CANCER STEM CELLS – CURRENT KNOWLEDGE AND TARGETING WITH NATURAL COMPOUNDS

NOWOTWOROWE KOMÓRKI MACIERZYSTE – AKTUALNY STAN WIEDZY I ICH ZWALCZANIE ZWIĄZKAMI POCHODZENIA NATURALNEGO

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Summary: Cancer stem cells (CSCs) constitute a distinct tiny subset of self-renewing, undifferentiated cells within the tumor, that are characterized by a particularly high carcinogenic potential. These cells play a fundamental role at every stage of cancer development from initiation through growth, relapse, and metastasis. CSCs have been identified *in vivo* in hematological tumors, in solid tumors as well as *in vitro* in continuous cultures of human tumor cells. Their identification is based on unique biological and molecular features associated with CSC phenotype including expression of specific surface/cytoplasmic markers and genes, overexpression of ABC transporters, or functional assays and xenotransplantation that determine the ability of CSCs to generate tumors. This review focuses on current knowledge of the biology and regulation of CSCs, emphasizing their role in cancer resistance to conventional chemotherapy and radiotherapy. The potential of some natural compounds to inhibit CSCs function damages these cells and/or reduces their resistance to cytostatic therapy and the common methods currently used in CSCs research are discussed.

Key words: Cancer stem cells (CSCs), hypoxia, epithelial to-mesenchymal transition (EMT), therapy resistance, CSCs identification, natural compounds

Streszczenie: Nowotworowe komórki macierzyste (NKM; ang. *Cancer Stem Cells*, CSCs) stanowią odrębną, niewielką część samoodnawiających się, niezróżnicowanych komórek w obrębie nowotworu, które charakteryzują się szczególnie wysokim potencjałem rakotwórczym. Komórki te odgrywają fundamentalną rolę na każdym etapie rozwoju nowotworu od inicjacji poprzez wzrost, nawrót i przerzutowanie. NKM zostały zidentyfikowane *in vivo* w nowotworach hematologicznych, w guzach litych, jak również *in vitro* w hodowlach ciągłych ludzkich komórek nowotworowych. Ich identyfikacja opiera się na unikalnych cechach biologicznych i molekularnych związanych z fenotypem NKM, w tym na ekspresji specyficznych powierzchniowych/cytoplazmatycznych markerów i genów, nadekspresji transporterów ABC oraz na testach funkcjonalnych lub ksenotransplantacjach,

które determinują zdolność NKM do generowania nowotworów. W pracy przedstawiamy najnowsze osiągniecia z zakresu biologii i regulacji NKM, podkreślając ich rolę w oporności nowotworów na konwencjonalną chemioterapię i radioterapię. Omówiono także potencjał niektórych naturalnych związków do hamowania funkcji NKM, uszkadzania tych komórek i/lub zmniejszania ich oporności na leczenie cytostatyczne oraz powszechne metody stosowane obecnie w badaniach NKM.

Słowa kluczowe: Nowotworowe komórki macierzyste (NKM), hipoksja, przejście epitelialno-mezenchymalne (EMT), oporność na leczenie, identyfikacja NKM, związki naturalne

CANCER STEM CELLS

Cancer has been defined as neoplastic tissue composed of heterogeneous cells populations with different biological characteristics and functions [14, 56, 72]. This intra-tumor heterogeneity was found in many types of cancer, including brain, renal, breast, leukemia, colorectal, melanoma, lung, sarcoma, and head-neck [56]. Recently, it has been proposed that the plasticity of cancer stem cells (CSCs) is responsible for the observed heterogeneity in the cellular morphology of tumors [35]. CSCs represent a distinct, aggressive subset of tumor cells with the exclusive ability to initiate and drive tumor growth and to maintain the tumorigenic potential through self-renewal. In addition, these cells display outstanding chemo- and radio-resistance that lead to malignant progression, metastasis and cancer recurrence [6, 35]. It was postulated that CSCs constitute a small percentage of quiescent, no differentiated cells, usually less than 5%, although several reports have also suggested that as many as 25% of cancer cells present CSC characteristics in some tumors [1, 17]. However, the current dynamic CSC model assumes that CSCs are related to the cell state rather than the cell type, and there is a bi-directional conversion between CSCs and differentiated non-CSCs. That means that CSCs have the ability to give rise to the differentiated tumor cells and any differentiated cancer cells can be reprogrammed to become CSCs through de-differentiation and reacquiring of self-renewal capacity (fig. 1) [11, 31, 72]. These inter-conversions between the CSCs and non-CSCs might explain the variation in the frequency of CSCs in different stages of diseases and various type of cancer. [53] The ratio of CSCs to non-CSCs depends on the rate of differentiation in relation to the CSCs turnover rate, i.e. the smaller the tendency to differentiate, the more aggressive the cancer and the higher CSCs frequency in it [73]. In addition it was postulated that CSCs are not homogenous within the tumor but constitute a heterogeneous population of cells which differ in their cell cycle, metabolism, redox status. Thus, CSCs can exist as a quiescent (or slow-cycling) cells or as proliferative cells that can generate differentiated progenies or undergo trans-differentiation into cells of different lineages. It means that intra-tumor heterogeneity is maintained by all CSCs: quiescent, proliferative and more differentiated [27].

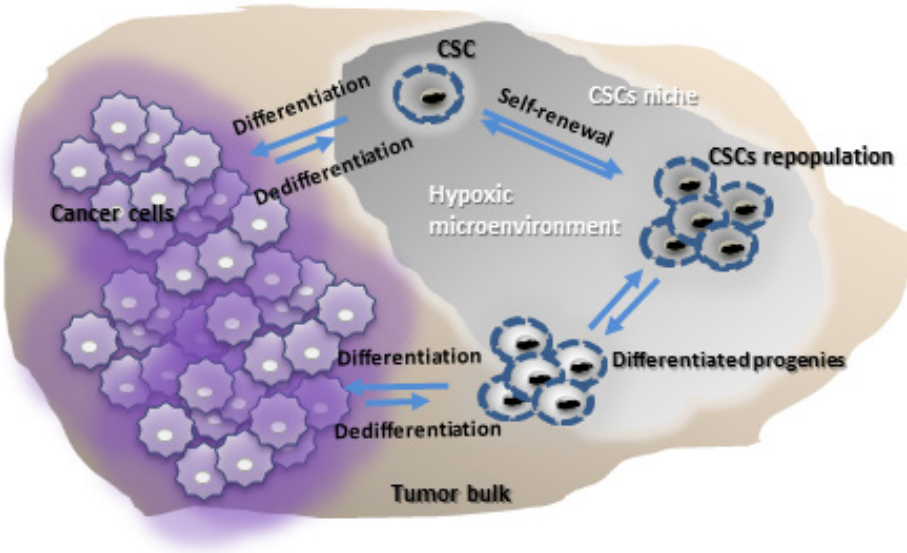

FIGURE 1. Schematic illustration of cancer stem cells model (CSCs): self-renewal, generation of differentiated progenies and tumor formation
RYCINA 1. Schematyczna ilustracja modelu nowotworowych komórek macierzystych (NKM): samoodnawianie, generowanie zróżnicowanych komórek progenitorowych i tworzenie nowotworów

CANCER STEM CELLS, HYPOXIA, AND EPITHELIAL-TO-MESENCHYMAL TRANSITION

The stem-like state of tumor cells is highly regulated by the microenvironment in which CSCs reside at the tumor site (cancer stem cell niche) (fig. 1). CSCs attempt for adapting to their microenvironment but also create their preferable condition in the niche [53]. In solid tumors, hypoxia and extracellular matrix composition are an essential component of CSCs microenvironment [31, 35]. Hypoxia conditions are beneficial for maintaining the CSCs functions and improving CSCs abilities of invasion and therapy resistance [53]. As was reported by Khan Z. et al., the microenvironmental changes, induced by both hypoxia and serum deprivation, contribute to the process of cellular dedifferentiation in U87-MG glioblastoma cell line, leading to the onset of CSC related feature [23]. Yeung T.M. et al. have demonstrated that hypoxic conditions inhibit differentiation and maintain stem-like phenotype in colorectal cancer cell line-derived CSCs [73]. Hypoxia signaling occurs through hypoxia-inducible factors (HIFs) that are considered to be critical regulators of the stem cell phenotype. Hypoxia-induced transient

HIF-1α activation with a subsequent switch to HIF-2α leads to an increase in the CSCs population. In addition, HIFs mediate CSCs adaptation to hypoxia through regulating the expression of genes involved in various biological processes such as cellular energy metabolism, survival, angiogenesis, EMT (epithelial-to-mesenchymal transition), invasion, and metastasis [5]. HIFs expression is also associated with upregulation of CSCs-specific markers like Oct4 (octamer-binding transcription factor 4), Nanog (homeobox domain transcription factor), Sox2 (sex determine region Y-box 2), CD133, CD44, VEGF-a (vascular endothelial growth factor a) [31, 36, 37, 55]. Interestingly, some of these markers have the capacity of modulating intracellular ROS level in CSCs. Similar to normal stem cells, the CSCs maintain lower intracellular ROS contents compared with non-CSCs which accounts for their self-renewal capacity and resistance to chemotherapy drugs and ionizing radiation. One of the mechanisms that contribute to the low ROS content is upregulation of free radicals scavenging systems such as glutathione (GSH) [10]. It was found that CD44 variant isoform (CD44v), a major molecular marker of CSCs, contributes to upregulation of GSH synthesis. The CD44v enhance intracellular GSH level in CSCs through direct interaction and stabilization of xCT (light chain subunit of cysteine-glutamate exchange transporter) thereby promoting the uptake cysteine that is essential for GSH synthesis [10, 20, 31, 44, 56].

Hypoxia and HIF-1α mediate remodeling of the CSCs niche architecture through the activation of extracellular matrix proteases (e.g. MT1-MMP, MMP-2) and induce epithelial-to-mesenchymal transition.[38] The EMT process is associated with alteration of epithelial-like characteristics of cells and acquisition of a mesenchymal phenotype, leading to (a) loss of cell polarity by downregulation of epithelial marker (E-cadherin), (b) secretion of proteases for extracellular matrix degradation, and (c) accelerated migration via upregulation of mesenchymal markers (N-cadherin and vimentin). Following hypoxia and EMT, cancer cells acquire the ability to escape tumor niche, migrate, and invade other tissues [24, 38]. Thus, the EMT plays a critical role in tumor metastasis and recurrence, which is tightly linked with the function of CSCs. It has been proposed that CSCs exhibit dynamic changes between epithelial and mesenchymal features [64]. In addition, recent data demonstrated the strong correlation between EMT and CSCs plasticity, i.e. it was shown that EMT process allows CSCs to self-renew and differentiate into all cell types represented in the tumor [24, 64, 65]. It was also suggested that partial EMT may be sufficient to initiate metastasis by CSCs. Moreover this hybrid (epithelial/mesenchymal phenotype) and the highly plastic multipotent CSCs can differentiate into both epithelial and mesenchymal lineages (bidirectional differentiation model) [46]. The association between EMT and CSCs has been observed in several cancers, including squamous cell carcinoma, breast, pancreatic, prostate and colorectal cancers [64]. In addition, EMT contributes to the generation of circulating CSCs subset (called EMT CSCs) that are disseminated in

the blood. The EMT CSCs exhibits both EMT and CSCs features including high invasiveness. It has been suggested that of all tumor cells only this subset is capable of an efficient metastasis leading to metastatic disease [1, 71].

DRUG RESISTANCE IN CSCS

CSCs display excellent ability to resist chemo- and radiotherapy. The cancer's and CSCs' resistance usually develops following prolonged exposition to these therapies and is associated with enrichment of CSCs. It has been postulated that such treatment induces reprogramming or dedifferentiation of normal tumor cells into tumor cells with enhanced CSC characteristics [33] or that progenitor cells, that were able to survive therapy, differentiate into lineages bearing assorted mutations exhibiting the resistance phenotype [43]. Resistance to a wide range of anticancer drugs is referred to as multidrug resistance (MDR). MDR phenotype of CSCs is mediated by several mechanisms, including increased drug efflux from the cells by ABC (ATP-binding cassette) transporters, reduced drug uptake and transport, enhanced drug metabolism or inactivation by enzymes, defective apoptotic pathways (fig. 2) [33, 34, 43].

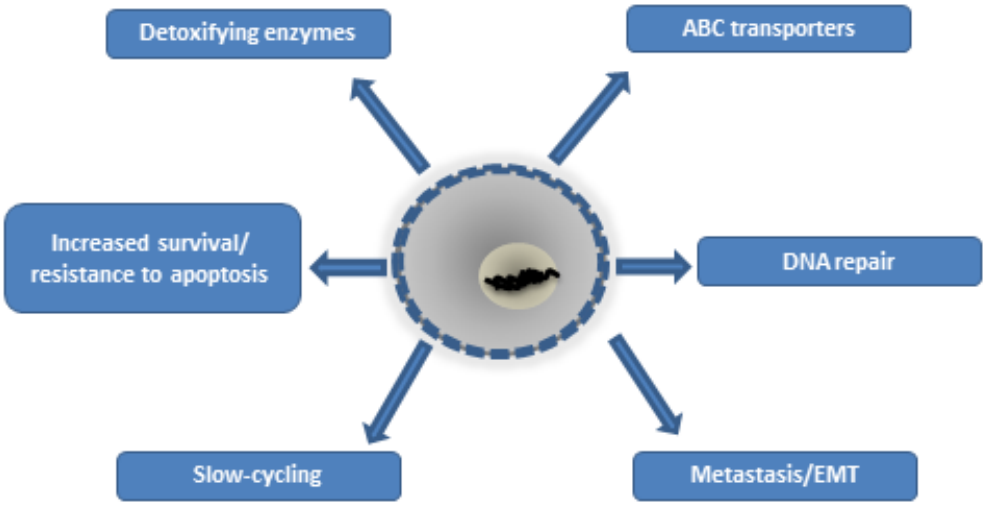

FIGURE 2. Schematic view of different mechanisms implicated in cancer stem cells (CSCs) drug resistance
RYCINA 2. Schematyczne przedstawienie różnych mechanizmów związanych z lekoopornością nowotworowych komórek macierzystych (NKM)

ABC transporters. ABC transporters are transmembrane proteins, which act as molecular pumps responsible for the transport of toxic compounds through cell membranes, thus protecting cells from their harmful effects. The energy needed for the translocation of drugs is provided from the hydrolysis of adenosine triphosphate (ATP) [40]. In CSCs, the ABC transporters, mainly P-glycoprotein (P-gp), multidrug resistance-associated protein 1 (MRP1) and breast cancer protein (BCRP/ABCGG2) are significantly upregulated. Their expression level is considerably higher compared to more differentiated cells making these cells the most resistant cells in the cancer bulk [12, 34]. Overexpression of ABC transporters plays a protective role, by contributing to the survival of CSCs (by efflux of chemotherapeutics), sustaining their proper performance and self-renewal features. In addition, their increased functional activity in cancer cells and CSC is associated with maintaining the aggressive characteristics of cancer cells [3]. Elevated expression of several ABC transporters has been found in the drug-resistant subpopulations of many cancers and cancer models, e.g. doxorubicin-resistant colon cancer cells (LOVO/DX) exhibit elevated mRNA and protein levels for P-gp, MRP1, MDR3, and LRP transporters compared to parental sensitive colon cancer cells (LOVO) [67].

The expression levels of ABC transporters can serve as biomarkers for identifying CSCs, e.g. in human melanoma cancer, CSCs are recognized by co-expression of BCRP and CD133 [33]. The functional activity of these transporters in CSCs can be detected and quantified by exclusion of fluorescent substrates, including hoechst 33342, rhodamine 123, calcein AM, PhenGreen SK diacetate [57]. The functional assays are frequently used to study the potential of natural or chemical compounds to reverse MDR via inhibiting ABC transporters. Molecules able to modulate MDR activity may sensitize the CSCs to conventional chemotherapy, and thus, when co-administrated with cytostatic agents, can increase the drug concentration in the CSCs and selectively kill them [60].

Detoxifying enzyme. The MDR phenotype of CSCs is also associated with the activity of detoxifying enzymes, such as aldehyde dehydrogenase (ALDH1A1). ALDH1A1 is responsible for detoxification of endogenous and exogenous aldehyde substrates through $NAD(P)^+$-dependent oxidation, including chemotherapeutic agents. ALDH1A1 is one of the main isotypes of ALDH1 enzyme overexpressed in several cancers including breast, lung, esophagus, colon, pancreatic, and gastric. Its high expression level is often associated with poor response to chemotherapy and cancer prognosis. In CSCs, ALDH1A1 is involved in self-renewal, differentiation, and self-protection. It has been used as a biomarker for identification and isolation of CSCs. Inhibition of ALDH1A1 enzymatic activity leads to chemosensitization of tumor cells to chemo- and radiotherapy [43, 60, 61].

The altered activity of other detoxifying enzymes such as cytochrome P450 (CYP3A4) or glutathione-S-transferase (GST) may result in a reduction in the

cytotoxic activity of chemotherapeutic agents regardless of their intracellular concentrations [9, 13]. This type of drug resistance mechanism may also be important in MDR phenotype of cancer cells and CSCs, however, it is not well characterized and require more extensive research.

Other mechanisms of drug resistance. Another mechanism associated with MDR phenotype of CSCs involve changes in the balance of proteins that control apoptosis [2, 34]. The BCL-2 (B-cell lymphoma-2) protein family has been found to be highly expressed in several CSCs, e.g. in breast CD44+ CSCs and CD133+ colon CSCs. BCL-2 protein mediates pro-survival effects via direct binding to proapoptotic proteins such as BAX (BCL-2-associated-X-protein) and BAK (BCL-2 antagonist killer) thus inhibiting proapoptotic pathway. In addition, BCL-2 family members may activate other signaling pathways required for CSCs survival. For example, they may induce Aurora-A, an oncogenic kinase that regulates the cell cycle. In colorectal cancers, CSCs co-expressed high levels of Aurora-A, BCL-2, MCL-1, and BCL-XL. Knockdown of Aurora-A led to downregulation of all these proteins [2].

The ability to evade apoptosis is tightly linked to a DNA damage response. The mechanisms that promote DNA damage repair are effective in protecting CSCs from DNA damaging radio- or chemotherapy [2]. CSCs are able to tolerate high levels of replication stress and resists to the harmful treatments [33]. Downregulation of DNA damage response factors result in chemosensitization of CSCs, e.g. inhibition of PARP (poly(ADP-ribose) polymerase-1 (PARP-1)) and ATR (ataxia telangiectasia and Rad3-related protein), enzymes activated in response to single strand DNA breaks, caused profound radiosensitization of glioblastoma CSCs [33]. Hypoxic conditions, that are present in the center of the tumor bulk and in CSCs niche, contribute to the induction of potent DNA damage response [2]. In addition, lower intracellular ROS level and high expression of oxidant scavengers support high resistance phenotype of CSCs and protect them from ROS induced damages caused by radiation or chemotherapeutics [33, 62].

Other mechanisms that may account for CSCs resistance include slow-proliferation rate of CSCs, upregulation of stem cell maintenance proteins (WNT/β-catenin, Notch, Hedgehog), constitutive activation of NF-κB pathway in CSCs, microRNA's (e.g. microRNA-22,27b,34), and the several mechanisms linked to the uncontrolled EMT process [2, 33, 54 ,62].

IDENTIFICATION AND ISOLATION OF CSCS

Cellular markers. The most common method of identifying CSC is the detection of specific cellular markers that define stem cell characteristics. The majority of those markers are cell surface proteins, but some of them are localized intracellular-

ly. The expression level and the set of specific, distinct markers are dependent on the type tumors, for example $CD34^+/CD38^-$ profile is characteristic for hematological malignancies whereas $CD44^+/CD24^-$ for breast cancer. An overview of the CSCs-related markers associated with different tumor types is presented in table 1 [1, 16, 62, 75].

TABLE 1. Marker patterns associated with CSCs in different cancer types
TABELA 1. Markery nowotworowych komórek macierzystych (NKM) w różnych typach nowotworów

	Tumor	Marker and marker patterns for CSCs
Hematological malignancies	AML	CD34+/CD38-; CD90+; CD123+, CD45RA+, CD33+; CD13+; CD44+, CD96+; CD47+;CD32+; CD25+; CLL1+; TIM3+
	B-ALL	CD34+/CD38-/CD19+; CD34+/CD10-
	T-ALL	CD34+/CD4-; CD34+/CD7-
	MM	CD34-/CD138-/CD27+

	Tumor	Marker and marker patterns for CSCs
Solid tumors	Bone sarcoma	Stro-1+/CD105+/CD44+
	Brain	CD133+; CD44+
	Breast	ESA+/CD44+/CD24-; $CD90^{low}$/CD44+; ALDH1A1+
	Colon	CD133+/CD44+; CD133+/ CD24+; ESA^{high}/CD44+; CD166+; EpCAM+; ALDH1A1+
	Colon (metastatic)	CD133+/$CD44^{low}$/CD24+; CD133-/CD44+/ CD24-
	Endometrial	CD133+, SP+
	Esophagus	CD44+; CD24+; CD133+; ABCG2+; CXCR4+; ALDH1A1+
	Gallbladder	CD133+/CD44+
	Gastric	CD44+; CD133+; CD24+; CD54+; CD90+; CD49f+; CD71+; EpCAM+; ALDH1A1+
	HNSCC	CD44+; CD133+; ALDH1A1+
	Liver	CD44+/CD133+; CD90+/CD45-; CD13+; EpCAM+
	Lung	CD44+; CD133+; CD166+; ALDH1A1+
	Melanoma	CD20+; CD133+; CD271+, ABCB5+
	Ovarian	CD133+/ALDH1A1+; CD44+/117+; CD24+; EpCAM+
	Pancreatic	CD44+/CD24+/ESA+; CD133+; ALDH1A1+
	Prostate	CD44+/CD24-/$\alpha_2\beta_1^{high}$; ALDH1A1+; SP+
	Renal	CD105+/CD133-/CD24-

The CD44, CD133 and ALDH1A1 are the most widely used CSCs markers. Both CD44 and CD133 are transmembrane glycoproteins that play important roles in malignant behaviors of several cancers, i.e. increased expression of these markers is associated with drug-resistant tumors and reduced sensitivity to chemotherapeutic drugs [48, 63]. It was shown that CD44 is involved in binding

to the extracellular matrix, cell migration, and differentiation, whereas CD133 is involved in cell growth and development [1, 75]. The ALDH1A1 is the main isoform of the aldehyde dehydrogenase that catalyzes the irreversible oxidation of cellular aldehyde in the cytoplasm, thus allowing the detoxification from drug and ROS. This indicates the implication of ALDH1A1 in CSCs chemoresistance. Other important examples of stemness-related proteins are in table 2 [75].

TABLE 2. Other stemness-related markers in CSCs
TABELA 2. Inne markery nowotworowych komórek macierzystych

Marker	Localisation	Function in stem cell	Expressed in Tumor types
OCT3/4	nuclear	self-renewal, pluripotency maintenance	bladder, brain, leukemia, lung, ovarian, pancreas, prostate, renal
SOX2	nuclear	self-renewal, pluripotency maintenance	brain, breast, lung, liver, prostate
KLF4	nuclear	self-renewal, pluripotency maintenance	brain, breast, leukemia, myeloma, prostate,
C-MYC	nuclear	self-renewal maintenance	brain, breast, colon, leukemia, lymphoma, pancreas, prostate, renal
Nanog	nuclear	self-renewal, pluripotency maintenance	brain, prostate
SALL4	nuclear	self-renewal and pluripotency maintenance, differentiation regulation	breast, colon, leukemia, liver, ovarian
Bmi-1	nuclear	self-renewal maintenance, invasion and metastasis, drug resistance	acute leukemia, breast ,colorectal, head and neck
Nestin	cytoplasmic	self-renewal maintenance, metastasis	breast, lung, osteosarcoma, ovarian, pancreatic
Musashi-1	cytoplasmic	CSCs survival, stemness	colorectal, lung, liver
TIM-3	cytoplasmic	immune checkpoint receptor	leukemia
CXCR4	surface	receptor for chemokine	brain, breast, pancreas
CXCR1, 2	surface	receptor for chemokine	breast, pancreas

Transcription factors. Several pluripotent transcription factors (TFs) are overexpressed in CSCs, such as OCT4, SOX2, NANOG [30, 75]. These TFs, promote stemness via upregulation of genes that are involved in pluripotency and self-renewal and downregulation of genes involved in differentiation [58]. It was found

that OCT4, SOX2, and NANOG are co-upregulated in many human cancers and hence they are called "core triad". The expression levels of these TFs mRNA transcripts are usually higher than surface CSCs markers [30]. The CSCs can be marked based on their activity. Several transcription factor-type reporter systems have been developed for enrichment and analysis of CSCs. Some of the reporter-based studies allow for determination of the CSCs state in real-time or track them *in vivo,* e.g. NANOG-GFP reporter, SOX2-EGFP reporter, OCT4-promoter, NOTCH-GFP repoter [26, 49, 58].

Side population. The CSCs can also be distinguished from non-CSCs based on their functional characteristics. An exclusive feature of CSCs is high ability to remove the chemotherapeutic drugs from their cytoplasm through ATP-binding cassette (ABC) transporters. It is well known that CSCs overexpressed these efflux pumps which confers them significantly high chemoresistance to anticancer treatment. Based on this property, a method of flow cytometric detection of the CSCs as a "side population" (SP) has been developed. SP cells actively efflux the Hoechst 33342 dye by ABC transporters forming a distinct cell population that show little or no fluorescence and fell to the "side" of the bulk of the positively stained cells in flow cytometric analysis plots (fig. 3). The SP cells possess higher tumor-initiating capacity, self-renewal, multipotentiality, and chemoresistance compared to non-SP cells from the same tumors. It is generally accepted that an SP is highly enriched in CSCs rather than represent the entire pool of these cells in a tumor. The SP cells have been identified in numerous cancer cell lines and tumors including breast, glioma, leukemia, colorectal cancer, endometrial carcinoma, neuroblastoma [1, 16, 18, 68].

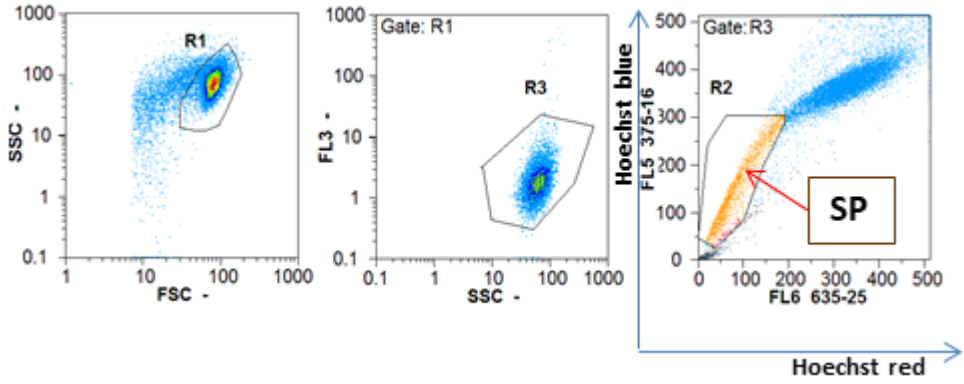

FIGURE 3. Example of Side Populatin (SP) analysis by flow cytometry and gating strategy used to analyze only singlet viable cells

RYCINA 3. Przykład analizy populacji bocznej (SP) metodą cytometrii przepływowej i strategii bramkowania stosowanej do analizy tylko pojedynczych żywych komórek

CSCs isolation and culture. There are two methods commonly used for CSCs isolation and purification from heterogenous population of tumor cells: flow cytometric sorting and antibody-conjugated magnetic beads separation (MACS). The MACS separation is a simple and fast methodology, based on specific surface markers, usually on only one. The flow cytometer-based sorting allows to isolate CSCs using multiple markers simultaneously i.e. surface markers, intracellular markers (like ALDH1A1), and/or SP phenotype. Compared to MACS, the purity of isolation is higher with flow cytometry sorting, however, the cells survival is higher with magnetic sorting [39, 49].

Regardless of the isolation methods, separated CSCs are usually further analyzed to confirm their properties and/or are subjected to other tests. The gold standard assay to validate the presence of CSC subpopulation involves serial transplantation of sorted cells in an immunocompromised mouse model (xenograft assays) based on the self-renewal (serially transplantable) and differentiation (generating heterogeneous lineages recapitulating an original tumor) characteristics [45]. In *in vitro* conditions, in a serum-free or soft agar medium, CSCs are characterized by the capacity to form tumor-spheres also called mammospheres or tumorosheres. Spheroid-formation assay is a three-dimensional (3-D) culture system that enables expansion of CSCs as a floating sphere which constitutes a surrogate to investigate the CSCs characteristics of solid tumors *in vitro* [19, 51].

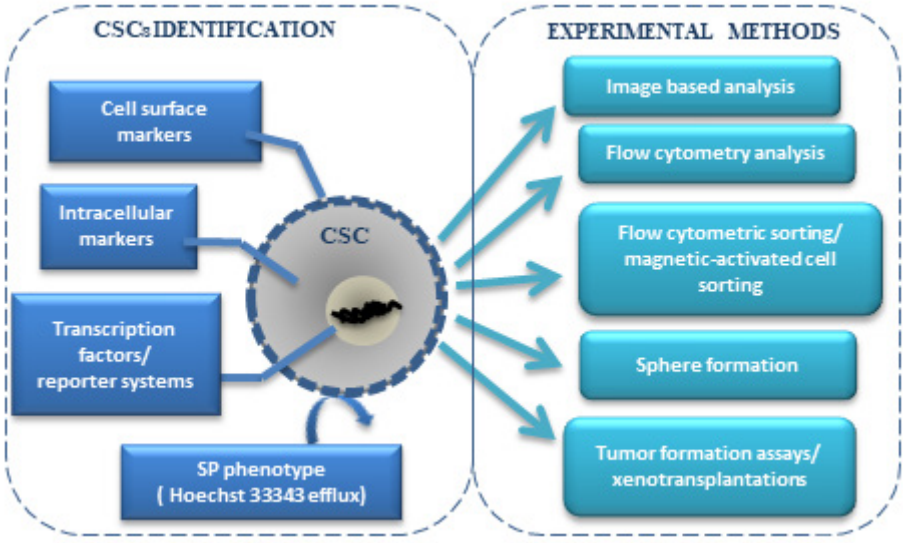

FIGURE 4. Diagram showing the markers used to identify cancer stem cells (CSCs) and experimental methods used to analyze and isolate CSC

RYCINA 4. Diagram przedstawiający markery używane do identyfikacji nowotworowych komórek macierzystych (NKM) i metody eksperymentalnych stosowanych do analizy i izolacji NKM

The current methodology used to detect and isolate CSCs population are summarized in figure 4. It is important to note that none of these methods used solely can guarantee pure CSC isolation, especially when based on cell surface markers, due to the plasticity of CSCs (interconversion between non-CSCs and CSC-like states). This underlines the importance of the simultaneous use of several complementary techniques.

NATURAL COMPOUNDS AND CANCER STEM CELLS

Over the last two decades, natural compounds have gained tremendous attention in the field of cancer research due to the limited efficacy of conventional chemotherapy and radiotherapy and frequent side effects [60]. This interest was also dictated by the observed effectiveness of various plants used in traditional medicine (e.g. traditional Chinese and Ayurvedic Medicine) in the treatment of many cancers. Up to now, the ability of various plant-derived compounds (referred to as phytochemicals) to inhibit tumor formation and growth has been confirmed *in vitro* and *in vivo* for various cancers [59]. Recently, it has been reported that some natural products and their derivatives in addition to killing normal cancer cells, forming the bulk of the tumor, have the ability to target CSCs. Naturally occurring phytochemicals are thought to possess privileged chemical structures that enable them to interact with various biological targets and thus disturb multiple cellular pathways simultaneously [60]. This property makes them excellent tools to combat CSCs that use many different survival mechanisms. For example, celastrol (tripterine), a natural pentacyclic triterpenoid isolated from the root extracts of *Tripterygium wilfordii Hook f* (a vine used in traditional Chinese Medicine, also called Thunder god vine) exhibits anticancer effects by affecting the different signaling pathways of tumor cells. It has been reported that celastrol affects the function of numerous important proteins in cancer cells through forming covalent adducts with nucleophilic thiol groups of cysteine residues [42]. The anticancer effects of celastrol were demonstrated for various cancers in *in vitro* studies. Recently, celastrol has also been shown to inhibit CSCs in ovarian cancer by suppressing the Pin1 (Peptidyl-prolyl cis-trans isomerase NIMA-interacting1) enzyme resulting in inhibition of cancer cells proliferation and migration, induction of apoptosis and cell cycle arrest [28]. In drug-resistant colon cancer, presumed to be enriched in CSCs, celastrol significantly decreases the size of SP cells, reduces drug resistance by inhibiting the functional activity of P-gp and induces apoptosis [42]. Triptolide, a second major active substance of *Tripterygium wilfordii*, demonstrates pro-apoptotic and tumor-suppressing effects in part initiated by topoisomerase and proteasome inhibition. In addition, it was shown to reverse hypoxia-induced epithelial-mesenchymal transition and stem-like features

in experimental models of pancreatic cancer stem cells. In CSCs isolated from patient tumors, triptolide was able to inhibit proliferation, downregulate CSCs markers and mesenchymal cells along with upregulation of markers for apoptosis and epithelial cells [32].

Targeting molecular pathways. The common molecular pathways in CSCs that are targeted by phytochemicals involve apoptotic pathway, the CSCs self-renewal, and signaling pathways and multidrug resistance mechanisms.

Targeting apoptosis. Many phytochemicals are able to trigger CSCs apoptosis e.g. resveratrol, curcumin, sulforaphane, celastrol, triptolide, gossypol, guggulsterone, green tea polyphenols (EGCG), 3,3'-diindolylmethane (DIM) in combination with herceptin, all-trans-retinoic acid (ATRA) [8, 59]. Tanveer S. *et al.* suggested that natural compounds exhibiting proapoptotic properties may kill both CSCs and non-CSCs using common mechanisms, thus could remain powerful chemotherapeutic agents [59]. However, this might not be a general property since it was demonstrated that sulforaphane (isothiocyanate extracted from broccoli) has been shown to primarily target CSC apoptosis instead of the bulk cancer cell population, through interfering with NF-κB anti-apoptotic signaling. This preference of sulforaphane for killing CSCs may be more significant for cancer chemoprevention [50]. Moreover, some natural products demonstrate selectivity against cancer cells without any toxic effects on normal cells, e.g. curcumin (a diarylheptanoid isolated from *Curcuma longa* Rhizomes root) and wogonin (a flavone derivative isolated from *Scutellaria baicalensis* Georgi root) [59]. It was suggested that this selectivity may be linked to phytochemical-induced repression of NF-κB activity which is known to be constitutively express in cancer cells and mediate cancer cells survival. NF-κB activity may also be suppressed by significantly increased levels of ROS, above the critical threshold, leading to cell death. Cancer cells and CSC exhibits poor ability to adapt to exogenous oxidative stress [59]. Importantly, many natural products demonstrate pro-oxidative activity under certain conditions such as at elevated concentrations. For example, sulforaphane showing the capacity to inhibit NF-κB transcriptional activity [69], also induces ROS-dependent apoptosis of 5637 cells [47]. The molecular mechanism of Celastrol and parthenolide (a sesquiterpene lactone derived from feverfew, *Tanacetum parthenium*) are also associated with apoptosis mediated by inhibition of NF-κB and increased ROS levels [4, 76].

Targeting stemness-related pathways. An increasing number of studies show that various phytochemicals are able to specifically affect the molecular pathways which regulate CSC maintenance, self-renewal, and differentiation. The major stemness-related pathways include the WNT/β-catenin, Hedgehog, Notch, and PI3K/AKT/mTOR signaling. In CSCs, these pathways are displayed aberrantly, leading to abnormal self-renew response and tumor progression [43]. Retinoic acid, the active form of vitamin A, has been shown to normalize the aberrant acti-

vation of the Wnt signaling through increasing proteasomal degradation of β-catenin. This modulation of impaired WNT/β-catenin signaling activity led to the induction of differentiation of promyelocytic leukemic cells to mature neutrophils, and to cancer regression. Thus, differentiation-inducing phytochemicals have the ability to switch undifferentiated CSCs to the harmless proliferative stage that is susceptible to chemotherapy [22, 59]. Other phytochemicals that were shown to inhibit Wnt signaling in CSCs include epigallocatechin gallate (EGCG), 6-gingerol, piperine, salinomycin, vitamin D_3, and quercetin [43]. Quercetin, a plant flavonol (found in many fruits, vegetables, leaves, and grains) as well as cyclopamine, steroidal alkaloids (extracted from *Veratrum californicum* Durant poisonous plant) have been shown to target the self-renewal properties of CSCs through inhibition of Hedgehog pathway [22 ,43]. Resveratrol, a stilbenoid (found in grapes, blueberries, raspberries, mulberries, and peanuts) decreases Notch protein expression and inhibits PI3K/AKT pathway which are both important in regulating apoptotic function. Inhibition of these signaling results in apoptosis in the CSCs population [22, 43]. Similarly, phytochemicals such as psoralidin and withaferin A were also shown to inhibit Notch signaling in CSCs [43]. Importantly, many phytochemicals are able to targets simultaneously several intracellular pathways. For example, the natural compounds like curcumin, delphinidin, genistein, lycopene, silibinin or ursolic acid can modulate the signaling of WNT/β-catenin, Hedgehog and Notch pathways [43].

Targeting multidrug resistance mechanisms. Various phytochemicals can be used as MDR modulators to reverse multidrug resistance in tumor cells and CSCs. They usually exhibit multiple activities against the MDR phenotype, by inhibiting the activity of ABC transporters and metabolic enzymes and/or inducing of apoptosis [13]. Overcoming MDR by inhibition of ABC transporter can sensitize CSCs to conventional chemotherapy. Up to now, the MDR modulatory activity was documented for several compounds belonging to flavonoids, stilbenoids, coumarins, carotenoids, terpenoids or curcumin derivatives [7]. Many naturally occurring compounds are shown to be a substrate for ABC transporters that may compete with cytotoxic drugs for binding to the active site of transporters leading to reduced drug efflux [7]. However, some phytochemicals are able to directly inhibit the functional activity of drug transporters. For example, celastrol was shown to exhibit significant chemosensitizing activities on drug-resistant colon cancer cells (LOVO/DX cell line) via direct binding to P-gp protein in cell membranes thus inhibiting its transport function. Blocking P-gp functional activity was associated with increased accumulation of rhodamine-123 (substrate of P-gp) and doxorubicin (standard cytostatic). In addition, celastrol caused a reduction in the frequency of the SP (side population; subpopulation of cancer cells enriched with cancer stem cells) and increased frequency of apoptosis [42]. Baicalein, a flavonoid from *Scutelariae Baicalensis* Georgi, has also been shown to inhibit P-gp

protein, which is manifested by the increase in intracellular rhodamine-123 concentration in adriamycin-resistant breast cancer cells (MCF-7/ADR) overexpressing P-gp [74]. The second main bioactive flavonoid from *Scutelariae*, wogonin is the ability to reduce the activity of MRP1 and Pgp in several cancers, as well as to increase the sensitivity of MCF-7/DX cells and K562/A02 to doxorubicin [74]. In addition, it was demonstrated that baicalein and wogonin significantly decrease the SP cells content in both MCF-7/WT (wild-type) and MCF-7/DX (doxorubicin-resistant) cells, in a dose-dependent manner [41]. Resveratrol mediates reversal of doxorubicin resistance in acute myeloid leukemia cells via downregulation of MRP1 expression [25]. In resistant human non-small cell lung cancer cells (NCI-H460), resveratrol significantly reduces mRNA expression levels of various ABC transporters genes (ABCB1/MDR1, MDR3, LRP, ABCC1, ABCC2, and ABCC3), decrease BCRP expression and inhibit of P-gp transport function in the rhodamine 123 efflux studies. Moreover, these effects were more pronounced in combination with paclitaxel, indicating the synergistic action of resveratrol with this cytotoxic drug [21]. In addition, In CSCs of nasopharyngeal carcinoma resveratrol diminished the expression of stemness-related genes (Oct4, Sox2, Klf4, c-Myc, Nanog, Lin28), reversed the EMT phenotype and increased the drug sensitivity via suppressing ABCG2 function [52]. Curcumin was demonstrated to restore drug sensitivity in cancer cells overexpressing the ABC transporters (P-gp, MRP1, ABCG2) by directly inhibiting their efflux function, thereby increasing the accumulation of the cytotoxic drug and inducing apoptosis [29]. Other examples of phytochemicals that have been implicated in the reversal of MDR phenotype by inhibiting ABC transporters include aposterol A, berberine, biochanin A, epigallocatechin, epicatechin, fumitremorgin C, genistein, icaritin, morin, piperine, phloretin, quercetin, and silymarin [60, 74].

Natural products can modulate multiple pathways that are related to MDR. Some phytochemicals may act synergistically with anticancer agents and reverse MDR in cancer cells without inhibition ABC transporters. For example, baicalein increases the cytotoxicity of cisplatin, an anti-cancer chemotherapeutic, by increasing gap junction intracellular communication in HeLa cells [66]. Genistein in combination with doxorubicin acts synergistically on MCF-7/ADR cells. Genistein reduces chemoresistance of MCF-7/ADR cells, leading to intracellular accumulation of doxorubicin and apoptosis. However, these effects were not caused by the inhibition of the P-gp function [70]. The supercritical fluid rosemary extract (SFRE) has a synergistic effect in combination with 5-FU (5-Fluorouracil) on colon cancer cells. SFRE sensitizes 5-FU-resistant colon cancer cells to the therapeutic activity of this drug, by downregulation of thymidylate synthetase (TYMS) and thymidine kinase (TK1) enzymes that are associated with 5-FU resistance [15]. It was also shown that ginkolic acids, dihydrometystycin, metysticin, hyperforin, and quercetin significantly inhibit the cytochrome P450

isoforms, enzymes that reduce the cytotoxic activity of drugs. Inhibition of drug metabolism enzymes can lead to increase in the cytotoxicity of cytostatic drugs and reverse MDR [7].

CONCLUSIONS

The CSC concept was initiated almost a century ago by British scientist John Beard, who is considered today the precursor of the current CSC theory. Since then, an enormous progress has been made in our knowledge of the role of CSCs in cancer development and their biology. It has now become evident that the main cause of cancer treatment failure and tumor recurrence is the unique ability of CSCs to evade anti-cancer therapy, repopulate the whole tumor and generate metastasis. Complete cure of tumors seems to be only feasible by targeting the "tumor root", thus by eliminating CSCs. Different approaches to selectively targeting the CSCs population are in the process of investigation, e.g. the use of nanoparticles loaded with chemotherapeutics as a direct drug delivery system or development of immunological strategies to specifically target CSCs. The use of photodynamic therapy for the selective killing of CSC is also a very promising approach. This strategy is based on the use of non-toxic dyes or photosensitizers that are effectively activated by light to produce cytotoxic effects. Based on the current CSCs biology knowledge, the most common anti-CSCs strategies involve targeting: 1. surface markers (like ALDH, CD44, CD133), 2. specific signaling pathways (WNT/β-catenin, Notch, Hedghog), 3. inhibition of ABC transporters and 4. tumor/CSCs niche microenvironment. Current chemotherapeutic agents mainly aimed at highly proliferating tumor cells and are not effective in eradicating CSCs, in part because they usually target a single molecular pathway. Naturally occurring compounds may bring better therapeutic effects due to their ability to simultaneously affect multiple signaling pathways involved in CSCs features. Several phytochemicals have already emerged as effective CSCs eliminating agents and became the leading compounds for the design of new analogs with improved antitumor effects. In addition to their ability to directly targeting the key CSC signaling, some of the phytochemicals can potentiate the anticancer activity of chemotherapeutic agents, and thus improve the effectiveness of conventional therapy. As natural products, the phytochemicals exhibit wide safety profile and are generally well tolerated, what makes them a good candidate for long-term cancer preventive cure. Due to the complexity of tumor and CSCs biology, it seems that the combination of all novel therapies targeting CSCs (including phytochemicals) with conventional radio- and chemotherapies, will still probably exert better therapeutic efficiency in the treatment of cancer.

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