Review

Future use of mitocans against tumour-initiating cells?

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Tumour heterogeneity has several important consequences including: (i) making their classification by morphological and genetic analysis more difficult because of the diversity within single tumours and the common majority of cells as the bulk of a tumour will dominate this classification whether or not these cells are critical for diagnosis or treatment, (ii) treatments may fail to eradicate tumours simply by failing to eliminate one of the cell subtypes within the tumour and (iii) differing abilities of the cell subtypes for dissemination and metastasis. Recently, a rare subpopulation of cells within tumours has been described with the ability to initiate and sustain tumour growth, to resist traditional therapies and to allow for secondary tumour dissemination. These cells are termed tumour-initiating cells (TICs). Understanding tumour heterogeneity will be critical for advancing treatments for cancer that target TIC subpopulations of cells in a tumour able to resist traditional treatments and eliminate them before metastatic disease occurs. It follows that the TICs will be the most important cellular components in the tumour target. Therefore, knowledge of the molecular mechanism(s) of resistance of TICs to treatment and overcoming this problem will be essential in order to develop effective drug strategies for cancer therapy.

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1 Introduction

The stem cell compartment maintains tissue homeostasis in an organism throughout life. Stem cells are undifferentiated cells that have several basic defining properties that they share in common with tumour-initiating cells (TICs) – the ability for self-renewal and the ability to give rise to multiple differentiated progeny (multipotency). The ability for self-renewal is a critical feature of stem cells and TICs. Through cycles of asymmetric cell division a stem cell gives rise to at least one daughter cell with the same characteristics as the parent and another daughter cell that becomes more differentiated [1]. Alternatively, stem cells may give rise through symmetric cell division to two daughter cells that retain the stem cell phenotype or two daughter cells that are more differentiated progenitor cells as transit-amplifying cells that can divide extensively, whilst differentiating into the mature cells of different lineages. Nonproliferative differentiated cells make up the bulk of the tissue and undergo apoptosis after a finite life span. The ability of TICs to perform self-renewal and give rise to multiple progeny can partially explain tumour heterogeneity. The relative quiescence of normal stem cells and TICs is another property they share in common and could explain the resistance of TICs to cell cycle specific therapies.

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Abbreviations: ABC, ATP-binding cassette; **AML,** acute myeloid leukaemia; **CSC,** cancer stem cell; **Hh**, hedgehog; **NOD,** nonobese diabetic; **PTL,** parthenolides; **SCID,** severe combined immunodeficiency; α**-TOS,** α-tocopheryl succinate; **TICs,** tumour-initiating cells

2 Properties of tumour-initiating cells

Stem cells and TICs also share other important characteristics. Both are long-lived with active anti-apoptotic path-

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ways and telomerase activity, allowing stem cells to survive whilst accumulating damaging mutations and genomic instability despite having active DNA repair mechanisms. Both cell types display a resistance to chemotherapeutic or radiation agents through a variety of mechanisms including a relatively quiescent phenotype and expression of the multi-drug resistance transporters. Stem cells and TICs also share the characteristics of anchorage independence and mobility, leading to migration and homing for stem cells and the potential for metastatic disease by TICs [2]. These characteristics of stem cells that are common to cancer cells suggests that fewer or different steps might be involved enabling stem cells to more readily transform into TICs by comparison to differentiated cells undergoing a similar path. It is thought that the unregulated self-renewal in the stem cell compartment can give rise to TICs and from there to tumour heterogeneity, although this does not rule out the possibility that malignant transformations may occur in transit-amplifying/progenitor cells or even differentiated cells. The theory for the clonal origin of tumours has been used to explain how tumours form, and postulates that cancer originates from a single mutation occurring in a few cells or a single cell that eventually leads to uncontrolled and unlimited proliferation of a population of clonally derived cells [3]. As the tumour progresses activation of proto-oncogenes into oncogenes and inactivation of various tumour suppressor genes occurs, ultimately giving rise to tumour heterogeneity. Building upon this model, there are two different theories for how tumour heterogeneity develops, the stochastic and hierarchical models. The stochastic model postulates that all or most cells in a tumour have the potential to be tumourigenic, capable of forming a new tumour through proliferation [4]. The hierarchical model postulates that only a rare proportion of the cells in a tumour have this tumourigenic capacity and that the rest of the cells represent terminally differentiated cells that lack the ability to proliferate and initiate tumours. The hierarchical model is in line with the cancer stem cell hypothesis, where the aberrant cancer stem cell (CSC) is the cell responsible for tumour initiation and maintenance and not the differentiated cells that make up the bulk of the tumour. The deregulation of signalling pathway mechanisms involved in self-renewal have been implicated in the formation of TICs [4].

An important characteristic of stem cells is the capacity for self-renewal and the regulation of the balance between self-renewal and differentiation. TICs share this property and the associated signalling pathways responsible include Notch, Wnt/β-catenin and hedgehog (Hh). The Notch family of transmembrane signalling proteins are expressed in stem cells and early progenitor cells and are involved in cell fate development [5]. Activation of Notch signalling by its ligand Jagged-1 was shown to be involved in the maintenance of self-renewal and plasticity for hematopoietic stem cells [6, 7]. In relation to TICs, Notch 4 was also shown to suppress the development of normal mammary glands and promote the development of mammary tumours *in vivo* [8]. This might indicate a role in the alteration of Notch 4 signalling in the transition from a stem cell to become a TIC in some types of solid tumours. The Wnt pathway is involved in stem cell self-renewal and cell fate determination in the hematopoietic system and several organs, and again, a pro-oncogenic role for β-catenin, a downstream target of Wnt signalling, was described [9]. Transgenic mouse models have shown that activation of the Wnt signalling pathway in stem cells can lead to epithelial cancers [10]. Specifically, it has been shown that over-expression of the Wnt signalling pathway in mouse mammary glands increases mammary tumour formation [11]. Taken together this indicates the involvement of Wnt signalling pathway members and β-catenin in the deregulation of self-renewal and tumourigenicity found in TICs.

The Hh/Patched pathway plays a role in embryonic growth and cell fate determination during development. Hh signalling has specifically been shown to play a role in self-renewal of hematopoietic stem cells [12]. The PITCH membrane protein, the product of the tumour suppressor gene Patched, is a receptor for the Hh family of signalling molecules, and has been implicated in a role in early embryonic tumourigenicity [13]. Alterations in the Hh pathway have been implicated in a variety of cancers including breast, prostate and lung cancer. Signalling pathways involved in TICs represent an important target for cancer therapy, as targeting the inappropriate signalling involved in self-renewal maintenance and tumour progression could represent a novel way to eliminate the cells responsible for maintaining the tumour.

It is important to further define TICs in terms of phenotype in order to identify TICs for therapeutic targeting. Stem cells and TICs are undifferentiated cells expressing low levels of lineage specific marker genes and hence are described as having a relatively lineage negative phenotype. One property shared by normal stem cells and TICs is the expression of the ATP-binding cassette (ABC)-G2 transporter. The ABCG2 is a class of drug transporters capable of pumping cytotoxic drugs out of the cell [14]. Expression of drug transporters could partially explain the protection of TICs from chemotherapy. These transporters have also been shown to specifically pump out the DNA-intercalating dye Hoechst 3334, the blocking of which leads to the identification of these cells by flow cytometry in the so-called side population location of the FACS scatter plot. Additional markers used to identify TICs are still being uncovered, and represent ways to isolate TICs for experiments and to target TICs for therapy of cancer. Some of these markers will be specific for cancers of specific organs. For instance, in humans breast cancer stem cells have been characterised as ESA^+, $CD44^+$, $CD24^-$/low, Lin^- [15]. Brain tumour stem cells have previously been shown to express CD133 and nestin, both of which are markers of undifferentiated neural stem cells [16]. Colon cancer stem

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cells have also been shown to express CD133 cells [17]. CD20 expression has been indicated in melanoma, CD133 has been further indicated for prostate and kidney carcinomas, and CD166 has been indicated for colon carcinomas [18]. For the case of acute myeloid leukaemia (AML), it has been shown that TICs in nonobese diabetic/severe combined immunodeficiency (NOD/SCID) mice exhibited a similar phenotype to normal hematopoietic stem cells of $CD34^+$, $CD38^-$ [19]. Progress in the phenotyping of TICs has been largely thanks to the development of the culture method referred to as the neurosphere assay, which uses serum-free media supplemented with EGF and basic fibroblast growth factor to grow neural stem cells as spheres [20]. This methodology has also found use in growing mammary stem cells and putative breast cancer stem cells [21, 22]. Further, defining markers for TICs will be an important goal for the development of rational therapies aimed at targeting these cells for elimination or for differentiation of the TICs away from the aberrant phenotype.

The gold standard for showing the existence of TICs relies on the ability to demonstrate self-renewal and generation of large numbers of differentiated progeny, which can be done firstly *in vitro*. In order to show the existence of a TIC *in vivo*, the gold standard is to demonstrate the sustained capacity for tumour initiation during serial transplantation of the putative TIC into NOD/SCID mice. The evidence for the existence of TICs responsible for the initiation and maintenance of cancer has recently been characterised for several tumours. During the 1990s, studies of AML were among the first to demonstrate that transformed human stem cell-like cells were capable of producing tumours [19, 23]. It was demonstrated that cells with the stem cell-like phenotype of $CD34^+$, $CD38^-$ could give rise to tumours in mice while those cells without a stem cell-like phenotype, $CD34^+$, $CD38^+$ could not [19]. Demonstrations of TICs were then confirmed in brain [24, 25] and breast cancers [15], demonstrating that TICs with a stem cell-like phenotype are involved in the initiation of some leukaemias and solid tumours. For the case of the breast cancer stem cell, it was shown that a tumourigenic subpopulation of cells could be serially passaged in mouse tumour transplants and that the newly generated tumour displayed the same phenotype as the TICs, in this case $CD44^+$, $CD24^{-/low}$, Lin^-, while also giving rise to differentiated nontumourigenic cells [15]. Furthermore, the $CD44^+$, $CD24^{-/low}$, Lin^- population could form a tumour in a NOD/SCID mouse when as few as 100 cells were injected, whereas no tumour was formed when 20 000 cells without this phenotype were injected, lending considerable evidence to the notion that tumour initiation could be driven by rare TICs. However, determining CSCs solely on cell surface phenotype might also be dangerous as no reliable cell surface phenotype can exclusively identify TICs and it has been pointed out that it will be critical to know more about TICs before therapies aimed at targeting them are brought to clinical trials [18]. For instance, a recent paper has shown that CD133 is not expressed in all colon cancers indicating that other cells besides the $CD133^+$ fraction could be putative TICs [26].

Mouse models of human tumour initiation are not without their caveats as best demonstrated by the notion that the microenvironment in an otherwise healthy immunocompromised mouse does not recapitulate the microenvironment in a human host with naturally occurring cancer. It has been suggested that the finding of a rare human AML cell capable of forming a tumour in NOD/SCID mouse might be the result of that cell being able to best adapt to growth in mouse tissue and did not necessarily reflect the exclusive ability of that cell to initiate a tumour. Recently, comparing tumour growth between rare subpopulations of murine cells with a stem cell-like phenotype and the tumour population as a whole was conducted in mouse models of lymphoid and myeloid malignancies, and revealed that tumour growth was not necessarily driven exclusively by the rarer TICs [27]. This latter report supports the need for more rigorous testing of the stem cell hypothesis, and the development of mouse models that more closely recapitulate the human microenvironment by incorporating human support cells into mice.

3 Markers of tumour-initiating cells

Cancer cells with stem-like properties as CSCs or TICs (also known as tumour maintaining cells) are being found not only in leukaemia where they were originally described, but also in tumours of epithelial or neural origin [28 – 30]. These cells often express specific cell surface markers such as prominin-1/CD133, CD44, ABCG2 transporter as well as other markers (Table 1) and often feature activated Hh, Notch or Wnt signalling pathways. Another promising CSC marker appears to be the Lgr5/Gprd49 G-protein-coupled receptor that is specifically expressed on intestinal and normal colon and cancer stem cells [31]. Like normal somatic stem cells, the cancer stem cells exhibit higher resistance to cell death triggering stimuli, are 'immortal' due to active telomerase and reside in a TIC niche formed by TIC-recruited blood vessels and support cells. Essential requirement for the formation of the TIC niche in tumourigenesis has been recently documented in tumours of neural origin and in AML [32, 33]. Coculture or co-implantation of $CD133^+$ meduloblastoma cells with primary human endothelial cells (PHEC) led to their vigorous growth and the formation of fast-growing, aggressive tumours [32]. This growth enhancement was aggravated by blocking the VEGF signalling. By contrast, forced EGF expression in glioblastoma TICs enhanced their tumourigenic potential and led to the massive expansion of vascular-rich glioblastoma, tumour-associated hemorrhage and high morbidity [34]. The stem cell supporting environment was also crucial

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Table 1. Cell surface proteins expressed in human cancer cells with stem cell-like properties

Cancer	Cell surface receptor	References
Breast carcinoma	$CD44^+$, $CD24^-$/low, Lin^-; $CD55^+$	[15, 59, 60]
Prostate carcinoma	$CD44^+$/a2b1hi/$CD133^+$	[61 – 63]
Brain tumours	$CD133^+$/$nestin^+$	[25, 64]
Colorectal carcinoma	$CD133^+$; $CD44^+$/EpCAMhi	[26, 65]
Pancreatic carcinoma	$CD44^+CD24^+ESA^+$	[66]
Hepatocellular carcinoma	$CD133^+$	[67, 68]
Head and neck tumours	$CD44^+$	[69]
Melanoma	$CD20^+$	[70]
Myeloid leukaemia	$CD34^+$/$CD38^-$	[19, 33]
Retinoblastoma	$ABCG2^+$/SCA-1^+	[71]
Ovarian carcinoma	$CD133^+$	[72]

for the growth of leukaemia cells from AML patients transplanted into NOD/SCID mice [33]. AML TICs were selectively eradicated *in vivo* by blocking of their homing to the supportive microenvironment (TIC niche) with the anti-CD44 mAb. Thus, by altering their stem cell fate in terms of homing, it was possible to eliminate the TICs.

4 Resistance of TICs to apoptosis

Intrinsic resistance of TICs to apoptosis and their sheltering in the vascular niche poses a serious obstacle for their efficient elimination. All three major signalling pathways (Wnt, Notch and Hh) that are required for phenotypic preservation of somatic stem cells also participate in maintaining the CSC phenotype in different tumours. Wnt-induced signalling activates expression of the IAP family protein, survivin or *via* PI3 kinase activation it indirectly enhances pro-apoptotic Bad phosphorylation and suppression of mitochondrial apoptotic signalling [35, 36]. Notch1-activated signalling is essential for survival of glioma or medulloblastoma $CD133^+$ cells [37, 38]. Blocking Notch signalling using siRNA or curcumin lead to apoptosis of pancreatic cancer cells accompanied by downregulation of Bcl-2 and Bcl-x_L expression [39]. Hh signalling is required for ovarian or pancreatic cancer tumourigenesis [40, 41] and promotes survival of medulloblastoma cells *via* upregulation of Bcl-2 [42]. Gene expression profiling of $CD133^+$ and $CD133^-$ glioblastoma cells revealed a number of differently expressed genes. In general, $CD133^+$ cells expressed more pro-survival proteins, including FLIP or Bcl-x_L, while their $CD133^-$ counterparts revealed the opposite [43]. These data suggest that resistance of $CD133^+$ cells is a general feature of CSC-like cells and helps to indicate which genes should be targeted to overcome such resistance in designing efficient strategies for eradication of TICs. We have recently found that CD133 high cells, including T-lymphoma Jurkat cells and breast cancer MCF7 cells also feature high level of FLIP expression and that downregulation of FLIP by siRNA sensitised the cells to the immunological inducer of apoptosis TRAIL [44].

5 Targeting of tumour-initiating cells with mitocans

Mitocans are a recently defined group of anticancer compounds which cause apoptosis in cancer cells by destabilising mitochondria. They have been classified into eight groups according to their precise targets in and around mitochondria. Group 8 mitocans include drugs without a defined mitochondrial target (mode of action) [45, 46]. Of these, the sesquiterpene lactones, epitomised by parthenolides (PTLs), appear to target TICs [47]. These agents have been isolated from the plant *Tanacetum parthenium* (feverfew), which used to be applied against migraine and arthritis. PTLs are compounds that induce apoptosis by targeting mitochondria, as shown in studies documenting oxidative stress-mediated apoptosis in cancer cells [48] *via* loss of $\Delta\Psi_{m,i}$ and induction of ROS accumulation [49 – 51]. Moreover, the apoptotic effect of PTLs appears selective for cancer cells [51]. Although the molecular mechanism of the TIC-targeting activity of PTLs has not been clearly defined yet, it is likely that these agents target mitochondria, since they give rise to ROS as shown for hepatocarcinoma [49], multiple myeloma [52] and pre-B leukaemia cells [53]. Evidence also points to PTLs as inhibitors of activation of the potent transcription factor NF-κB [54 – 56].

Recent data clearly point to PTLs as compounds that efficiently kill TICs, as documented in at least two different types of the neoplastic disease. Guzman *et al*. [47] showed that PTL-induced apoptosis in human AML stem cells, while not affecting normal hematopoietic cells. Further, the compound suppressed AML in xenografts in NOD/SCID mice. Recently, PTL was reported to target breast cancer stem cells using the mammosphere model of TICs derived from the human breast cancer cell line MCF-7 [56]. The data suggest that PTL preferentially kills mammary TICs.

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Since a similar effect was also observed for the antioxidant pyrrolidinedithiocarbamate, it can be speculated that the NF-κB inhibitory activity is likely to be one key mechanism by which PTL could suppress breast cancer stem-like cells, and the mitochondrial effect of PTL is likely to significantly contribute to the overall efficacy of the compound. The promise of the natural compound PTL as an agent efficient against cancer stem cells is further supported by results from a study which showed that combination of the anticancer drug vinorelbine capable of killing nonstem-like breast cancer cells and PTL may be a promising strategy to eradicate both cancer and cancer stem cells. This is based, for example, on the observation that vinorelbine is not capable of killing the stem-like side population of the breast cancer cells, which is a target of PTL, and a follow up experiment in which mice with breast cancer xenografts were treated with the combination of stealthy liposome-encapsulated vinorelbine and PTL, which resulted in complete disappearance of the tumours [57].

We have recently found that the mitocan α-tocopheryl succinate (α-TOS) [58] suppresses mammosphere formation using the human breast cancer cell line MCF7, suggesting an inhibitory effect on breast TICs (Neuzil *et al*., unpublished data). However, following preparation of mammospheres, α-TOS was not capable of killing the cells, although it efficiently killed the adherent MCF7 cells. Intriguingly, a newly synthetised analogue of α-TOS that accumulates in mitochondria (Neuzil *et al*., unpublished data), killed the mammosphere cells more efficiently than the adherent MCF7 cells. At present, we do not know the molecular basis for this surprising finding, although we may speculate about the role of mitochondrial targeting in more differentiated, fast proliferating cancer cells and the corresponding TICs. These data indicate that mitocans may hold promise as drugs against TICs, although we are only at the very beginning here and much more has to be done in this area. Notwithstanding, we believe that mitocans, epitomised by the mitochondrially targeted analogue of α-TOS, hold a great promise as agents targeting TICs.

6 Conclusion

Characterisation of TICs and determination of factors involved in forming TICs will have an important impact on the therapy of cancer. Current treatments of cancer have shown efficacy in removing the bulk of differentiated cancer cells, while failing to eliminate the TICs responsible for tumour relapse. Additionally, it will be important to target those cells that are capable of metastasising to form new tumours before the dissemination occurs. Common mechanisms involved in stem cell self-renewal and cancer initiation are starting to be uncovered. The challenge now will be to find ways to eliminate those rare TICs by targeting the common growth signalling pathways, while sparing normal stem cells. Understanding these differences, perhaps through gene array profiling, will go a long way towards developing a rational approach to eliminate the rare cells responsible for tumour initiation, maintenance and relapse.

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