum in the reprogramming-differentiation model of cancer genesis and progression. The metabostemness hallmark not only dictates the plasticity of the original differentiated cell that is targeted so as to be reprogrammable by early or late genetic and/or epigenetic hits, but, by removing or remodeling the potential barriers between non-CSC and CSC cellular states, it can also operate as a bona fide accelerator of the reprogramming process, allowing cells to rapidly progress toward the acquisition of a CSC-like status. Because gains and losses of the metabostemness trait can shift the balance of the non-CSC-to-CSC interconversion in one direction or another, it then follows that only certain cell metabolotypes will be compatible with the operational properties exclusively owned by CSC cellular states; conversely, certain metabolotypes will impose cellular modes refractory to CSC reprogramming. In other words, the molecular logic behind the conversion of non-CSC into CSC can be understood in terms of cellular metabolotypes that operate as pathways or roadblocks by facilitating or impeding, respectively, the transcriptional events and signal transduction pathways that lastly coordinate the reprogramming paths to CSC cellular states. Since metabolic (like epigenetic) programs, and unlike most genetic actors involved in stemness (e.g., loss-of-function mutations in tumor-suppressors, the activation of non-catalytic transcription factors), can be erased, manipulated, and reinitiated, the inclusion of the metabostemness hallmark as a new-dimensional cancer driver opens an entirely new framework for cancer prevention and treatment based on CSCs' unique metabolic dependencies. Because small perturbations in a particular metabolic pathway or metabolite might have drastic consequences on the formation, maintenance, and evolution of CSC, an unambiguous elucidation of a putative metabo-stem connection remains mandatory before we could develop a new generation of therapies directed against CSCs' metabolic dependencies. Accordingly, anti-metabolic reprogramming strategies begin to provide a roadmap for the generation of novel "metabo-stemotoxic" therapies that metabolically target CSCs in biologically aggressive tumor types; in this regard, perhaps it is not surprising that the potential applications for biguanides in oncology (184) closely relate to their differential metabolic effects during the cellular transformation and CSC stages (185). A growing number of studies have demonstrated that the biguanide metformin selectively ablates CSCs, as evidenced by the decreased expression of pluripotency-associated genes, CSC-associated surface markers, and other CSC-specific properties including tumor-initiation (83, 84, 166, 185–204).

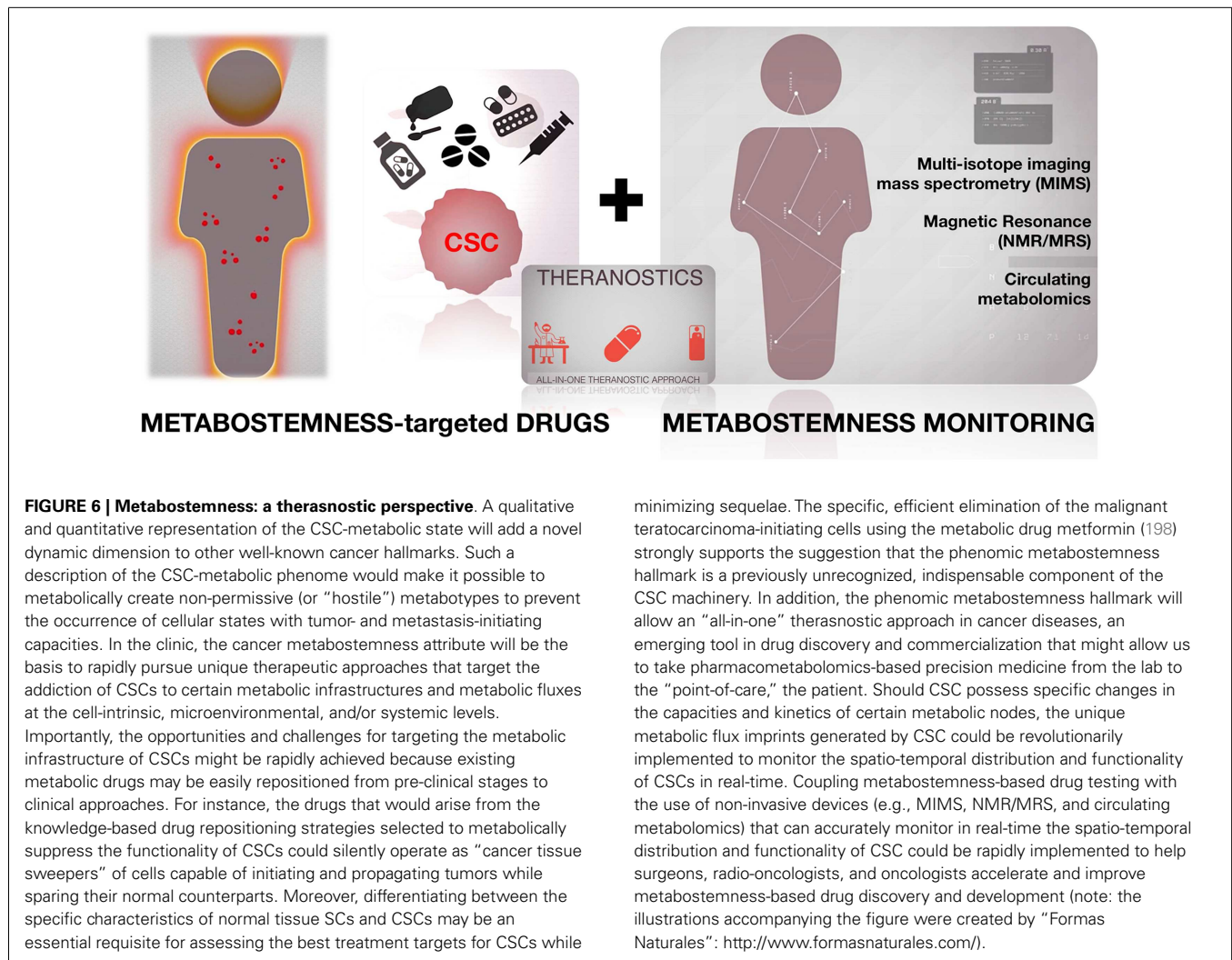
Moreover, a definitive elucidation of the metabolo-phenomic maps of CSC cells may reveal an unforeseen route to the development of therapies against not only the intrinsic metabolic energy-generating machinery of CSC cells, but also their nutritional niches. The emerging discipline of nutritional genomics or nutrigenomics includes both the study of the effects of diet on an individual's gene activity and health and the study of how genetic composition affects nutrient metabolism (205–208). Through an understanding of the unique roles of specific nutrients and their

possible roles in boosting CSC-like cell phenotypes, it might be possible to customize "smart foods" or systemic "metabolic nichotherapies" tailored to the specific nutritional phenomes possessed by cells with CSC cellular states. Nevertheless, because new techniques such as multi-isotope imaging mass spectrometry (MIMS) permit the high-resolution tracking of heavy isotope-labeled molecules upon utilization by specific types of cells (209, 210), the unique metabolic fluxes generated by the catabolic energy-producing machinery of CSC could be implemented in a novel manner to monitor the spatio-temporal distributions and functionality of CSC cellular states in real-time.

COROLLARY

In view of recent groundbreaking studies showing that aging-related decline of certain metabolites can set the metabolic stage for later mutations to drive tumorigenesis (i.e., the geroncogenesis hypothesis) and that the epigenetic fate determination of differentiated cells might be converted to pluripotency by microenvironmental cues, it is time to paradigmatically rethink our perception of the regulatory role of metabolic reprogramming in cancer cell-fate decisions. Frustrated by the gene-centric guidelines that usually govern conventional approaches against CSC, we recently envisioned that the incorporation of robust metabolic phenomics, i.e., the systematic acquisition and objective documentation of cancer metabolic data at the level of CSC cellular states, might revolutionize the advancement of CSC-related cancer precision medicine. Our current proposal of the metabostemness cancer hallmark should forge a new and different path to treating and monitoring cancer through the metabolic phenome of CSC cellular states. Under the assumption that the molecular biology of the stemness transformation itself would be the sole credible target in CSC, a systematic combination of biology, biochemistry, pharmacology, genetics, fluxomics, and mathematical approaches should unambiguously provide the first qualitative and quantitative phenomic representation of the metabolic state of CSC, thus unlocking an almost unexplored field of discovery in cancer research. By generating a robust product engine to interrogate the differential cellular metabolism of CSCs relative to normal and non-CSC tumor cells, we might discover an unforeseen new phenomic hallmark of cancer that we have called cancer metabostemness.

Previously, scientists believed that metabolic changes were a mere consequence of aberrant cancer cell growth. We propose that metabolic reprogramming of CSC has cancer-causing activity. This proposal suggests an alternative explanation and offers the possibility of delineating a metabolic roadmap for the acquisition and maintenance of CSC cellular states that might not even require pre-existing mutations or rearrangements of well-established "cancer genes." Moreover, identifying the metabolotypic infrastructure of CSCs will add a novel dynamic, phenomic dimension to well-recognized cancer hallmarks. Should CSC possess specific changes in the capacities and kinetics of certain metabolic nodes, the unique metabolic flux imprints generated by CSC could be revolutionarily implemented to monitor CSCs' spatio-temporal distribution and functionality in real-time. Once in the clinic, this approach can be used to pursue unique and potentially more rapid clinical development pathways by theranostically focusing on cancer populations defined by specific



CSC-metabolites or CSC-metabolic imprints. Indeed, we will be able to pharmacologically establish cell metabolotypes that are protected against the reprogramming events leading to CSC cellular states, thus providing the molecular bases to accelerate and diversify our current therapeutic capacity in new clinical trials designed to metabolically prevent and target CSC cellular states. The metabostemness cancer hallmark generates a shifting oncology theory that should guide a new era of metabolo-epigenetic cancer precision medicine. The tremendous health and social impacts of this paradigmatic shift in the origins of CSC-driven cancer heterogeneity are suited to becoming the platform for new biopharmaceutical strategies based on CSC-metabolic phenomics and dedicated to the research and development of the almost unexplored field of CSC-metabolic theranostics (CSC-metabolic diagnosis + CSC-metabolic treatment; **Figure 6**) in cancer diseases.

ACKNOWLEDGMENTS

This work was financially supported by the Ministerio de Ciencia e Innovación (SAF2012-38914), Plan Nacional de I+D+I, MICINN, Spain.

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Received: 03 August 2014; accepted: 07 September 2014; published online: 29 September 2014.

Citation: Menendez JA and Alarcón T (2014) Metabostemness: a new cancer hallmark. *Front. Oncol.* 4:262. doi: 10.3389/fonc.2014.00262

This article was submitted to Molecular and Cellular Oncology, a section of the journal *Frontiers in Oncology*.

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