Chapter 9

On the Far Side of Telomeres: The Many Roles of Telomerase in the Acquisition and Retention of Cancer Stemness

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Additional information is available at the end of the chapter

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Abstract

It is well recognised that upregulation/reactivation of telomerase, the telomere-lengthening enzyme, is the *sine qua non* of cellular immortalisation and malignant transformation. But there is also convincing evidence that telomerase stands at a crossroads where several developmental signalling pathways meet and that its upregulation/reactivation has effects beyond maintaining telomere length, such as altering energy metabolism and modulating gene expression. We believe that it is important to realise that, in a pathological context, such extratelomeric effects of telomerase are related to the emergence and persistence of the cancer stem cell (CSC) phenotype. Given the common conception of cancer stemness as a major contributor to therapy resistance and tumour relapse, a more complete annotation of biological mechanisms for its regulation by telomerase will provide the opportunity to develop telomerase-targeted anticancer therapies which kill or differentiate CSCs effectively.

Keywords: telomeres, telomerase, TERT, cancer stemness, CSCs, targeted anticancer therapy

1. Introduction

Telomeres are specialised structures that define the very ends of linear eukaryotic chromosomes and provide for their stability by protecting against degradation or end-to-end fusion. In mammals, telomeres are localised throughout the nucleus and associated with the nuclear matrix. Telomeric DNA of human cells is composed of a long (5 to 15 kb) stretch of the repeating hexanucleotide sequence 5'–TTAGGG–3' on one strand (the G-rich strand) and the complementary 5'–CCCTAA–3' on the other (the C-rich strand). The G-rich strand has a short (35 to

600 nt) single-stranded overhang at its 3' end (the G-overhang) which folds back and base pairs with the C-rich strand, forming a T-loop [1]. In humans, hexameric telomere repeats act as binding sites for various telomere-binding proteins collectively termed the shelterin complex, a dynamic ensemble of interactions that allows the cell to distinguish between natural chromosome ends and DNA double-strand breaks, preventing the cell's DNA damage response (DDR) from improper activation [2]. Telomeres undergo progressive shortening with each cell division as a result of incomplete lagging strand synthesis, less widely known end-processing events, and oxidative damage [3, 4]. This so-called telomere erosion operates as a kind of mitotic clock that determines ageing of the whole organism and suppresses malignant transformation of its constituent cells. The biological function of telomeres is heavily regulated and relies on both a minimal length of telomeric DNA and the proper functioning of the associated shelterin complex. A unique enzyme termed telomerase assists in replicating linear chromosomes through *de novo* synthesis of telomeric repeats, thereby counteracting the progressive telomere erosion that would otherwise occur in its partial or complete absence. In addition to its role in telomere length homeostasis, telomerase also performs telomere length-independent functions such as modulation of gene expression. In a pathological context, telomerase's new talents are intimately related to tumour development and progression to metastatic disease. This chapter summarises the newly discovered extracurricular activities of telomerase and describe how these are involved in regulating cancer stemness, the stem-like component of human tumours.

2. Telomerase and the cancer connection

Telomerase is a conserved RNA-dependent DNA polymerase canonically responsible for the maintenance of telomere length above a critical threshold. Human telomerase is primarily localised in the nucleus, as deducible from its role in telomere biology, but it can also be found in other cellular compartments such as the cytosol and mitochondria [5]. Telomerase is ribonucleoprotein in nature and consists minimally and essentially of a protein catalytic subunit (telomerase reverse transcriptase, TERT) and a large RNA subunit (telomerase RNA, TER). Active human telomerase has a bilobal architecture where one TERT subunit and one TER subunit participate in the formation of each lobe and a hinge region connects the two lobes [6]. This conformationally flexible, dimeric structure of the human enzyme undoubtedly has profound functional implications with respect to the catalytic cycle. Firstly, during the synthesis of telomeric DNA by telomerase, the 3′ end of the G-overhang is positioned in the active site of TERT and aligned by base pairing with the 3' end of the RNA template in TER. Secondly, TERT catalyses the addition of deoxyribonucleotides to the chromosome substrate through reverse transcribing TER into hexameric telomere repeats until the 5' end of the RNA template is reached. Lastly, telomerase translocates and realigns with the newly synthesised 3' end of the chromosome substrate to restart the catalytic cycle [7, 8]. In spite of the fact that TERT and TER are the two subunits that provide the catalytic core of telomerase, there are several other molecules that associate with telomerase and are involved in its biogenesis, trafficking, recruitment, and activation. Some of the most well-known telomerase-associated

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proteins include the nucleolar protein dyskerin [9], the three other nucleolar proteins NOP10, NHP2 and GAR1 [10], the two AAA+ ATPases pontin and reptin [11], and the WD40-repeat protein TCAB1 [12]. It should be noted that not all cells necessarily rely upon telomerase to maintain telomere length. Some telomerase-negative immortalised cell lines and tumours are able to elongate their telomeres by the much rarer alternative lengthening of telomeres (ALT) pathway. In contrast to telomerase, which utilises an RNA template to *de novo* synthesise telomeric repeats, the ALT pathway utilises a DNA template for DNA copying in an inter- or intramolecular recombination event [13].

Cancer is usually an age-related genetic disease, manifesting only when normal cells develop genomic instability over a reasonable period of time and acquire unlimited replicative potential that leads to the generation of macroscopic tumours. Telomerase upregulation/reactivation is observed in at least 85% of advanced human tumours, strongly suggesting a crucial role during human tumour pathogenesis [14, 15]. The most widely accepted multistep model of general tumourigenesis for explaining the part played by telomerase in telomere maintenance and cellular immortalisation is provided in section 3.2. Besides being found in primary tumours, telomerase activity is also detected in circulating tumour cells in, for instance, breast [16], ovarian [17] and prostate [18] cancers. Telomerase is upregulated/reactivated in premalignant cells by five common mechanisms: (i) increased transcriptional activation of *TERT* and/or *TER*; (ii) loss of transcriptional repressors of *TERT*; (iii) mutations in the *TERT* gene promoter/enhancer region (which result in the transactivation of this gene); (iv) several kinases (which phosphorylate and thus enhance the activity of TERT); and (v) gain of copy number of *TERT* and/or *TER* [13]. Somatic mutations in the *TERT* gene promoter region are frequent events in cancers of the bladder, central nervous system, skin (melanoma) and thyroid (follicular cell-derived) [19]. Two mutually exclusive and highly recurrent *TERT* promoter mutations are C250T and C228T [20, 21]. Although both mutations create a similar binding motif for E-twenty-six (ETS) transcription factors, they are functionally distinct in such a way that the the C250T *TERT* promoter but not the C228T *TERT* promoter additionally requires non-canonical NF-κB signalling in order to be transcriptionally driven [22]. Collectively, these findings highlight the contribution of *TERT* promoter mutations and non-canonical NF-κB signalling to tumourigenesis and decipher a fundamental mechanism for the reactivation of TERT in various tumours.

3. Cancer stemness

Cancer cells within a single tumour often exist in distinct phenotypic states which differ in functional attributes. This so-called intratumoural heterogeneity originates from a myriad of cell types recruited to the tumour as well as from genetic, epigenetic and metabolic differences amongst the cancer cells themselves and may result in variable or unpredictable responses to treatment [23]. Postulated to be the driving force behind tumour maintenance, hypermalignant stem-like cells called cancer stem cells (CSCs) represent a unique dimension of intratumoural heterogeneity. This often-small subpopulation of cancer cells is thought to play pivotal roles

in tumour initiation and progression, spreading, therapy resistance, and recurrence, all of which lead to poor prognosis.

3.1. Definitions and measurements

CSCs have critical implications for nearly, if not quite, all types of cancers, including leukaemias [24–26], lymphomas [27], melanomas [28, 29], sarcomas [30], and various carcinomas such as brain [31], skin [32], head and neck [33], lung [34, 35], liver [36], gastric [37], colorectal [38, 39], bladder [40], pancreatic [41, 42], prostate [43], breast [44] and ovarian [45] cancers. The functions assumed by CSCs in human cancers are diverse, ranging from sustaining tumour growth and dissemination to treatment failure and tumour relapse. The three clear-cut features that contribute to the aforementioned functions of CSCs, aka cancer stemness traits, are: (i) their unrestricted ability to self-renew; (ii) their aberrant ability to differentiate into mixed populations of tumour cells; and (iii) their high ability to transition from a proliferative to a quiescent state. In spite of the fact that these operational characteristics are, to a large extent, shared by both CSCs and physiological stem cells, CSCs are the ones that are known to be related to several malignant phenotypes, including induction of invasion and metastasis and resistance to apoptosis. In addition, CSCs are distinguished from bulk tumour cells by their capacity to form nonadherent spheres when cultured in stem cell media, their propensity to found fresh tumours when transplanted into severe combined immunodeficient mice and their expression of a selected repertoire of stem cell-surface markers [46]. Therefore, it is important here to realise that cancer stemness, no matter how it is measured, stresses the ways in which CSCs differ from bulk cancer cells as well as from physiological stem cells. The cancer stemness phenomenon is of considerable clinical importance and significance because it prognosticates that successful anticancer therapy must involve strategies that will eradicate CSCs, as these cells are able to dominate any residual tumour cells that survive conventional anticancer therapies.

3.2. Determinants and signatures

In the past, the cancer stemness model was widely seen as a static one which suggested a stable CSC population and a hierarchical organisation of cell division and differentiation. In recent times, however, the cancer stemness model has evolved into a dynamic one where CSCs are rather accepted as a functional subpopulation of cancer cells and can also be formed by the process of dedifferentiation from mature cancer cells under proper environmental conditions [47]. Accumulating data have revealed that cancer stemness is governed by genetic changes (such as oncogene activation and oncosuppressor gene inactivation), epigenetic changes (such as miRNA targeting and promoter DNA hypomethylation/hypermethylation) and metabolic changes (such as the shift to aerobic glycolysis) concomitant with changes in the tumour microenvironment, especially the CSC niche (Figure 1). These changes are a prerequisite for the oncogenic transformation and cellular (nuclear and metabolic) reprogramming of non-CSCs to CSCs and precipitate a spectrum of drastic cellular consequences, including overproduction of certain oncoproteins, disruption of certain oncosuppressor proteins, upregulation/reactivation of telomerase, reactivation of the epithelial-to-mesenchymal transition (EMT)

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programme, modulation of energy metabolism, stimulation of a number of embryonic/oncogenic signalling pathways, and differential expression of several microRNAs (miRNAs).

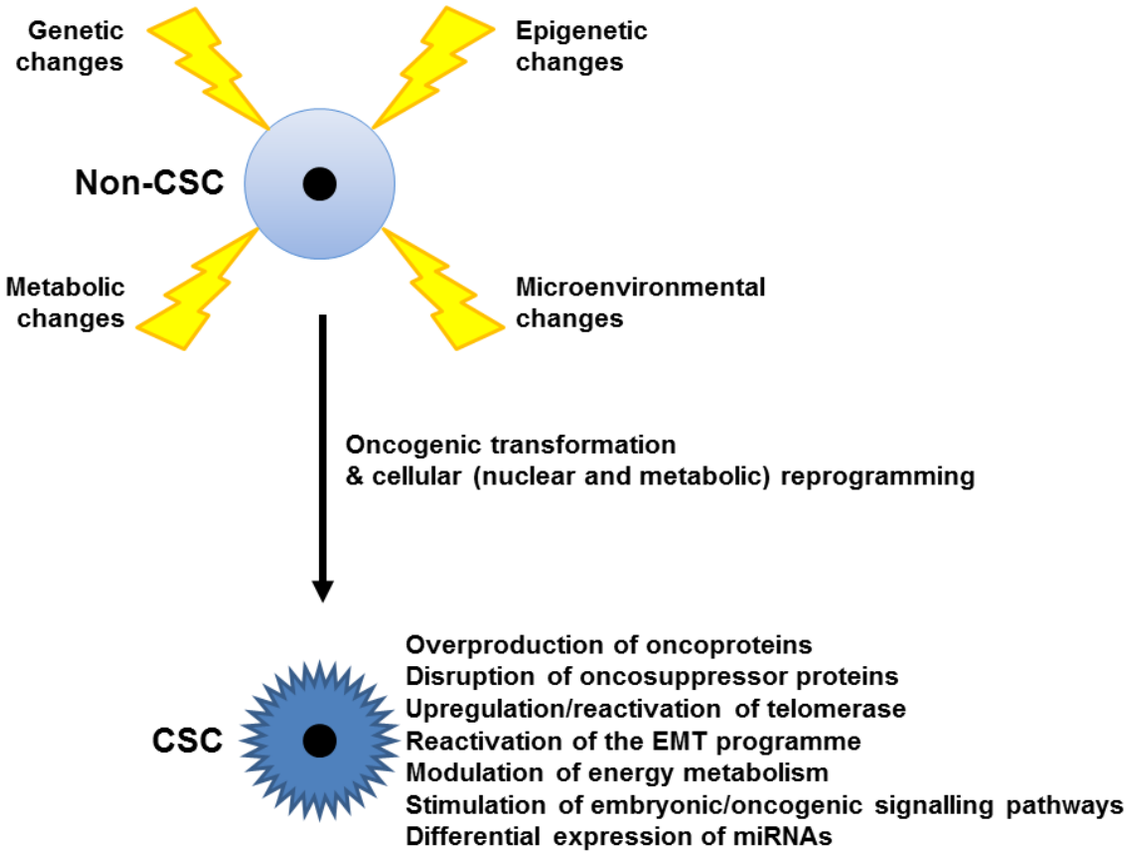

Figure 1. How do CSCs arise? The joint impact of genetic, epigenetic, metabolic and microenvironmental factors is believed to determine the conversion of non-CSCs (which could be normal stem cells, mature cancer cells, or others) to CSCs. This process somehow is a dynamic one and CSCs are a functional and not merely territorial subpopulation of cancer cells.

Disruption of diverse oncosuppressor proteins with antiproliferative, prodifferentiative and/or proapoptotic effects accounts for an early molecular event accompanying the emergence of cancer stemness traits. p53, pRB, PTEN, and $p16^{INK4A}$ are by far among the most commonly inactivated oncosuppressor proteins in advanced human tumours [48]. Their inactivation permits premalignant cells to avoid replicative senescence, apoptosis, or both, thereby continuing to divide and accumulating further tumourigenic alterations like genomic (chromosomal) instability that follows telomere erosion [49]. The subsequent upregulation/reactivation of telomerase compensates for telomere erosion (which would otherwise trigger entry of cells into a period of crisis with massive cell death), suppressing genomic (chromosomal) instability and allowing premalignant cells to proliferate for a virtually infinite number of cell divisions. Additionally, and surprisingly, there is accumulating evidence that telomerase upregulation/reactivation provides susceptible cells with cancer stemness traits. The many functions of telomerase in the development and maintenance of cancer stemness will be addressed in more detail in the next sections of this chapter.

A further means by which non-CSCs acquire cancer stemness traits is through the EMT process. Physiologically, EMT causes cells to change from a stationary epithelial to a motile mesenchymal morphology, thereby allowing for wound healing, tissue regeneration and organ fibrosis in adults and cell migration and tissue remodelling in developing embryos [50]. In the context of epithelium-derived carcinoma, however, the reactivation of the EMT programme contributes to the evolution of primary tumours towards increasingly aggressive phenotypes. The complex molecular, cellular and morphological alterations linked to pathological EMT are generally mediated by the joint action of the signals from the tumour microenvironment that induce the EMT programme (for example, TGF-β signalling, inflammatory cytokines, and hypoxia), the transcription factors that coordinate the EMT programme (for example, SNAI-1, SNAI-2, ZEB-1, ZEB-2, TWIST-1, and TWIST-2), and the effector proteins that execute the EMT programme (for example, low levels of E-cadherin and high levels of vimentin, N-cadherin, fibronectin, CD44, and MMPs) [51]. Such cooperation between the cell-extrinsic signals and the cell-intrinsic regulators is fully important and primarily responsible for endowing epithelial tumour cells with CSC-like properties, ranging from cell motility to invasiveness to cell survival, which are indispensable to metastatic dissemination from the primary tumour site, secondary tumour growth at a distant site, and resistance to therapy, respectively. Although CSCs originating from bulk tumour cells within epithelium-derived carcinomas achieve their final stemness state possibly *via* EMT, the degree to which they resemble or depart from CSCs originating from adult stem cells has yet to be fully explored.

Metabolic reprogramming is also an obvious mechanism of intervening and redirecting the cell fate of differentiated (normal or non-CSC tumour) cells. Traditionally, energy metabolism was widely accepted as a passive process that generated ATP and building blocks to meet the demands of the specialised cell types of the body in response to extra- and/or intracellular signals. Today, however, the modulation of energy metabolism and build-up of oncogenic metabolites are viewed as the harbingers of cancer stemness [52]. Typically, cancer cells are dependent more on glycolysis for energy production, even in the presence of sufficient oxygen to support oxidative phosphorylation. This phenomenon of aerobic glycolysis is commonly referred to as the Warburg effect and appears to fulfil the requirement of proliferating cancer cells to rapidly yield ATP and to provide anabolic substrates (such as amino acids, nucleotides, and phospholipids) for their daughter cells [53]. Besides, increased lactate generation during aerobic glycolysis provokes the acidification of the tumour microenvironment, ultimately giving rise to motile, invasive/metastatic and drug-resistant cells [54]. In agreement with this, a recent report confirmed and substantiated the need for a metabolic switch to glycolysis in the emergence of EMT-driven CSC-like characteristics in basal-like breast cancer cells [55]. Another report utilising nasopharyngeal carcinoma as a model system established that behaviourally-selected and accordingly-assayed CSCs, as distinct from their differentiated progenies, exhibit a metabolic shift from oxidative phosphorylation to glycolysis for ATP supply [56]. Nevertheless, contradictory evidence on the metabolic profile of CSCs has also been presented; two independent research groups reported that the bioenergetic and biosynthetic demands of quiescent/slow-cycling CSCs are likely to be met by oxidative phosphorylation, not by glycolysis [57, 58].

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CSCs maintain their prolonged residence in the stemness state through diverting and co-opting elegant signalling pathways that are normally active during embryonic development. The embryonic/oncogenic signalling pathways operating in CSCs include Notch, Hedgehog (HH), Wnt/β-catenin, cytokine receptor-mediated JAK/STAT, TNF-α receptor-mediated NF-κB, growth factor receptor (receptor tyrosine kinase)-mediated PI3K/AKT/mTOR, TGF-β/BMP receptor-mediated SMAD, and Hippo-YAP/TAZ [59–61]. Sustained activation of and crosstalk between these cascades ultimately enhance the expression of cell-surface proteins (for example, CD133, CD44, integrins, and CXCR4), prosurvival proteins (for example, BCL-2, BCL-XL, MCL-1, survivin, and MIC-1), induced pluripotency-associated transcription factors (for example, BMI-1, OCT-3/4, SOX-2, MYC, and NANOG), EMT-associated proteins (for example, MMPs, vimentin, N-cadherin, SNAI, TWIST, and ZEB), glycolysis-associated proteins (for example, GLUTs and glycolytic enzymes), treatment resistance-associated proteins (for example, GSH, ALDH1, ABCB1, ABCC1, ABCG2, CHK-1, and CHK-2), proangiogenic factors (for example, VEGF and COX-2), and proinflammatory cytokines (for example, IL-6 and TNF-α) [60, 62, 63].

Lastly, several miRNAs have been observed to support the emergence of cancer stemness traits through targeting signalling elements and gene groups implicated in CSC biology. miRNAs are a fast-growing class of short (19 to 22 nt), noncoding, regulatory RNA molecules that customarily bind to the 3′-untranslated region (3′-UTR) of their target transcripts to induce translational repression, degradation, or destabilisation. Although miRNAs generally help regulate the transitions between different stages of development, they are also linked with tumourigenesis. As such, CSC-specific miRNA expression profiles may be useful for prognostic purposes. Those miRNAs that are highly expressed in CSCs of a specific tumour are termed oncomiRs; those that are excluded from CSCs of the same tumour are known as tumour-suppressor miRs. Breast CSCs were the first CSCs in which differential expression of miRNAs was demonstrated [64].

4. Regulation of cancer stemness by telomerase

Most adult somatic cells do not or only transiently express telomerase and undergo telomere shortening with every cell division until the cell eventually dies. Most tumour cells, including CSCs, however, display high levels of telomerase activity and possess the ability to continually regenerate their telomeres [65]. As a result, telomerase upregulation/reactivation serves as an important mechanism for CSCs to attain indefinite (or at least extremely long) replicative lifespans. In fact, in reality, telomerase undertakes roles that significantly diverge from its normal role in elongating telomeres, as suggested by contemporary research on manipulation of telomerase expression and/or function in cells representing potential targets for oncogenic transformation and cellular (nuclear and metabolic) reprogramming. Central to the extratelomeric roles of telomerase (particularly of TERT) is its interaction with key downstream components of the main embryonic/oncogenic signalling pathways or with other macromolecules (such as DNA and transcription factors) by which gene expression is regulated. The presence of a few to several hundred copies of TERT, which are not assembled into telomerase,

in human immortalised cell lines reasonably provides a molecular basis for the formidable power of TERT as a transcriptional cofactor in oncogenic transformation and cellular (nuclear and metabolic) reprogramming, irrespectively of its TER-dependent DNA polymerase activity [66]. A very recent systemic review of the literature by us disclosed that most of the non-canonical responsibilities of telomerase identified so far strongly relate to the control of cancer stemness traits [67]. Telomerase/TERT-controlled aspects of the CSC phenotype involve proliferation, survival, therapy resistance, induced pluripotency, motility, glycolytic metabolism, and niche establishment and integrity (Figure 2). Equally strikingly, there seems to be a positive feedback loop between a number of gene products targeting TERT and TERT expression itself, plausibly amplifying the effects of central oncogenes and oncogenic pathways associated with the generation and/or maintenance of cancer stemness traits in a cell-autonomous manner. Although some of the observed cell-intrinsic/microenvironmental changes may require a catalytically active enzyme, there are several examples of oncogenic alterations brought about by catalytically inactive telomerase, as in the case of alternatively spliced (AS) TERT variants. To date, as many as twenty different AS TERT variants have been identified [68]. These variants tend to occur more frequently in cancer cells than in normal cells, indicating that they may be evolutionarily favoured in the context of pathology.

4.1. Stimulation of CSC proliferation

Given their role in the expansion of a tumour cell population, CSCs must display extensive proliferative capacity. Cell proliferation is both a matter of progression through the cell cycle and an issue necessitating cell growth (biosynthesis). There is a wealth of information in the literature on the promotive role of telomerase, independent of its telomere-elongating function, in cell proliferation. In an early study of the association between telomerase and cell proliferation, telomerase was shown to support the proliferation of human mammary epithelial cells through elevated EGFR signalling (even though it is quite ambiguous whether this effect is telomere length-independent or not) [69]. Moreover, TERT confers CSC characteristics to glioma cells by inducing EGFR expression, disconnectedly from its role in telomere biology [70]. Interestingly, telomerase upregulation was found to be closely linked to EGFR expression in actively proliferating normal human epithelial cells [71]. These observations imply the existence of a feed-forward loop that involves telomerase/TERT and EGFR. A plausible mechanism linking the EGFR–telomerase axis to cancer is that aberrant EGFR signalling may render CSCs less dependent on exogenous mitogens/growth factors and reinforce the persistent expression of telomerase in CSCs, thus playing a critical role in tumour development and progression.

Expanding these findings, one research group demonstrated that TERT promotes the proliferation of mammalian tissue progenitor cells *via* transcriptional control of a MYC- and Wnt-related developmental program [72]. To be more precise, TERT physically occupies the promoters of Wnt/β-catenin target genes, including those encoding cyclin D1 and MYC [73]. Cyclin D1 is a cell cycle control protein with oncogenic potential and has both enzymatic and nonenzymatic activities which are of great significance in tumour cells [74]. An additional molecular component involved in cyclin D1 expression in proliferating cells is nucleolar

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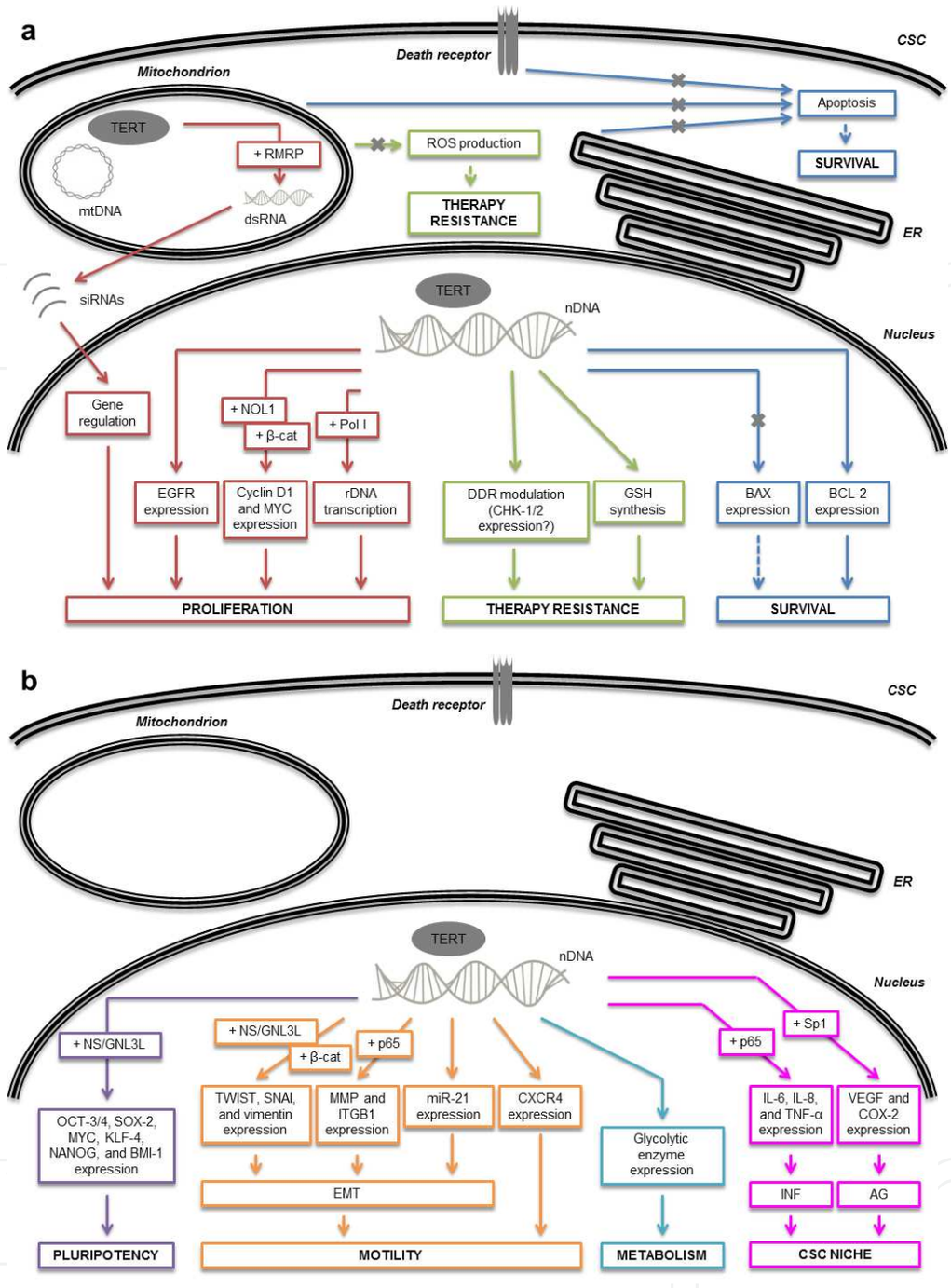

Figure 2. Emergence and persistence of cancer stemness by telomerase/TERT (figure adapted from [67] with permission). Clearly and unmistakably, telomerase/TERT is a powerful regulator of many aspects of the CSC phenotype, including: (a) proliferation, survival, therapy resistance; and (b) induced pluripotency, motility, glycolytic metabolism, niche establishment and integrity. This multifaceted ribonucleoprotein complex exerts its telomere-independent tumour-promoting effects partly by diverting and co-opting developmental signalling pathways and modulating gene expression. A cross symbol denotes an inhibition (blockage). A dashed arrow indicates that a given cancer stemness trait is not a direct consequence of the process shown in the preceding box, but of the inhibition of that process. AG, angiogenesis; DDR, DNA damage response; dsDNA, double-stranded DNA; dsRNA, double-stranded RNA; GSH, glutathione; INF, inflammation; mtDNA, mitochondrial DNA; nDNA, nuclear DNA; rDNA, ribosomal DNA; RMRP, RNA component of mitochondrial RNA-processing endoribonuclease; ROS, reactive oxygen species; siRNAs, small interfering RNAs

antigen p120 (NOL1), as suggested by the results of a very recent study [75]. In this study, telomerase was found to interact, in a TER-dependent fashion, with NOL1 and activate the transcription of the gene coding for cyclin D1. The relationship between TERT and cyclin D1 expression was corroborated also by other scientists [76–79]. Further support for TERT involvement in the stimulation of Wnt signalling-mediated cell proliferation was evidenced by an independent approach employing the Δ4–13 AS variant of human TERT that is devoid of reverse transcriptase activity [80]. In this approach, ectopic expression and small interfering RNA (siRNA)-mediated knockdown of the Δ4–13 AS variant in ALT cells, transformed telomerase-positive cell lines and telomerase-negative normal cells unquestionably proved that the proliferative effect of TERT is not coupled to telomerase activity. Because β-catenin is known to modulate TERT expression in stem cells and tumour cells [81], it is tempting to speculate that telomerase and Wnt/β-catenin signalling may act together in a positive feedback circuit to actively encourage the proliferation of CSCs.

Intriguingly, one group failed to find evidence that TERT promotes Wnt signalling in human breast cancer cells, indicating that TERT's effect on Wnt signalling is possibly context- and cell type-dependent [82]. Their findings are in the same direction as those from a former study on telomerase-null mouse models, where TERT loss-of-function in a physiological setting was reported to have no evident effects on Wnt signalling [83]. An alternative mechanism of action of telomerase on cell proliferation, as deduced by reverse genetics in human mammary epithelial cells, is that TERT-induced cell proliferation may result primarily from decreased levels of the RNA component of mitochondrial RNA-processing endoribonuclease (RMRP), not from increased Wnt signalling [78]. TERT and RMRP form a definite ribonucleoprotein complex that exhibits RNA-dependent RNA polymerase activity and, using RMRP as a template, produces double-stranded RNAs that can be later processed into siRNAs in a Dicer-dependent fashion [84]. siRNAs regulate gene expression at the posttranscriptional level as well as at the level of chromatin structure; therefore, it is reasonable to question whether their mutations or altered expression correlate with human cancers.

Aside from activating Wnt signalling and regulating gene expression (in the presence of RMRP), telomerase also stimulates ribosomal biogenesis through increased Pol I-directed ribosomal DNA transcription, exerting a positive influence on cell cycle and proliferation dynamics [85]. This may ultimately improve the protein synthesis capacity of CSCs for unrestrained growth. The molecular mechanism behind telomerase-induced ribosomal biogenesis was investigated in a very recent report where a MYC-driven oncogenesis model was proposed [79].

4.2. Promotion of CSC survival

Apoptosis is a form of cell death induced by miscellaneous stimuli and mediated by a subset of cysteine proteases termed caspases. A cancer cell's ability to evade death signals, thus preventing self-destruction by the activation of an apoptotic programme, is regarded as one of the hallmarks of cancer [86]. Several pieces of information suggest that telomerase exerts antiapoptotic effects in cancer cells through telomere-independent mechanisms. In the case of CSCs, telomerase-mediated inhibition of apoptosis may contribute to the enhanced and

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continued survival of these cells in tumours. In keeping with its cytoprotective function, TERT was revealed to inhibit cell death by blocking the death receptor-initiated (or extrinsic) apoptotic pathway in acute promyelocytic cells [87]. Similarly, yet in a mechanistically different way, TERT was also shown to block the mitochondrion-initiated (or intrinsic) apoptotic pathway in colon and cervical carcinoma cell lines [88]. A more thorough probing of the molecular mechanism behind telomerase-mediated suppression of the intrinsic apoptotic pathway found that TERT overexpression upregulates the expression of the antiapoptotic mitochondrial protein BCL-2, downregulates the expression of some proapoptotic mitochondrial proteins (for example, BAX) and reduces the activation of some caspases (for example, caspase-9) in ovarian surface epithelial cells [89]. Given the capacity of BCL-2 to increase telomerase activity in human colorectal and cervical carcinoma cell lines [90], it is conceivable that telomerase and BCL-2 are engaged in a positive feedback loop that impedes apoptosis. In this connection, it is well to add that the introduction of a constitutively expressed TERT construct into colon carcinoma and Burkitt's lymphoma cell lines was, independently of telomerase activity, associated with the reversion of a transcriptional programme coordinated by p53, a potent and common activator of both the intrinsic and extrinsic apoptotic pathways [91].

Apart from suppressing mitochondrion-initiated cell death, overexpression of TERT was also found to suppress, in a telomere-independent manner, endoplasmic reticulum (ER) stress-induced cell death in murine primary neural cells and human cancer cell lines [92]. ER stress arises as a result of perturbations in ER function and elicits the unfolded protein response (UPR), a conserved signal transduction pathway for dealing with misfolded proteins. When the UPR-induced mechanisms fail to alleviate ER stress, both the intrinsic and extrinsic apoptotic pathways may become activated [93]. Reciprocally, specific activation of ER stress was demonstrated to upregulate TERT expression in a breast cancer cell line [94]. It therefore seems reasonable to suggest that TERT and ER stress are involved in a dynamic interplay supporting CSC survival in abnormal metabolic conditions such as glucose starvation.

4.3. Induction of pluripotency

Restoration of the molecular circuitry that forms the necessary base of pluripotency in embryonic stem cells (ESCs) strongly correlates with the gaining and retention of cancer stemness. In ESCs, this circuitry is made up of special transcription factors and function as a repressor of differentiation. Takahashi and Yamanaka were the first to demonstrate that a cocktail of four transcription factors (namely OCT-3/4, SOX-2, MYC, and KLF4) are necessary and sufficient for nuclear reprogramming into an ESC-like state [95]. In CSCs, the so-called Yamanaka factors, besides driving the induction of pluripotency, are additionally involved in inhibiting apoptosis [96]. A valued piece of work documented that TERT forms a ternary complex with the nucleolar GTP-binding protein NS/GNL3L and the chromatin remodelling factor BRG1 and that the resulting NS/GNL3L TERT BRG1 complex is required for NS/GNL3L-induced upregulation of the nuclear reprogramming factors OCT-3/4, MYC, and KLF-4 [97]. The likely part played by TERT/telomerase in contributing to the pluripotent character of CSCs is also congruous with the later finding that siRNA-mediat-

ed hTERT depletion in gastric CSCs downregulates the induced pluripotency-associated transcription factor OCT-4 [98].

4.4. Increase of CSC motility and invasiveness

Migrating CSCs and EMT-phenotypic cells have the ability to disseminate from their primary site and are thus present in the invasive front of tumours. The initial evidence for telomerase/TERT participation in cell migration came from experiments measuring the *in vitro* migration rate of telomerase-positive progenitor cells and cancer cell lines [99, 100]. Later experiments aiming at uncovering the molecular mechanism behind this positive trend showed that telomerase reconstitution boosts cell migration through the activation of Rho family members and the SDF-1–CXCR4 axis [101]. With respect to CSC motility, expression of the chemokine receptor CXCR4 may enable CSCs to migrate along a gradient of the ligand SDF-1 and thus help facilitate their spread. Therapeutic strategies intended to interfere with the SDF-1–CXCR4 axis can possibly have useful clinical relevance and application in the prevention of metastatic disease.

Differentiated epithelial cells that have undergone EMT may as well exhibit augmented motility and invasiveness leading to metastasis. The ternary complex containing TERT, BRG1, and NS or GNL3L (see section 4.3) acts in a telomere-independent mode to activate the EMT programme *via* NS/GNL3L-induced upregulation of vimentin, SNAI, and TWIST, three of the mesenchymal cell markers, in genetically defined cancer cells [97]. TERT additionally stimulates EMT in gastric cancer cells through directly regulating the expression of Wnt/β-catenin target genes like those coding for vimentin and SNAI-1 [98]. Equally important is the fact that TERT, in a telomere-independent manner, regulates the expression of several MMP family members, such as MMP-9, *via* the NF-κB pathway [102]. MMPs are the key mediators promoting extracellular matrix (ECM) degradation and remodelling, both of which pave the way for EMT and subsequent metastasis. The indirect involvement of TERT in dissemination was also highlighted by a separate set of data which documented that changes in the motility and invasiveness of malignant cells are likely to result from the TERT-induced upregulation of the metastasis-implicated proteins RhoC and MMP-9 [103]. Interestingly, MMP-9 silencing was shown to downregulate TERT expression *via* ITGB1-mediated FAK signalling in glioma xenograft cells [104]. It is worth mentioning here that a very recent report found that ITGB1 itself is regulated by TERT and that TERT may promote the invasion and metastasis of gastric cancer cells by enhancing ITGB1 protein levels [105]. Collectively, these findings reinforce the notion that there is an indirect, metastasis-favouring interaction between TERT, MMP-9 and ITGB1 in cancer cells. Another study discovered that TERT upregulates the levels of MAC2BP, a metastasis-related secreted ECM glycoprotein, in gastric cancer cells [106]. MAC2BP is believed to support metastasis through interacting with galectins and altering cell–cell and cell–matrix adhesion properties [107].

Another contribution to knowledge came from a very recent report in which TERT was found to stimulate the expression of oncomiRs, including miR-21, in human leukaemia and HeLa cell lines [108]. Extant research identifies miR-21 as being among the most frequently upregulated miRNAs in epithelial cell-derived solid tumours [109] and also as having a

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decisive role in the conservation of CSC phenotype *via* the AKT and ERK1/2 signalling pathways targeting PTEN [110]. The centrality of miR-21 to cancer stemness was confirmed in a recent study on the antisense oligonucleotide-mediated inhibition of miR-21 in two different anaplastic thyroid carcinoma (ATC) cell lines, where the knockdown of miR-21 disturbed the stemness state of ATC cells, as assessed by a decreased expression of the genes encoding OCT-4 and ABCG2 [111].

4.5. Modulation of energy metabolism

Apparently, genetic, epigenetic and microenvironmental changes that regulate the transition to a CSC-like state cannot occur without the presence of a favourable metabotype. In general, stimulation of aerobic glycolysis promotes metabolic reprogramming, whereas inhibition of glycolytic enzymes impairs metabolic reprogramming. In harmony with the concept that metabolism is involved in the control of cancer stemness, a microarray-based gene expression profiling study elucidated that ribozyme-mediated targeting of telomerase in murine melanoma cells downregulates the expression of more than a few glycolytic pathway genes such as those coding for phosphofructokinase and aldolase C [112]. Additionally, a very recent report showed that siRNA-mediated knockdown of TERT in human lymphoma cells lowers the expression of MYC-regulated target genes such as those coding for the glycolytic enzymes lactate dehydrogenase, hexokinase 2, and pyruvate kinase M2 isoform [79]. Due to the fact that MYC is a well-established oncogenic transcription factor activating TERT expression [113], a feed-forward mechanism for the rewiring of glucose metabolism in CSCs is likely to prevail between TERT and MYC.

4.6. Contribution to therapy resistance

CSCs are notorious for their resistance to existing cancer treatment regimens, including radiotherapy and chemotherapy. Both cell-intrinsic and microenvironmental factors appear to contribute to the emergence of therapy resistance in CSCs. Radiotherapy works by directing ionising radiation toward tumours to induce the generation of reactive oxygen species (ROS) which react with and cause damaging of DNA. By the same token, a number of chemotherapeutic agents such as platinum-based antitumour drugs are known to bind to and cause crosslinking of DNA. In this regard, implementation by CSCs of fast and efficient DNA repair mechanisms as well as potent antioxidant/scavenger systems may prove vital to circumvent the deleterious effects of irradiation and several classes of antitumour compounds.

Growing evidence points to a role for telomerase in modulating DDR and contributing to DNA repair. In a prior report, TERT was proposed to, independently of its effect on telomere length, set in motion a transcriptional programme leading to enlarged ribonucleotide (NTP) pools, enhanced DNA repair, and increased chromosomal stability [114]. A circumstantial investigation into the enhanced DNA repair capability of telomerase-expressing cells suggested that TERT/telomerase increases DNA end-joining repair and accelerates nucleotide excision repair through recruiting proteinaceous factors to sites where DNA damage is occurring [115]. The aforementioned findings are consistent with a newer report which showed that TERT expression affords a means of protecting human transformed cells against double-stranded DNA-

damaging drugs and increases their endurance to chromosomal instability [116]. Specifically expressing TERT mutants lacking catalytic activity in ALT cells, the authors of the same report reached the conclusion that the observed cytoprotective effect of telomerase is distinct from its function in telomere biosynthesis. In a separate study providing evidence for an epigenetic component to telomerase-induced treatment resistance, stable short hairpin RNA (shRNA)-mediated suppression of TERT expression was demonstrated to, in a telomere length-independent way, diminish the response of human fibroblasts to DNA double strand breaks, most likely through a mechanism altering the overall state (that is to say, configuration) of chromatin [117].

Telomerase upregulation/reactivation also seems to be involved in counteracting oxidative stress-induced intracellular injury that often follows therapy, as evident from several experimental studies. The initial study examining the extratelomeric function of telomerase under oxidative stress found that mitochondrially-localised TERT decreases cellular peroxide levels and mitochondrial superoxide production, increases mitochondrial membrane potential and protects mitochondrial DNA from oxidative damage in human lung fibroblasts [118]. The observation that telomerase provides resistance to oxidative stress was validated and extended in an ensuing study where TERT was shown to bind to mitochondrial DNA and accordingly protect it and its function against damage [119]. An alternative explanation for telomerase-induced resistance to oxidative stress came from a more recent study in which TERT overexpression in cancer cells was demonstrated to alleviate basal ROS levels and intracellular ROS production through potentiating the effects of endogenous antioxidants or free radical scavengers such that the proportion of reduced to oxidised glutathione (GSH/GSSG) is increased and peroxiredoxin is replenished in the interior of the cell [120]. Apart from serving to keep mitochondrial DNA damage-free, mitochondrially-localised telomerase also guards nuclear DNA against oxidative attack through decreasing mitochondrial ROS production [121]. Finally, it is appropriate to mention that the β-deletion variant, a catalytically defective AS variant of TERT, localises to both mitochondria and the nucleus and, distinct from the canonical role of TERT in telomere extension, protects three basal breast cancer cell lines from cisplatin-induced apoptosis, endowing breast tumours with chemotherapy resistance [122].

4.7. Establishment and integrity of the CSC niche

The tumour microenvironment (TME) is an umbrella term that encompasses all cellular and non-cellular components surrounding a tumour. These components include tumour-adjacent stromal cells (for example, endothelial cells and fibroblasts), diverse effectors of the immune system (for example, lymphocytes and mesenchymal stem cells), ECM elements, proteases, and networks of cytokines, growth factors and other soluble factors. Specifically, both the immediate TME (cell–cell and cell–matrix connections) and the extended TME (for example, vascular bed) are thought to be implicated in tumour progression. The TME is also capable of creating a niche for CSCs, in which they remain in an undifferentiated state until stimulated to differentiate into non-CSC tumour cells and form tumour bulk. Modulation of gene expression and metabolism by telomerase in CSCs may recondition the CSC niche in favour of the hypermalignant (that is to say, highly metastatic, therapy-resistant) nature of these cells.

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It is in this regard that a recent study demonstrated that telomerase binds to p65 and localises to promoters of NF-κB target genes, such as those encoding IL-6, TNF-α, and IL-8, proinflammatory cytokines that are the critical triggers of inflammatory responses [123]. Inflammation is considered an enabling characteristic of cancer for the reason that it supplies bioactive molecules (for example, growth factors and EMT-inducing ligands) to the TME and primes cells to release ROS and other chemicals that drive the mutagenesis, and hence genetic evolution, of nearby tumour cells toward hypermalignancy [86]. Since NF-κB is known to transcriptionally upregulate telomerase levels [124], this finding implies that a positive feedback loop between telomerase and NF-κB may explain the grounds for the coexistence of chronic inflammation and sustained telomerase activity in neoplastic lesions.

Furthermore, it was reported that TERT activates the transcription of VEGF, an endothelial mitogen and master orchestrator of angiogenesis, independently of telomerase activity in HeLa cells [125]. Further dissection of the underlying regulatory mechanism led to the conclusion that TERT upregulates VEGF expression through its interaction with the specificity protein 1 (Sp1) transcription factor [126]. Angiogenesis is the process of growth or formation of fresh blood vessels from the pre-existing vasculature, and its induction is widely considered an essential attribute of tumour growth as well as metastasis as solid tumours larger than 1 cm^3 have to develop their own blood supply to circumvent necrotic cell death. Given the prior discovery that VEGF stimulates the production of TERT [127], it may be that there is a positive feedback circuit between TERT and VEGF. This regulation may account for the combined and continuing contribution of these two proteins to the maintenance of CSCs in solid tumours. Moreover, siRNA-mediated knockdown of TERT was shown to downregulate the prostaglandin-synthesising enzyme COX-2 in pancreatic cancer cells [128]. COX-2, like VEGF, is a proangiogenic factor that has the potential to establish a selective niche favourable to the preservation of CSCs. Subsequent studies showed that COX-2 stimulates the expression of TERT in cervical cancer cells [129]. Collectively, these data indicate that a feed-forward regulation, which could be important in carcinoma growth and progression, occurs between TERT and COX-2.

5. Prospects for telomerase-targeted anticancer therapies

There is little doubt that telomerase is not only an effector in human tumour pathogenesis but also a regulator of essentially all aspects of malignant behaviour, including spreading to secondary sites. This being the case, specifically and sensitively measuring telomerase activity in clinical samples represents both an early diagnostic marker and a negative prognostic factor for patients with malignant disease. The obvious capacity of telomerase to directly regulate an ever-expanding number of tumour-promoting genes and pathways renders this gifted ribonucleoprotein particle an attractive and almost universal target for human cancers. Given the integral role played by cancer stemness in disease recurrence, selectively targeting and eradicating CSCs with low toxicity to somatic cells and with minimal side effects holds the promise of a full cure for cancer. Besides being pursued with vigour in the hope of improving patient outcomes, successfully suppressing cancer stemness may also provide the ultimate

evidence for the CSC concept. As summarised in this chapter, telomerase contributes to carcinogenesis most likely through the emergence and persistence of conspicuous CSC qualities. Accordingly, telomerase inhibition in CSCs is predicted to: (i) shrink telomeres; (ii) restrain anchorage-independent growth and inhibit proliferation by cell cycle arrest; (iii) induce CSC death; (iv) induce CSC differentiation; (v) inhibit CSC migration and reverse the EMT programme; (vi) deteriorate glucose metabolism; (vii) enhance radio- and chemosensitivity; and (viii) disrupt the CSC niche. Serious telomere shrinkage is assumed to be a long-term effect of telomerase inhibition in CSCs so the tumour mass will continue to expand for a time after treatment until its constituent cells enter crisis and begin to die in large numbers. The rest of the aforementioned effects, however, are likely to occur after short-term exposure of CSCs to telomerase inhibitors, inducing relatively rapid initial responses to treatment. Natural telomerase inhibitors (phytochemicals) and small-molecule telomerase inhibitors, antisense oligonucleotides and chemically modified nucleic acids, immunotherapeutic agents, and telomerase-directed gene therapy are promising treatment options and may play a larger role in the near future [130]. Imetelstat (GRN163L), which was designed by Geron Corporation in 2003, is the first telomerase inhibitor to advance to clinical development. It is a lipid-conjugated 13-mer (5'–TAGGGTTAGACAA–3') antisense oligonucleotide that is complementary to and binds with high affinity to TER, thereby directly inhibiting telomerase activity and interfering with telomere length homeostasis. It is perhaps safe here to assume that Imetelstat impairs the regulatory role of telomerase in CSC biology not only through telomere shortening but also through negatively influencing its telomere length-independent tumour-promoting functions. In support of this, short-term (72-hour) Imetelstat exposure was shown to promote the differentiation and inhibit the colony-forming ability of multiple myeloma CSCs through a telomere length-independent mechanism [131]. Similarly, *in vitro* Imetelstat treatment was found to deplete breast and pancreatic CSCs, as measured by the reduced proportion of ALDH-positive and CSC-surface marker-expressing cells, through a mechanism of action independent of telomere shortening [132]. Although Imetelstat is known to form thermodynamically stable and sequence-specific duplexes with TER, the possibility that even less thermodynamically stable tetraplexes of Imetelstat may bind to and interfere with some other, yet to be identified, proteins (particularly those that interact with telomerase) should not be excluded [133]. There also exists a possibility that telomerase inhibitors like Imetelstat may be coupled with conventional therapies such as surgical (debulking) therapy, radiotherapy, and chemotherapy, all of which have their own weaknesses and inadequacies. Such combination therapy is predicted to result in rapid and durable clinical responses in broad tumour types (Figure 3). As shown by the sources provided earlier in this chapter and elsewhere in the literature, the principal signalling pathways governing CSC biology operate in physiological stem cells as well. This complicates telomerase inhibition therapy because of the risk of telomerase inhibitors exerting an adverse influence on the size of the physiological stem cell pool and/or on the integrity of the physiological stem cell niche. The notion that physiological stem cells only transiently express telomerase and have relatively long telomeres [65], however, means that there is likely to be a narrow but safe therapeutic window where only CSCs will be depleted by telomerase inhibitors and normal stem cells will remain unaffected. Furthermore, rational approaches that disrupt the interactions of telomerase with important downstream components of embryonic/oncogenic signalling pathways (Wnt/β-catenin and NF-κB being the most prominent of all so far) may be conceived and executed as therapeutic tactics to

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specifically target and eliminate CSCs. As far as targeting protein–protein interactions is concerned, a future challenge is to add to existing pathways or identify new ones where telomerase, independently of its role in preventing telomere loss, intervenes and contributes to CSC phenotype. The essential nature of telomerase in the promotion and maintenance of cancer stemness provides a logical basis for believing that CSCs will not develop resistance to any of the aforementioned telomerase-focused therapeutics, in contrast to other targeted anticancer therapies whose targets are likely to be compensated for by functionally equivalent gene products and signalling pathways.

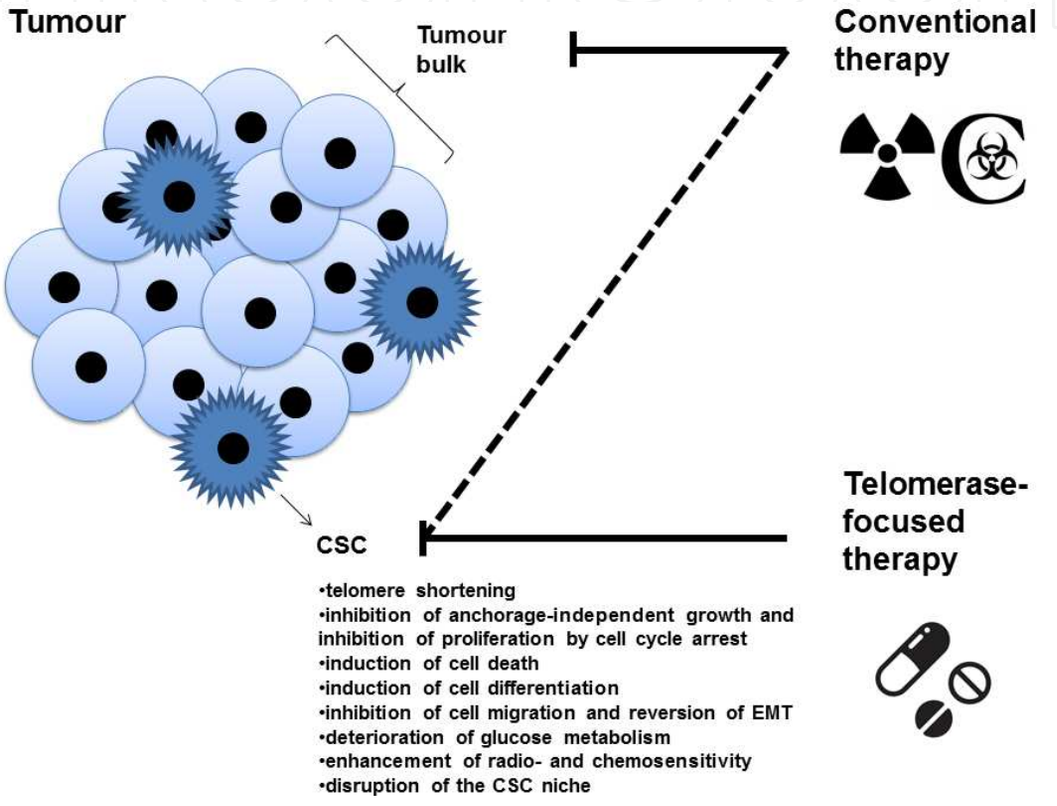

Figure 3. Combination therapy for predicting rapid and durable clinical responses in broad tumours. Here, telomerase-focused therapy is proposed as a strategy to effectively target CSCs. Combining telomerase-focused therapy with conventional therapy may provide scope for the elimination of bulk tumour cells while preventing recurrence by simultaneously eradicating the stem-like component of the tumour. Combination therapy may also prevent non-CSC tumour cells from acquiring cancer stemness traits *via*, for example, EMT. A bar-headed solid line denotes a strong inhibitory (negative) effect. A bar-headed dashed line denotes a weak inhibitory (negative) effect.

6. Abbreviations

ABC, ATP-binding cassette; Akt, protein kinase B; ALDH1, aldehyde dehydrogenase 1; BAX, BCL-2-associated protein X; BCL, B-cell lymphoma family protein; BMI-1, B lymphoma Mo-MLV insertion region 1 homolog; BMP, bone morphogenetic protein; BRG1, Brahma-related gene 1; CD, cluster of differentiation; CHK, checkpoint kinase; COX-2, cyclooxygenase-2;

CXCR4, C–X–C chemokine receptor type 4; EGFR, epidermal growth factor receptor; ERK1/2, extracellular signal-related kinase 1/2; FAK, focal adhesion kinase; GLUTs, glucose transporters; GNL3L, guanine nucleotide-binding protein-like 3-like; IL, interleukin; ITGB1, integrin beta-1; JAK, Janus kinase; KLF-4, Kruppel-like factor-4; MAC2BP, Mac-2-binding protein; MCL-1, myeloid cell leukaemia-1; MIC-1, macrophage inhibitory cytokine-1; MMPs, matrix metalloproteinases; MYC, v-myc avian myelocytomatosis viral oncoprotein homolog; mTOR, mammalian target of rapamycin; NANOG, Nanog homeobox transcription factor; NF-κB, nuclear factor-κB; NS, nucleostemin; OCT-3/4, octamer-binding transcription factor-3/4; PI3K, phosphoinositide 3-kinase; PTEN, phosphatase and tensin homolog; SDF-1, stromal cell-derived factor-1; SMAD, small mother against decapentaplegic homolog; SNAI, snail family zinc-finger transcription factor; SOX-2, SRY (sex determining region Y)-box 2; STAT, signal transducer and activator of transcription; TAZ, Tafazzin; TGF-β, transforming growth factor-β; TNF-α, tumour necrosis factor-α; TWIST, twist family bHLH (basic helix-loop-helix) transcription factor; VEGF, vascular endothelial growth factor; Wnt, Wingless ligand; YAP, Yes-associated protein; ZEB, zinc-finger E-box-binding homeobox family protein

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