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FORUM REVIEW ARTICLE

The Potential Role of CD133 in Immune Surveillance and Apoptosis: A Mitochondrial Connection?

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Abstract

Significance: Recent research has shown that tumors contain a small subpopulation of stem-like cells that are more resistant to therapy and that are likely to produce second-line tumors. ***Recent Advances:*** Cancer stem-like cells (CSCs) have been characterized by a variety of markers, including, for a number of types of cancer, high expression of the plasma membrane protein CD133, which is also indicative of the increase of stemness of cultured cancer cells growing as spheres. ***Critical Issues:*** While the function of this protein has not yet been clearly defined, it may have a role in the stem-like phenotype of CSCs that cause (re-)initiation of tumors as well as their propagation. We hypothesize that CD133 selects for CSC survival against not only immunosurveillance mechanisms but also stress-induced apoptosis. ***Future Directions:*** High level of expression of CD133 may be a useful marker of more aggressive tumors that are recalcitrant toward established therapies. Compelling preliminary data indicate that drugs targeting mitochondria may be utilized as a novel, efficient cancer therapeutic modality. *Antioxid. Redox Signal.* 15, 2989–3002.

Introduction

Carcinogenesis results from accumulation of mutational and genetic changes that alter normal cell growth control and proapoptotic/survival pathways. The newly formed malignant cells are morphologically distinct: they are able to evade apoptosis; proliferate out of control; invade the surrounding tissue; induce angiogenesis; and eventually metastasize (48). During the early stages of carcinogenesis, a variety of intrinsic tumor suppressor mechanisms are ready to trigger apoptosis, repair, or senescence if cellular proliferation and division become abnormal (108). Apoptosis can be triggered in response to a variety of signals induced by cell stress and injury, with mitochondria as key intracellular mediators (21), or in response to ligation of cell-surface death receptors, such as Fas or the tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) (88).

Not all malignant cells are destroyed during the early stages of carcinogenesis. Transformed cells that escape the intrinsic cellular controls for abortive suicide are then subjected to extrinsic tumor suppressor mechanisms. Thus, the immune system functions as an extrinsic tumor suppressor by detecting and eliminating malignant cells on the basis of tumor-specific antigens (108, 112). Cancer immune surveillance, whereby the immune system identifies cancerous and/or precancerous cells and eliminates them before they can cause harm, is now known as the elimination phase of a broader process that has been termed cancer immunoediting (27, 28). This process is based on the concept that the immune system not only protects the host against cancer formation but also promotes cancer development, and the "three Es" of cancer immunoediting comprise the elimination phase (cancer immunosurveillance), equilibrium phase, and escape phase (29).

In the elimination phase, both the innate and adaptive arms of the immune system work together to detect and eliminate cancer cells that have developed as a result of failed intrinsic tumor suppressor mechanisms (107). The initial stage mainly engages the innate immune system, including macrophages and natural killer (NK) cells that may recognize developing tumors *via* receptor-mediated recognition processes, some of which involve tumor antigen presentation (108). The tumor-activated immune cells then utilize cytotoxic effector mechanisms to kill and eliminate the transformed cells (or cells undergoing malignant transformation). The immune cells

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also secrete interferons (IFNs), which control tumor growth and amplify the immune response (108). Initially, IFN-γ that is released at the tumor site induces production of chemokines, which recruit cells like macrophages that produce interleukin-12 and NK cells producing more IFN-γ at the tumor site (5). IFN-γ-activated macrophages can induce cancer cell death by releasing products such as reactive oxygen species (ROS) (98). Activated NK cells induce apoptosis in cancer cells by triggering TRAIL- or perforin-dependent pathways (50, 106, 113).

The tumor necrosis factor family member TRAIL is one of the major mediators of antitumor immunity. TRAIL is predominantly expressed by immune cells, particularly of the myeloid lineage, where it is either present on the cell surface as a membrane protein or is secreted in a soluble form to induce apoptosis by binding to cognate death receptors on target (tumor) cells (63, 118). In contrast to other mediators, TRAIL induces apoptosis in various cancer cell types, being generally nontoxic toward normal cells (38). Cancer cell resistance to TRAIL-mediated cell death may be due to upregulation of the antiapoptotic FLICE-inhibitory protein (FLIP) (40, 55, 123, 132).

ROS are formed as a natural by-product of normal oxygen metabolism. Increase in the level of ROS creates oxidative stress, which causes cell damage with ensuing cell death by way of activating various signaling pathways that, in many cases, converge at apoptosis induction (15, 67). While the signaling mechanisms have not been completely unraveled, mitochondria play a key role in the oxidative stress-induced apoptotic pathways (72). Mitochondria, organelles vital for cellular energy homeostasis, produce ROS in the mitochondrial respiratory chain (13). Recent studies have showed that ROS generation involves complex II of the respiratory chain (1, 71), rendering it very important for apoptosis induction and the ensuing death of cancer cells (3, 23, 25, 75). We have recently designed novel anticancer drugs from the family of mitocans (81, 83) that specifically target mitochondria (the mitochondrial complex II) and that are much more efficient than the corresponding mitochondrial-untargeted compounds, while preserving selectivity for cancer cells (23, 25). These anticancer drugs showed very high efficacy against fast proliferating breast cancer cells as well as their cancer stem-like counterparts, which in our study were represented by spheroid cultures, that is, multicellular structures with stem cell-like characteristics (see below).

If the transformed cells are not fully destroyed during the elimination phase, they then enter into a temporary state of equilibrium with the immune system (28, 29). Here, cancer cells can either remain dormant or continue to evolve by accumulating additional changes, which would allow them to escape detection by the immune system (108, 112).

Taken together, the immune system is for the most part very effective in suppressing cellular transformation and cancer growth *via* the diverse extrinsic mechanisms as described above such that cancer, on average, occurs less than once in a lifetime. This is the case even though many cells in the body exist as potential targets for significant mutational and genetic changes. Nevertheless, cancers do arise in immunocompetent individuals, suggesting that clonally derived populations of cells are able to escape the immune system. These cells that emerge from the selective pressures of the immune system are resistant to immune-induced killing due to reduced immunogenicity. Moreover, such cells are often also resistant to many of the currently used anticancer therapies.

The Significance of Emerging Cancer Stem-Like Cells

In the past decade or so, the cancer stem cell hypothesis has been experiencing a resurgence. Various studies have suggested that stem cells may play a vital role in carcinogenesis because cancer stem-like cells (CSCs) (also referred to as tumor-initiating cells) share many crucial characteristics with normal stem cells that have the capacity to repopulate whole tissues (104, 123). These features include the ability to self-renew and differentiate, high levels of telomerase activity, greater capacity for DNA repair, activation of antiapoptotic pathways, and increased membrane transporter activity including the ATP-binding cassette drug transporters (providing for an increased level of drug resistance). In addition, CSCs are able to migrate and metastasize, hence forming secondary tumors that are, as a rule, refractory to established therapeutic modalities (2, 92, 104, 123).

To date, CSCs have been identified in a range of different cancers, including leukemias (8, 62), multiple myeloma (86), neoplasias of the nervous system (90, 104), colorectal, prostate or hepatocellular carcinomas (18, 94, 127), breast cancer (4), melanoma (32), and osteosarcoma (41). Within these tumors the CSCs represent only a small subpopulation, sometimes referred to as the side-population because these cells can actively exclude fluorescent DNA-staining dyes (53). The main problem with CSCs is that, similar to normal stem cells, they share properties that render them relatively resistant to current cancer therapies such as chemotherapy and radiation therapy, thereby promoting their survival. For these reasons they present a considerable problem in cancer management (6, 36, 47).

A variety of markers have been used to characterize CSCs (Table 1). In particular, these include CD133 and CD44 surface proteins and several intrinsic markers (*e.g.*, Oct-4 or ALDH1). Overexpression of CD44 has been found in the CSC population in breast (4), prostate (18), and pancreatic cancer (64). The CD44 protein is involved in cell–cell and cell–matrix interactions, and $CD44^+$ breast cancer cells exert more pronounced transforming growth factor-β signaling (102).

CD133 (Fig. 1), also known as prominin-1 (20, 74), is emerging as an important marker of CSCs identified in many different studies (73, 77, 85, 94, 104, 110, 124). CD133 is highly expressed by a variety of tumor types, including medullo-

Table 1. Examples of Markers for Different Types of Tumors

Tumor type	*CSC markers*	*References*
Brain	Musashi-1^+, nestin$^+$, $CD133^+$	105
Breast	$CD44^+$, $CD24^{+/low}$, $ALDH1^+$, $CD133^+$	4, 42, 124
Colon	$CD133^+$	84, 94
Hepatocellular	$CD133^+$	110
Leukemia	$CD34^+$, $CD38^-$, $CD44^+$	11
Melanoma	$CD20^+$, $CD133^+$, $ABCG2^+$	32, 77
Pancreatic	$CD133^+$, $ABCG2^+$	85
Prostate	$CD44^+$, $\alpha_2\beta_1^{high}$, $CD133^+$	18, 73, 87

CSC, cancer stem-like cells.

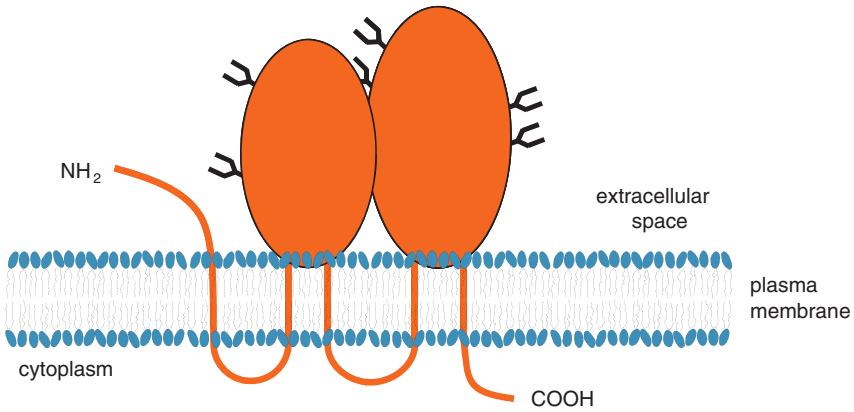


FIG. 1. The proposed structure of CD133 (Prominin-1) showing the glycosylation sites. (Adapted and modified from refs. 20, 33, 34, 69, 74, 122.) (To see this illustration in color the reader is referred to the web version of this article at www.liebertonline.com/ars).

blastoma (104), glioblastoma (96), colorectal (84), prostate (18), and breast carcinomas (124), as well as mesotheliomas (J.N. *et al.*, unpublished). Given its high level of expression, CD133 has received considerable attention. For example, it has been shown that $CD133^+$ cells purified from cultured cancer cells or tumors and transplanted into immunocompromized NOD/SCID mice give rise to carcinomas phenotypically similar to the primary tumors from which they were derived (84, 94, 127). In addition, whereas as many as 10^7 cultured hepatocarcinoma cells were required to form tumors in NOD/SCID mice, only 10^3 or less $CD133^+$ cells were required to form tumors (127). Similar results have been obtained using breast CSCs (124). Shmelkov and colleagues have recently demonstrated that $CD133^-$ cells can also form tumors in NOD/SCID mice. However, in this study they also showed that $CD133^+$ cancer cells have much greater metastatic potential than their $CD133^-$ counterparts (103). Even if CD133 may not always be a *bona fide* marker of CSCs, it has a role for suggesting an increase of the level of stemness of cell cultures grown as spheres or, in other words, as a marker of enrichment of sphere cultures in CSCs. Table 2 documents some of the reports proposing a role of CD133 in tumorigenic properties of cancer cells as well as those opposing this notion.

Table 2. Reports Documenting the Tumorigenic Properties of $CD133^+$ Cells and Those Opposing This View

Type of tumor	*Positive*	*Negative*
Brain tumors	105	
Gliomas, glioblastomas	57, 65, 96	
Medduloblastomas	104	
Breast tumors	124	
Colorectal tumors	35, 84, 94	22, 103
Endometrial cancer	80	
Gallbladder carcinomas	101	
Hepatocellular carcinoma	59, 110, 127	97
Melanomas	77	
Mesotheliomas	J.N. *et al.*, unpublished	
Non-small-cell lung carcinomas	117	
Oral squamous cell carcinomas	128	
Pancreatic cancer	85	
Prostate cancer	18, 73	89
Ovarian cancer	44	

CD133, Avoidance of Immunosurveillance, and Resistance to Stress-Induced Apoptosis

In this review, we hypothesize that CSCs, escaping elimination by the immune system, are sculpted to efficiently form tumors. This is based on the immunoediting theory and the cancer stem cell theory as well as related studies supporting these paradigms as described in the previous sections. As a result, surviving transformed cells are likely to be selected to exert enhanced levels of stemness. It is also possible that during immunoediting, the pressure of the forces of the immune system causes modifications in cancer cells such that they acquire cancer stem cell-like characteristics, which enable them to survive. This would suggest that CSCs feature low levels of antigen-presenting capacity as well as low levels of NK ligands. In this regard, Wu and colleagues investigated the immunogenicity of $CD133^+$ cells in two human astrocytoma and two glioblastoma multiformae samples by flow cytometry and revealed that the majority of $CD133^+$ cells do not express detectable major histocompatibility (MHC) Class I or NK cell-activating ligands (125). While MHC Class I expression is required for $CD8^+$ T cell-mediated cytotoxicity, the converse is true for NK cells, which recognize and attack cells lacking MHC class I ligands (61, 121). Although $CD133^+$ cells do not express MHC Class I ligands, which would make them susceptible to NK-mediated cytotoxicity, the absence of NK cell-activating ligands on $CD133^+$ cells could contribute to their escape from immune surveillance by these innate immune cells.

Accordingly, studies on embryonic stem (ES) cell immunogenicity revealed that ES cells are immune-privileged as well and are readily transplanted across MHC barriers without or with only minimal immunosuppression (26, 31, 59). Hence, it would appear that CSCs share properties similar to stem cells, including ES cells. It is, therefore, also possible that the immune system fails to recognize these cells as a potential threat since their malignant status remains undetected.

CSCs and the Role of CD133

CD133 expression is not just restricted to CSCs in that, for example, both $CD133^+$ and $CD133^-$ metastatic colon cancer cells are equally capable of initiating tumors (103). It would appear more likely that CD133 expression represents a transient state that CSCs are capable of undergoing. Thus, the $CD133^+$ population in the normal intestinal crypt is part of a larger stem cell/transit amplifying progenitor compartment that is susceptible to neoplastic transformation after which

CD133 expression reduces to a small percentage of cancer cells (131). Notwithstanding, whether CD133 expression is essential for the emergence of tumors remains unclear.

Thus far, the exact role of CD133 in CSCs has not been resolved. However, CD133 family molecules show a highly restricted localization on plasma membrane protrusions of epithelial and other cell types (hence the name "prominin-1"), where they are associated with cholesterol-rich lipid rafts, indicating a role in the organization of plasma membrane topology (19, 20, 76, 95, 122). The inclusion in cholesterol lipid rafts, which are important for cell growth signaling, is consistent with the association of CD133 with important stem cell growth signaling pathways. As well as being a stem cell marker (74), CD133 has been proposed to be involved in several signaling pathways (76, 115), including the Notch pathway (30, 111) and the related sonic HEDGEHOG/GLI pathway (17), which are of significant importance in normal stem cell as well as CSC proliferation and self-renewal. A role for CD133 in signal transduction is consistent with the observations that the protein is phosphorylated on cytoplasmic tyrosine residues 828 and 852 of CD133 in human cancer cells by Src and Fyn tyrosine kinases (10). Recently, a link between the Notch pathway and CD133 has been corroborated by findings that blocking the Notch pathway (using an inhibitor of γ-secretase) promoted down-regulation of CD133, which then resulted in prevention of such cells to form spheres and tumors in immunocompromized animals (30, 111). Moreover, we have observed that CD133-positive mesothelioma cells, grown as mesospheres, are also typified by increased Notch expression. We also found higher expression of CD47 in mesospheres (J.N. *et al.*, unpublished), a marker of resistance of cancer cells to be phagocytosed by macrophages that may protect CSCs from the immune system (14, 56), further suggesting a link between CD133 expression and the propensity of such cells to evade the immune surveillance.

CD133 is likely also involved in adaptive changes in cellular bioenergetic metabolism, providing the CSCs with increased survival advantages. Thus, CD133 expression is greatly increased in the presence of high glucose, whose uptake is facilitated by CD133 (130). It has now been documented that CD133 also represents a state of hypoxic induction in CSCs and may be indicative of a side population of stem cells, thereby transient in nature depending on the prevailing microenvironment and conditions these cells are exposed to and influenced by (68, 109). Consistent with this role, CD133-expressing CSC populations have been commonly observed to show greater proliferative potential than their $CD133^-$ counterparts (7, 125). Further, increased exposure to hypoxia results in stabilization of the hypoxia-inducible factor-1α (HIF-1α) and is linked to the increase in the level of CD133 (43, 70, 109). Hypoxia-induced activation of HIF-1α and the increase in the expression of CD133 requires activation of the kinase Akt. Another piece of evidence linking CD133 to Akt signaling comes from a recent article by Takenobu *et al.* (114), who showed that knocking down CD133 in neuroblastoma cells deregulated the Akt as well as mitogen-activated protein (MAP) kinase pathway and, interestingly, resulted in much slower growth of tumors in nude mice than found for the parental cells. In these studies, CD133 was also found to enhance the survival of the neuroblastoma cells in spheres, further linking CD133 to the stemness phenotype. Since Akt is a central regulator of the survival pathways, promoting survival and proliferation while suppressing apoptosis, we propose an emerging picture linking the high level of CD133 and increased resistance to apoptosis (Fig. 2). Further, the increased activity of HIF-1α and higher expression of CD133 connects this phenotype to the mitochondrial bioenergetics, since HIF-1 is known as a regulator of a variety of mitochondrial bioenergetics pathways as well as a sensor of oxygen (93, 99).

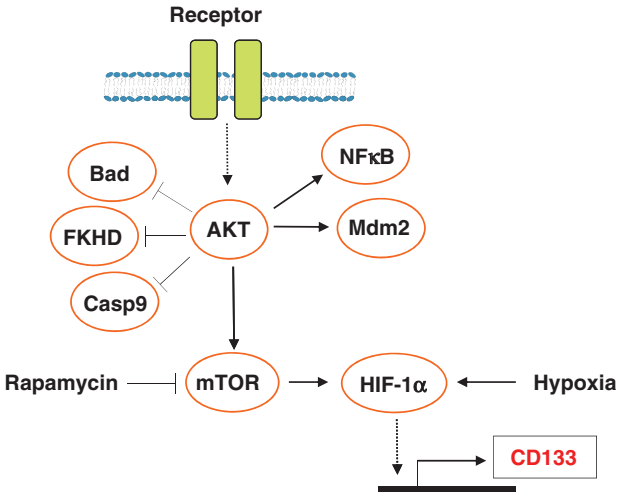


FIG. 2. Possible link between resistance of cancer stem-like cells (CSCs) to apoptosis and the level of CD133. Within the hypoxic microenvironment of pretumorous mass or carcinoma, HIF-1α becomes stabilized, which is linked to increased expression of CD133. This process requires the activation of the central prosurvival kinase Akt, which is responsive, generally, to mitogenic signaling. Akt itself suppresses the activity/activation of the proapoptotic pathways *via* phosphorylating the Bcl-2 family protein Bad, caspase-9, and members of the family of the forkhead (FKHD) transcription factors, such as FoxO1 that is involved in transcriptional activation of the Bcl-2 family member Noxa (120). Akt also activates several important prosurvival pathways, involving the transcription factor NF-κB, the Mdm2 protein suppressing the function of p53, and the molecular target of rapamycin (mTOR). mTOR positively controls the function of hypoxia-inducible factor-1α (HIF-1α), further promoting the expression of CD133. Although not completely understood at present, these notions provide a possible explanation of the association of CD133 with the prosurvival/antiapoptotic status of CD133-positive cancer cells (adapted from ref. 68). (To see this illustration in color the reader is referred to the web version of this article at www.liebertonline.com/ars).

As indicated above, high levels of CD133 expression have been associated with resistance of CSCs to apoptosis (6, 17, 36, 37). This is directly supported by the observation that down-regulation of CD133 sensitizes cancer cells to apoptosis (17). Analysis of $CD133^+$ and $CD133^-$ glioma cell lines prepared from primary human glioblastoma cells identified a number of genes differentially expressed in the two subpopulations (65). The analysis showed that the antiapoptotic proteins Bcl-2, Bcl-x_L, FLIP, and members of the inhibitors of apoptosis family of proteins (c-IAP1, c-IAP2, XIAP, and survivin) were highly up-regulated in $CD133^+$ cells, whereas the expression of the proapoptotic protein Bax was slightly down-regulated when compared to their $CD133^-$ counterparts. Of all the up-regulated genes, FLIP exerted the highest expression with up to 300-fold increase in the $CD133^+$ cells when compared to the $CD133^-$ cells (Table 3). This is of great interest, since FLIP (as

Table 3. Examples of Differentially Expressed Apoptosis-Associated Genes in $CD133^+$ and $CD133^-$ Glioblastoma Cells (Adapted from Ref. 65)

Gene name	*Fold change between $CD133^+$ and $CD133^-$ cells*
FLIP	294 ± 26
cIAP1	39 ± 3.5
XIAP	21.9 ± 2.2
Bcl-2	13.9 ± 1
Bcl-x_L	5.6 ± .4
cIAP2	3 ± 0.3
Survivin	1.6 ± 0.1
Bax	0.33 ± 0.03

discussed above) is an inhibitor of death receptor-induced apoptosis (55, 116). Its increased expression could be a consequence of acquisition of the malignant phenotype of cells, requiring an advantage to survive the tumor immune surveillance.

Further support for the survival-promoting effects of CD133 on CSCs stems from studies where $CD133^+$ cells were also found to be resistant to cytotoxic chemotherapeutic drugs, including etoposide, paclitaxel, temozolomide, and carboplatin, whereas their autologous $CD133^-$ counterparts were susceptible to these agents (65). Despite the new insights into CSCs, the reasons for their resilience to therapy are not well understood. However, recent research has indicated possible molecular determinants underlying this resistance (119). Several studies have shown that CSCs, epitomized in this case by $CD133^+$ cells, exhibit increased expression of antiapoptotic proteins (FLIP, Bcl-2, Bcl-x_L, and the IAP family members) and a low level of expression of proapoptotic proteins (including Bax) (57, 65, 100). We reported that $CD133^{high}$ subpopulations of cancer cells (including both Jurkat T lymphoma and breast cancer cells) possess high levels of the antiapoptotic protein FLIP relative to their $CD133^{low}$ counterparts, which makes them resistant to TRAIL-mediated apoptosis (132). By contrast, we found that $CD133^{low}$ cells were more susceptible to killing by TRAIL. This result can be reconciled with the notion that TRAIL-induced apoptosis is one of the key mechanisms by which the immune system eliminates transforming cells. In addition, the TRAIL-dependent apoptotic cascade primarily affects malignant cells and, as a rule, has only marginal effects, if any, on normal (nonmalignant) cells (63). However, this is not the case for CSCs (or at least for $CD133^{high}$ cancer cells), as we have demonstrated. Nevertheless, down-regulation of FLIP in $CD133^{high}$ Jurkat T lymphoma or breast cancer cells caused their sensitization to apoptosis induced by TRAIL in both cell types (132). This finding is further corroborated by results of another report documenting that down-regulation of FLIP in TRAIL-resistant melanoma cells was sufficient to make these cells susceptible to the apoptogen (40).

In a separate study on CSCs, we observed that stress in the form of nutrient deprivation, cell crowding, or hypoxia results in apoptosis induction (J.N. *et al.*, unpublished). Interestingly, the surviving population showed an increased expression of CD133 in agreement with similar findings of others (43). We also observed that exposure of cancer cells growing in monolayer cultures to the same stresses results in rapid up-regulation of CD133 such that the resulting cells were also protected from apoptosis for extended periods of time. These processes, involving differential expression of CD133, may signify more general properties of CSCs, since we observed similar findings with diverse cancer cells, including Jurkat T lymphoma, breast cancer, and mesothelioma cell lines (J.N. *et al.*, unpublished). This suggests that either the cells that survive already display the phenotype of CSCs or that the pressure of the stressful environment favors cancer cell populations acquiring increased cancer stem cell-like properties to survive. This latter possibility has been re-

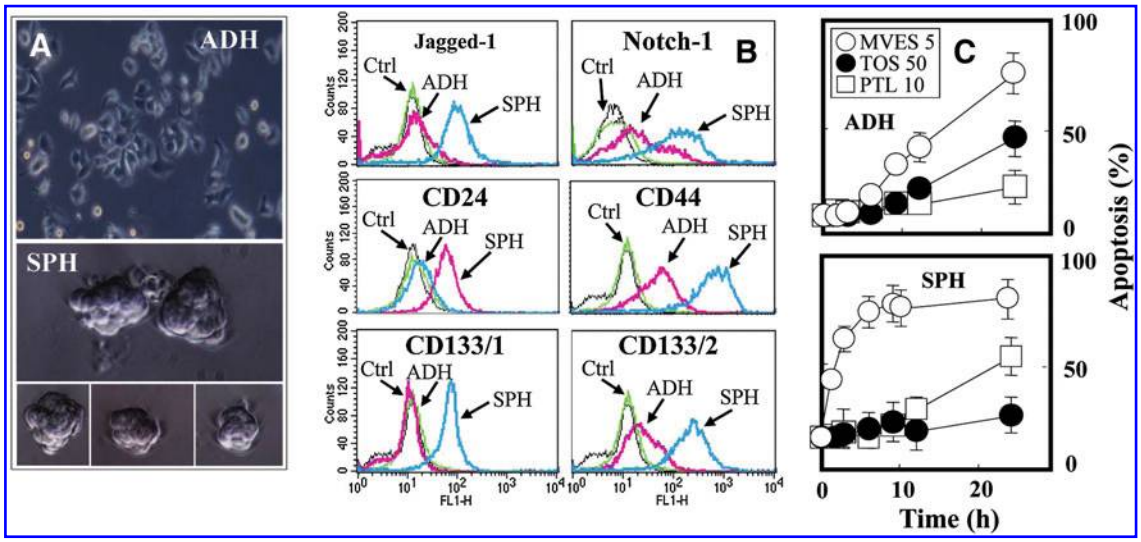


FIG. 3. Breast cancer stem-like cells are susceptible to mitochondrially targeted vitamin E succinate (MitoVES). MCF7 cells were grown as adherent cells (ADH) or spheres (SPH) **(A)** and assessed for the expression of several CSC markers, including CD133 (both isotypes), CD44, CD24, Jagged-1, and Notch-1 **(B)**. The adherent and sphere cells were exposed to α-tocopheryl succinate (α-TOS), its mitochondrially targeted analog (MitoVES), or pathenolide (PTL) at the concentrations (μM) and times shown, and assessed for apoptosis induction **(C)**. (To see this illustration in color the reader is referred to the web version of this article at www.liebertonline.com/ars).

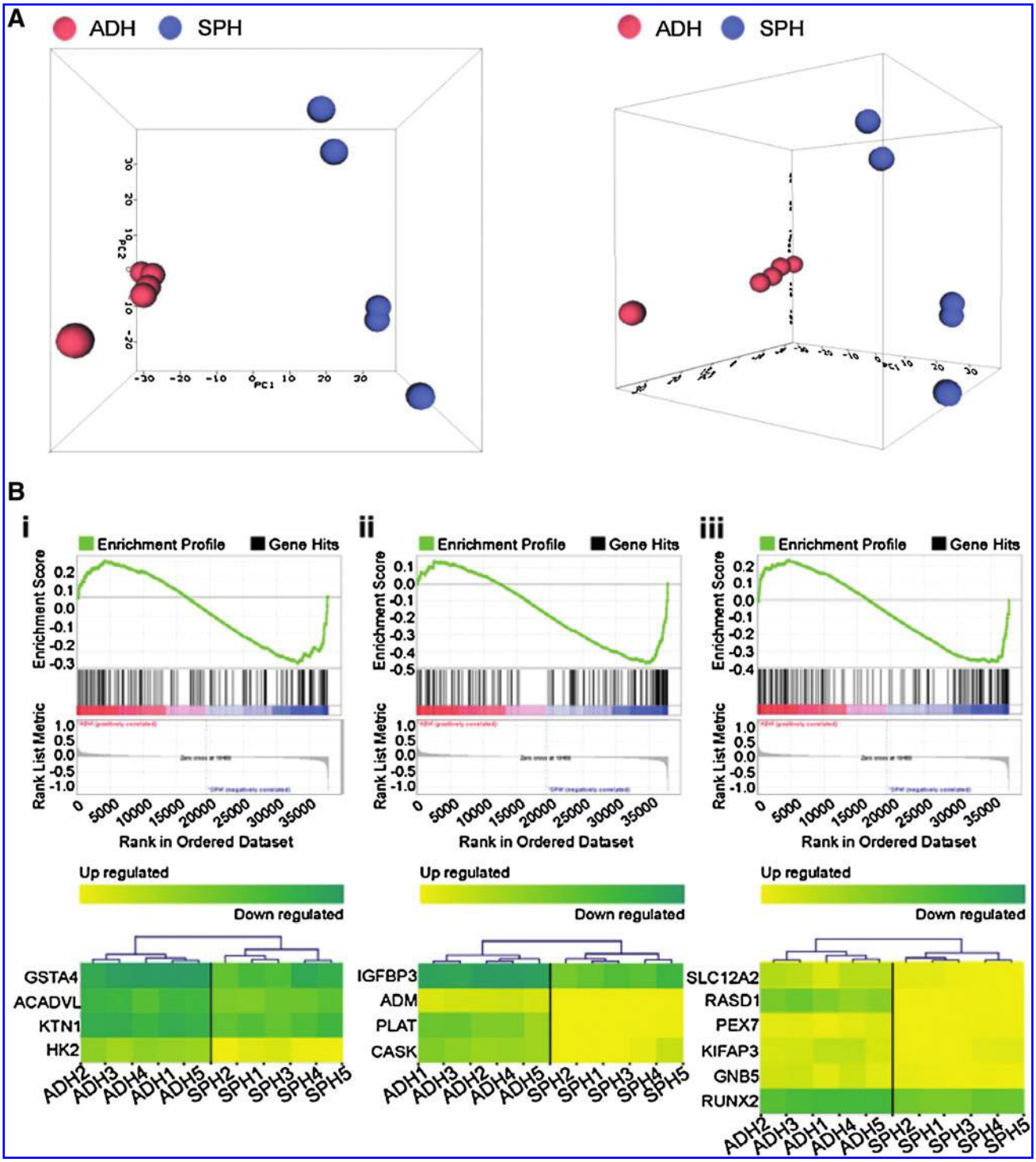


FIG. 4. Microarray data characterize mammospheres as a phenotype with increased stemness. (A) Principle components analysis of adherent (ADH) and mammosphere (SPH) MCF7 cells shows that each phenotype clusters together. Principle components analysis projections are represented in a two-dimensional (left) and a three-dimensional (right) manner. **(B)** Gene set enrichment analysis plots show enrichment of **(i)** embryonic stem cell ($p = 0.044$, false discovery rate [FDR] = 0.046), **(ii)** neuronal stem cell ($p = 0.001$, FDR = 0.006), and **(iii)** hematopoietic stem cell ($p = 0.075$, FDR = 0.085) gene sets in mamosphere but not adherent cultures. Each *vertical line* on the enrichment plot represents a probe in the corresponding gene set. The left to right position of vertical lines indicates the relative position genes from ESC, NSC, and HSC gene sets within the rank-ordered list of the 37,805 probes present on the HumanHT-12 BeadChip. The first probe on the left represents the most upregulated probe in adherent samples and the last on the right represents the most upregulated probe in the sphere forming samples. Probes in the middle are not differentially expressed. (To see this illustration in color the reader is referred to the web version of this article at www.liebertonline.com/ars).

cently supported by the studies of Griguer and colleagues, who showed that CD133 expression was up-regulated in response to stress by hypoxia or by mitochondrial respiratory inhibition using rotenone (43). This finding can be reconciled with the reported high level stabilization of HIF-1α under normoxic conditions in $CD34^+$ and $CD133^+$ hematopoietic stem cells, probably *via* increased levels of (mitochondrial) ROS generation, suggesting that the cells are undergoing adaptation to promote their enhanced survival propensity (91).

Hence, cancer cell selection by the immune system or prevailing conditions (*e.g.*, oxidative or hypoxic stress) leads to their acquisition of increased level of stemness, epitomized by elevated CD133 expression, being more difficult to efficiently eliminate using current therapies. These cells are also capable of forming second-line tumors, clonally derived from the resistant $CD133^+$ CSCs, and such carcinomas are expected to be much more difficult to treat.

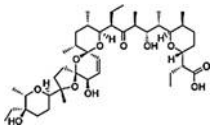
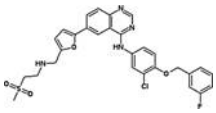
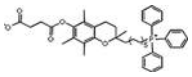
Where to from Here?

As stated above, CD133 may be either a factor that selects cancer cells (possibly with high levels of stemness) for survival, or readily increases when cancer cells are challenged by stressful/unfavorable selection pressures leading to increased CD133 expression associated with greater resistance of cancer cell subpopulations to the stress (in very broad terms). It is unclear at this stage, though, whether there is a causal (or, better, functional) link between the level of CD133 and resistance of cancer cells to stress, most likely involving mitochondria. Based on the evidence presented here, we more strongly favor a role for expression of CD133 in the induction of apoptosis-regulating genes such that CSCs exhibit greater likelihood of survival. We also believe it more plausible that malignant cells develop resistance during immunoediting to escape immunosurveillance first, and then CSCs with initially low levels of CD133 emerge that rapidly increases when the cells are challenged by the ensuing unfavorable or stressful growth conditions. However, although unlikely, current data cannot completely rule out a concomitant increase of CD133 and acquisition of a resistant phenotype.

It is unlikely that the selection of CSCs that survive the challenge (including immunosurveillance or, later on, stress) is a random, uncoordinated process. However, the molecular basis is not known whereby CSCs are selected for survival, and one would reason that CD133 is not the only key element that dictates the very early cellular responses to the selection pressure(s). Understanding how cells are selected is not only of fundamental importance for our understanding of those events occurring within cancer cell populations, but it also has far-reaching implications for clinical applications to anticancer therapy. Thus, we propose that CD133 remains a valid marker for predicting subpopulation of cancer cells that will show enhanced survival to immune and other stressful challenges. This is supported, for example, by findings that $CD133^+$ cells isolated from tumors show much greater resistance to apoptosis than their $CD133^-$ counterparts (12, 16, 65). Approaches that focus on targeting CD133, such as therapy resulting in its down-regulation and the consequential enhanced susceptibility of these cells to apoptosis, both mediated *via* the death receptor and mitochondrial mechanisms (17), are warranted.

Gene therapy is, scientifically, a very elegant, although challenging approach. Silencing of the expression of the *CD133* gene in experimental animals, such as transgenic mice with spontaneously generated tumors, may prove superior in preventing selection of $CD133^+$ cancer cell resistance to apoptosis during the process of immunoediting. On the practical level, however, this can be difficult to achieve due to potential effects on other bystander cells that are not the desired target. Such approaches ought to be first tested in animals with experimental tumors, such as transgenic, immunocompetent mice with spontaneously arising tumors. In these models, stable transfection with a plasmid or a viral

Table 4. Examples of Compounds That Kill Cancer Stem-Like Cells

Name	*Structure*	*Type of tumor*	*Mechanism of action*	*Reference*
Parthenolide		Leukemia Breast cancer Prostate cancer	Inhibition of NFκB	46, 49, 58
Salinomycin		Breast cancer	Potassium ionophore	45
Metformin		Breast cancer	Effect on energy metabolism	52
Lapatinib		Breast cancer	Dual inhibitor of receptor tyrosine kinases	60
MitoVES		Breast cancer, mesothelioma	Generation of ROS by targeting complex II	J.N. *et al.*, unpublished

MitoVES, mitochondrially targeted vitamin E succinate; ROS, reactive oxygen species.

vector carrying *CD133* shRNA targeted to the tumor-prone tissue could be tested. This approach, if successful, will provide direct evidence for the pathophysiological role of CD133 and indicate a possibility of gene therapy to eliminate malignant $CD133^+$ cells.

Another way for dealing with $CD133^+$ cancer cells is to design pharmacological agents that would kill such cells with high efficacy and selectivity without affecting normal cells. To date, only several compounds have been published and shown to possess these features, a prime example being parthenolide (PTL), a drug isolated from the plant feverfew with NFκB inhibitory activity (51). PTL was found to be relatively efficient in killing leukemia stem cells (46, 49) and, also, breast CSCs represented by mammospheres (MS) derived from breast cancer cell lines (129). The latter include MS derived from the human breast cancer cell line MCF7, which were resistant to a range of established anticancer agents. We observed similar susceptibility of MCF7 cell-derived MS to PTL in our laboratory, and have also found that these MS exhibited high level of expression of both isotypes of CD133 relative to their adherent counterparts (J.N. *et al.*, unpublished). High-throughput screening using $CD44^{high}/CD24^{low}$ cells uncovered salinomycin as an efficient agent that exerted selective toxicity toward breast CSCs, although a relationship to the level of CD133 has not been documented (45).

We have recently designed novel anticancer drugs from the family of mitocans (81–83), epitomized by the mi-

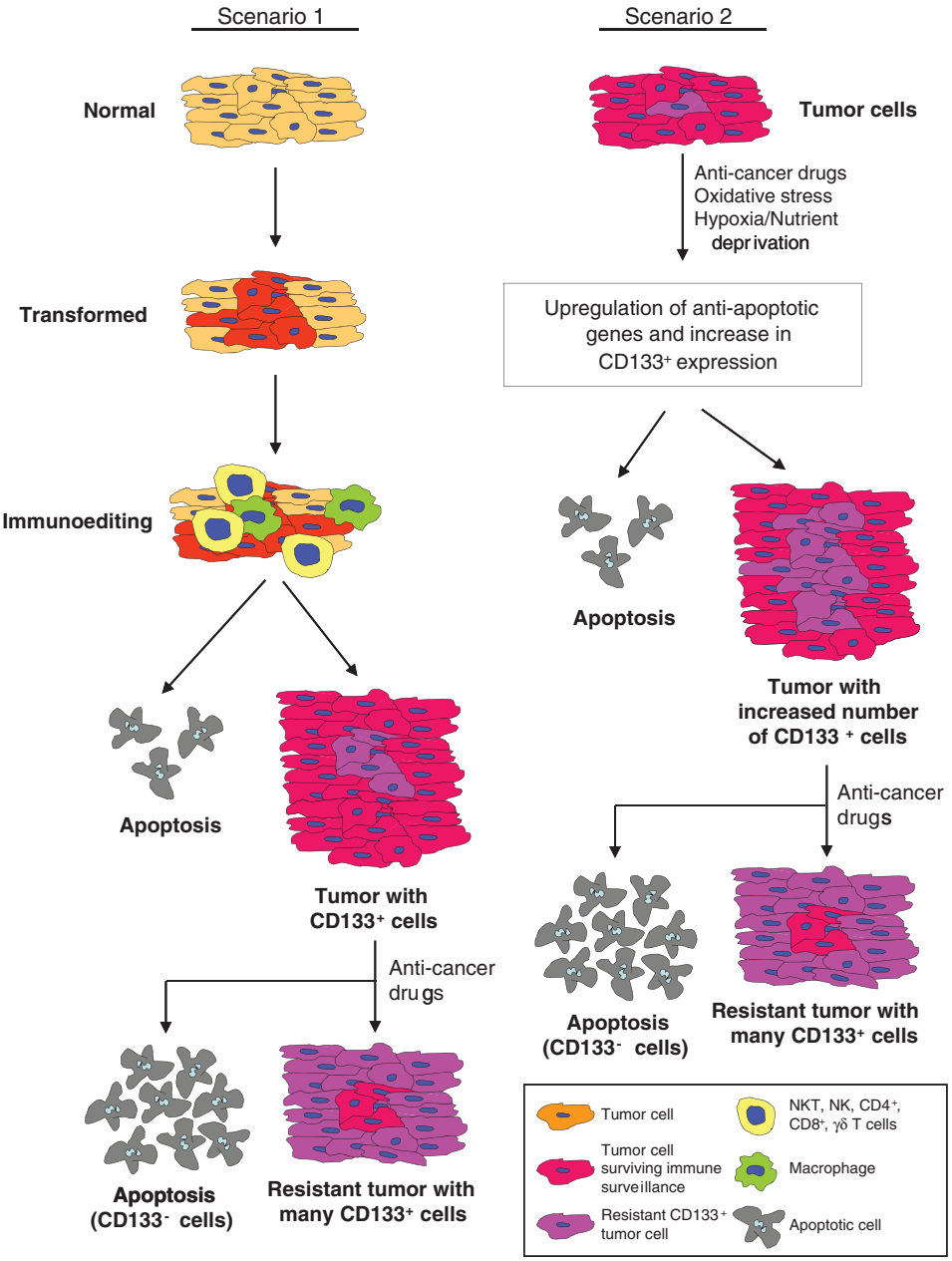


FIG. 5. CD133 selects cancer cells for resistance to apoptosis. We propose two scenarios describing the origin of the resistant, $CD133^+$ cancer (stem-like) cells in tumors. Scenario 1 involves the role of the immune system, which, *via* the process of immunoediting, selects cells that are resistant to immune surveillance or escape being intercepted and eliminated by the immune system. These cells form a tumor with low number of $CD133^+$ cancer cells. Upon challenge of the tumor with anticancer drugs, the $CD133^-$ cells are killed *via* apoptosis, unless they can respond rapidly by up-regulating CD133 and antiapoptotic genes (plus, most likely, down-regulating proapoptotic genes). The $CD133^+$ cells survive and the resulting tumor will be highly enriched in these apoptosis-resistant cells. Scenario 2 is very similar to Scenario 1. However, it is based on results obtained largely with cell lines. Accordingly, tumor cells, when under the pressure of oxidative stress, anticancer drugs or hypoxia (most likely in combination with nutrient deprivation), rapidly up-regulate expression of CD133 in a subpopulation of the cells, and this is associated with differential regulation of apoptosis-modulating genes. Therefore, in the forthcoming therapy, the intervention causes killing of non-resistant cells, whereas the resistant ($CD133^+$) cells survive and constitute the majority of cells within the resulting tumor, highly recalcitrant to anticancer agents (adapted from ref. 108). (To see this illustration in color the reader is referred to the web version of this article at www.liebertonline.com/ars).

tochondrially targeted vitamin E succinate. These agents specifically target mitochondria and are much more efficient than the corresponding mitochondrial-untargeted parental compounds, while preserving selectivity for cancer cells (24). Mitochondrially targeted compounds exert very high efficacy not only against fast proliferating cancer cells, but also against their cancer stem-like counterparts typified by high levels of CD133 expression and grown as spheres (Figs. 3 and 4) (J.N. *et al.*, unpublished). Therefore, mitocans show substantial promise to be developed into anticancer drugs that can efficiently kill the recalcitrant $CD133^+$ cells, and we are currently investigating the molecular mechanism of susceptibility of CSCs to certain mitocans, with a special focus on mitochondrially targeted vitamin E succinate. A representative list of compounds that have been suggested to kill CSCs, often with increased level of expression of CD133, is shown in Table 4, with some of these agents acting at the level of mitochondria.

Even though CD133 seems to be the most important marker of wide interest at the moment for many CSC types, there is a possibility that other CSC markers may play significant roles in carcinogenesis and in the evasion of the immune system. Nevertheless, we believe that the escape mechanisms of cells presenting CD133 or CD44 and other markers should be very similar if not the same as that of the cancer cells (CSCs) that we have discussed here as being capable of evading the immune surveillance. Targeting of these CSCs [found, *e.g.*, in breast cancer (4), prostate cancer (18), and pancreatic cancer (64)] should also be the same as for CSCs that express the CD133 marker. Since not much is known about the function of CD44, in particular its role in CSCs, CD44 might not be the best target. However, targeting CSCs with pharmacological agents should have a similar affect (31). For example, in a recent study, we found that mitocans (23, 81, 83) showed a considerable efficacy against $CD44^+$ CSCs represented by MS (J.N. *et al.*, unpublished). Therefore, this approach also appears promising for CSCs that are characterized by markers other than CD133 (78). Collectively, while CD133 appears the most wide-spread marker of CSCs and a potential target for therapeutic approach (or, rather, targeting mitochondria of CD133-positive cells), other markers should not be excluded.

Conclusions and Future Perspectives

We present here a hypothesis according to which the surface receptor CD133 selects cancer cells to survive immunosurveillance as well as stress-induced apoptotic challenges. This hypothesis is two-pronged (Fig. 5): (i) Precancerous cells are under immunosurveillance, and the cells that emerge from the process of immunoediting are resistant to killing by apoptotic inducers, many of which are established anticancer agents. This population of CSCs shows high level of CD133 expression. (ii) Within cancer cells (including cancer cell lines), there are subpopulations (or a subpopulation) that, upon being challenged with inducers of apoptosis (stress, anticancer agents, *etc*), will rapidly increase CD133 expression and, associated with this, up-regulation of antiapoptotic and down-regulation of proapoptotic genes.

Regardless of whether CD133 is functionally linked to resistance of cancer cells to apoptosis or is co-upregulated in cancer cells with a more pronounced stemness phenotype, typified by the propensity to escape killing by apoptosis, high levels of CD133 expression may be considered a marker of more aggressive and more resistant tumors (126). To overcome such resistance, new methodological approaches need to be developed. One approach that appears plausible, given the initial results, is mitochondrial targeting. A class of novel agents is being developed that act on mitochondria and that are modified so that they accumulate in these -organelles. These agents, tagged with cationic group (9), are being proved as more efficient in killing cancer cells than their untagged counterparts (9, 24). Whether such compounds are efficient in killing CSCs represented by $CD133^{high}$ populations, as indicated by our initial results (either alone or in combination with other agents), is yet to be fully documented, although our pilot data are encouraging.

One plausible approach to efficient tumor therapy can be documented by the following example. We have used microarray analysis to characterize the stemness of several types of cancer cells grown as spheres, including breast and prostate cancer as well as malignant mesotheliomas, all with increased level of CD133 in the sphere phenotype (J.N. *et al.*, unpublished). This resulted in the confirmation of increased stemness in all three types of cancer. We found that of the upregulated pathways the three types of CSCs shared the tryptophan metabolism (represented by increased expression of indoleamine-2,3-dioxygenase, IDO). This finding would imply that CSCs deplete their neighborhood of tryptophan. In this manner, they may protect themselves from being killed by cells of the immune system (66, 79). Thus, inhibitors of IDO are being tested as anticancer agents (39, 54). We dare speculate that a highly efficient way to kill CSCs with high levels of CD133 may be accomplished by a combinatorial treatment of such cells with agents like mitochondrially targeted compounds (mitocans) and inhibitors of IDO. In other words, a drug that has high propensity of killing cancer cells by targeting their mitochondria in combination with agents that may enhance the tumor immune surveillance may present the magic bullet of optimal anticancer therapeutic modality, hitting the Achilles' heel of cancer.

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Abbreviations Used

CSC = cancer stem-like cell
ES = embryonic stem
FDR = false discovery rate
FLIP = FLICE-inhibitory protein
HIF-1α = hypoxia-inducible factor-1α
IDO = indoleamine 2,3-dioxygenase
IFN = interferon
MHC = major histocompatibility
MitoVES = mitochondrially targeted vitamin E succinate
MS = mammospheres
NK = natural killer
PTL = parthenolide
ROS = reactive oxygen species
TOS = tocopheryl succinate
TRAIL = tumor necrosis factor-related apoptosis-inducing ligand

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