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Review

Cancer Stem Cells and Individualized Therapy

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Abstract. *The concept of individualized cancer chemotherapy emerged three decades ago from the observation that a small fraction of cells in primary tumors can form colonies in soft agar similar to stem cells of the hematopoietic system. In a series of retrospective and prospective clinical studies, clonogenic tumor growth and effects of anticancer agents on the putative cancer stem cells were assessed as predictive factors. The results of these trials showed that clonogenic growth is associated with poor outcome and drug resistance. Recent breakthroughs enabling isolation and the molecular classification of cancer stem cells have renewed interest in cancer stem cells as a therapeutic target. Here, we provide a current overview of cancer stem cell biology and highlight possibilities for targeted intervention with existing and novel experimental anticancer agents.*

Despite drastic improvements in the use of existing therapeutic modalities and the advent of "molecularly targeted agents", the outlook for most cancer patients, particularly those with advanced solid tumors, is still poor. Even though many of the new anticancer drugs can result in tumor shrinkage, their impact on survival has been modest (1). Resistance to cancer chemotherapeutic agents, both new and old, remains a major problem (2).

There is emerging evidence that a rare and biologically distinct subpopulation of cancer cells has the ability to self-renew, self-protect, and proliferate indefinitely. The characteristics of this population are analogous to normal stem cells and therefore these cells have been described as "cancer stem cells". Most conventional chemotherapies affect differentiated cancer cells that make up the bulk of a tumor, but are often ineffective against cancer stem cells (3, 4). As a consequence, an initial reduction in tumor bulk is frequently followed by rapid relapse of the tumor. One of the characteristics of cancer stem cells is a high expression of drug efflux pumps. Thus, tumors repopulating after relapse are frequently composed of chemotherapy resistant cells. The current challenge in anti-cancer drug development is therefore to find means to selectively target and eradicate cancer stem cells. In this article, we will review the historical background of stem cells and cancer stem cells, as well as the role of targeting cancer stem cells for individualized cancer therapy (5).

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The Stem Cell Concept

The stem cell concept was proposed in the early 1900s. However, not until the early 1960's stem cells were actually identified. Two Canadian scientists, Ernest A. McCulloch and James E. Till, pioneered the field by injecting bone marrow cells into irradiated mice. Visible nodules were observed in the spleens of the mice and were described as "spleen colonies". They initially speculated that each nodule arose from a single marrow cell and perhaps a stem cell. Later on, they were able to demonstrate that each nodule did indeed arise from a single cell (6, 7). Hence, stem cells are cells with the exclusive ability to self-renew, and develop into multiple lineages through differentiation (8). During embryonic development and adult life, stem cells divide in a particular pattern. Polarity is the unequal spatial distribution of cellular constituents. For instance, there are two types of polarity in simple epithelia: planar cell polarity and apicobasal polarity. Stem cells are capable of dividing asymmetrically and possess an axis of polarity. After asymmetric division, one daughter cell will retain stem cell properties and remain undifferentiated (Figure 1). The other daughter cell will become a committed progenitor. There is evidence that disruption of the process of

asymmetric cell division and loss of polarity can induce a cancer-like state in neuronal stem cells (9, 10). In addition to their capacity for self-renewal, recent work has demonstrated that stem cells can "self-protect" through high expression of drug efflux pumps including BCRP (ABCG2), p-glycoprotein, and MRP (11).

Cancer Stem Cells

Stem cells and cancer cells are similar in many aspects including their self-renewal ability, the ability to differentiate, their limitless proliferate capacity by expression of telomerase, the ability to activate anti-apoptotic pathways, increased membrane transporter activity, resistance to cytotoxic agents, and their ability to migrate. It was formerly believed that cancer is a homogenous disease and that each cancer cell can give rise to the entire tumor. However, recent studies have demonstrated that only a rare and biologically distinct subset of cancer cells is capable of extensive proliferation. In the 1960s, Bruce and Van Der Gaag (12) found that only 1 in 130 (0.77%) murine lymphoma cells were capable of forming a colony in the spleen. Subsequently, McCulloch (13) illustrated that 1 in 10,000 to 1 in 100 mouse myeloma cells was able to form colonies in clonal *in vitro* colony-forming assays. This subset of cells was described as leukemic stem cells (LSC). McCulloch also proposed that in order to be successful in controlling the tumor growth, these tumor stem cells must be eradicated. Nevertheless, based on these early observations it remained still possible that all leukemic cells are clonogenic, but may have been unable to proliferate under the assay conditions used. In order to demonstrate that cancer cells are in fact heterogeneous and only a distinct subset of cells has clonogenic capacity, it was essential to separate different populations and define one subset of cancer cells that has extensive proliferating capacity. This has been recently accomplished by John E. Dick *et al.* (14). The latter evaluated cell surface markers that are used to identify hematopoietic stem cells in acute myeloid leukemia (AML) and demonstrated that only cells with a $CD34^+/CD38^-$ phenotype were able to initiate human AML in non-obese diabetic/severe combined immunodeficient (NOD/SCID) mice. These cells were capable of self-renewal, proliferation and differentiation *in vivo* into the original leukemic blast, indicating leukemic stem cells. More recently, it was demonstrated that leukemic stem cells are not functionally homogeneous but consist of a distinct hierarchically arranged class similar to normal hematopoietic stem cells (15).

Importantly, the pathways that regulate the self-renewal of normal stem cells (16-18), including Wnt, Notch, and Hedgehog, have been found to play an important role in human carcinogenesis (18). Dysregulation of these pathways has been reported in colon, pancreatic, gastric, prostate (19), cervical, leukemic, skin (20) and breast cancers (18, 21, 22).

Cancer Stem Cells in Solid Tumors

Similar to hematological malignancies, solid tumors have been demonstrated to possess a phenotypically distinct and rare population of clonogenic cancer cells (23, 24). Merely 1 in 1,000 to 1 in 5,000 lung cancer, ovarian cancer or neuroblastoma cells were able to form colonies in soft agar (8, 25). Recently, Al-Hajj *et al.* (26) described a distinct cell population with a $CD44^+/CD24^{-/low}/lineage^-$ phenotype in breast cancer. As few as 100 cells of this particular population were capable of generating a phenotypically diverse breast tumor similar to the original patient tumor in NOD/SCID mice, whereas as many as 20,000 cells with $CD44^+/CD24^+$ phenotype were unable to generate a tumor (18, 26). Singh *et al.* (27, 28) demonstrated that a $CD133^+/Nestin^+/Lineage^-$ population from human brain tumors was capable of generating clonal tumor spheres in suspension culture. Again, only 100 cells of this explicit subpopulation could produce the phenotypically diverse tumor resembling that from the patient in the NOD/SCID mouse repopulation assay, while 10^5 of $CD133^-$ cells engrafted but were unable to form a tumor. Matsui *et al.* (29) demonstrated that a distinct $CD138^-$ population in multiple myeloma cells was clonogenic *in vitro* and in NOD/SCID mice. Furthermore, Collins *et al.* (30) illustrated that only the population with $CD44^+/\alpha_2\beta_1^{hi}/CD133^+$ phenotype in prostate cancer was capable of self-renewal and recapitulation of the original tumor. In contrast to the report by Singh in brain tumors, the fraction of this population in prostate cancer was relatively small and constant regardless of tumor grade. In addition, cancer stem cells were also recently described in lung cancer (31), head and neck (32), pancreas (33) and colon (34) carcinomas. Interestingly, several stem cell surface markers are shared by cancer stem cells in different tumor types. These markers include CD44, α_6 integrin, β_6 integrin, and CD133. Owing to the exponentially increasing reports on the identification and definition of cancer stem cells in the scientific literature, it is possible that cancer stem cells will be identified for every known tumor type.

Changes in the Cancer Treatment Paradigms

The vast majority of the over 100 registered and clinically utilized anticancer drugs are "classical" cytotoxic agents. Their common mechanism of action is the preferential killing of rapidly dividing cells by either interruption of the mitotic spindle apparatus or by targeting DNA. Many cancers have a relatively higher proportion of proliferating cells than most normal tissues, and consequently are more sensitive to cytotoxic agents, creating a therapeutic window. Even though some tumors may initially show measurable response to these treatments, the reduction in bulk tumor mass is often short lived and associated with severe side

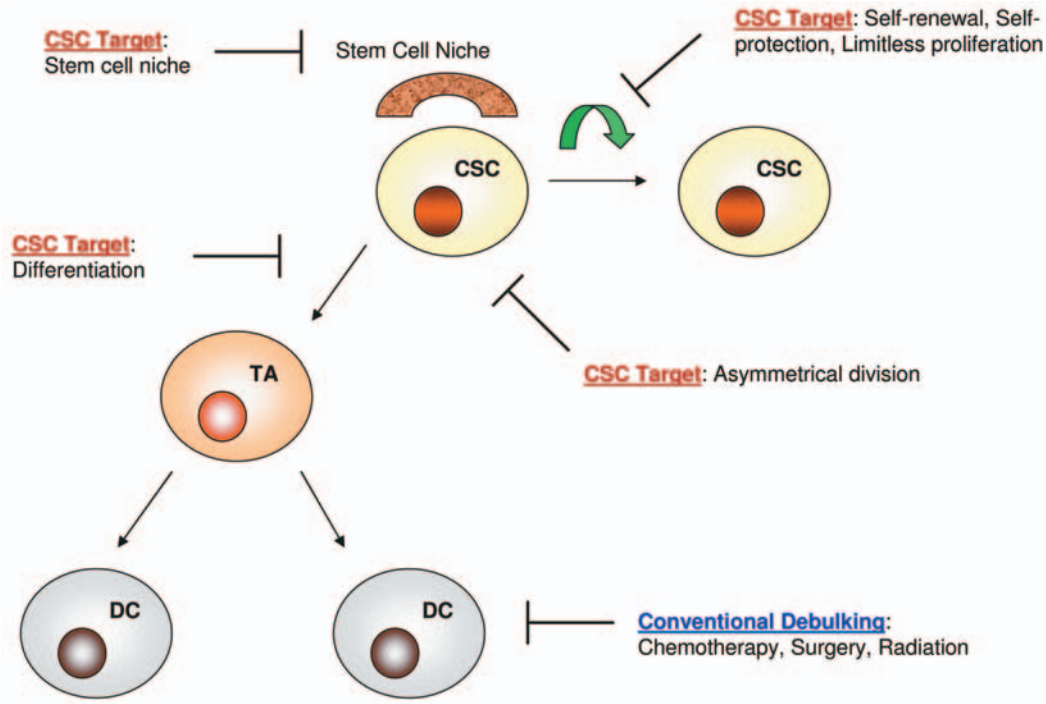

Figure 1. *Cancer stem cell (CSC) hierarchy and possibilities for therapeutic intervention. Attractive CSC targets include the self-renewal pathway, asymmetrical division, the cancer stem cell niche and differentiation. Transient amplifying cancer cells (TA) and differentiated cancer cells (DC) can be treated by conventional debulking methods such as chemotherapy, surgery and radiation.*

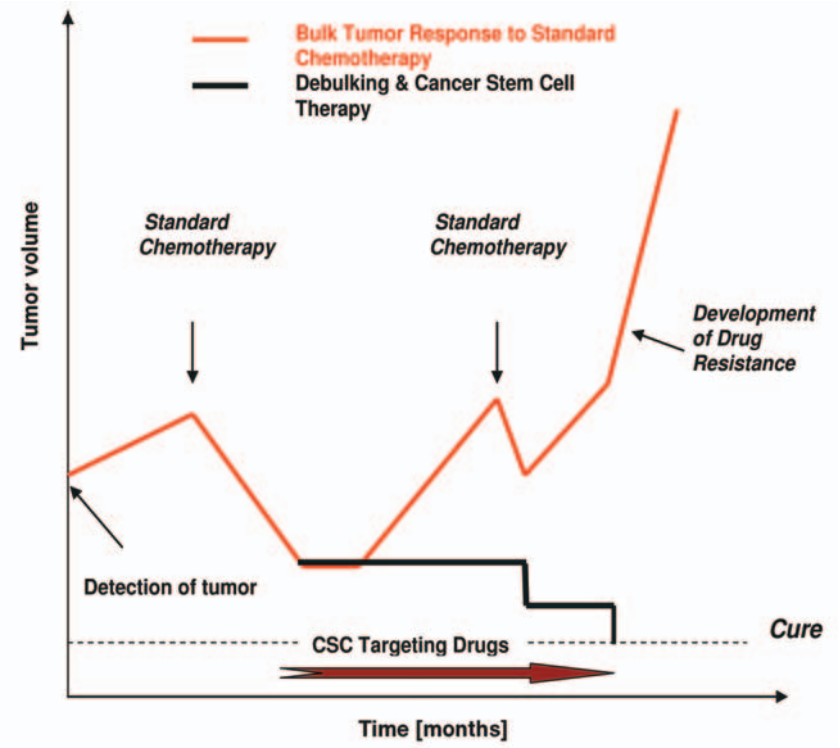

Figure 2. *Cancer treatment paradigms. The two curves demonstrate the typical courses of disease for more than 95% of tumors (red) if treated with currently available cytotoxic chemotherapy alone, and the potential impact of cancer stem cell-directed therapies (black) on the curability of cancer.*

effects (Figure 2). Due to the associated toxicities, cytotoxic agents are often given intermittently to allow recovery of normal tissues. However, this also can lead to tumor regrowth, and repopulation of tumor mass with drug resistant cancer cells. To date, only about 4% of advanced cancers requiring systemic treatment are curable (35).

"Molecularly targeted agents", *i.e.* drugs designed to inhibit specific pathways critical for the maintenance and proliferation of cancer cells, have raised much hope that advanced cancer could become a chronically treatable condition, instead of an acutely fatal disease. In fact, several new approved therapies inhibiting a disease-specific target have shown substantial clinical benefit. The best-known illustration is chronic myeloid leukemia (CML), a disease defined by constitutive activation of a tyrosine kinase as a result of a chromosomal translocation. The BCR-ABL kinase can be inhibited by the tyrosine kinase inhibitor, imatinib (1, 36). Imatinib has become the standard-of-care in newly diagnosed CML (37). However, despite producing higher response rates or even complete cytogenetic responses, imatinib has not led to a durable clinical response (38). Patients who achieve complete cytogenetic response will eventually relapse after discontinuation of imatinib or progress on the drug due to evolving resistance (2, 39, 40). Jones *et al.* (36) have recently demonstrated that although imatinib is remarkably active against differentiated CML progenitors, it has limited activity against primitive CML progenitors. Thus, the dramatic response to imatinib (38) is probably due to its activity against the differentiated CML progenitors which make up the bulk of leukemic cells. Since imatinib has only limited activity against primitive CML progenitors/stem cells, relapse is inevitable after discontinuation of the drug. This highlights the imperative of devising new approaches designed to both eradicate cancer stem cells as well as reduce tumor bulk in order to achieve cure (Figure 2).

The Human Tumor Stem Cell Assay (HTCA) and Individualized Patient Chemosensitivity Testing

There are multiple and potentially complementary strategies in development to target cancer stem cells (Figure 1). In an approach analogous to the culture and sensitivity assays used for the management of microbial infections, Salmon and Hamburger pioneered the concept of targeting cancer stem cells and concomitantly individualizing cancer therapy in the late 1970s (41). Single cell suspensions prepared from cancer patients' tumor tissues or effusions were cultured in multilayered soft agar and treated with anticancer drugs. This technique was termed "clonogenic assay" or "human tumor stem cell assay" (HTCA). Salmon and Hamburger based their hypothesis that tumor stem cells grow in soft agar on the fact that tumor cells in this semi solid matrix had a low plating efficiency (0.001 to 0.1%), similar to the extent of colony formation by bone marrow. Additionally, the homogenous appearance of tumor colonies as well as the staining of these colonies with cell type specific dyes was consistent with a stem cell hypothesis (41, 42). Moreover, the secondary colonies that arose when primary HTCA colonies were disaggregated and replated, were observed to be similar to the primary colonies in size, morphology, and culture requirements demonstrating stem cell-like self-renewal properties (43). The HTCA was subsequently used in a first approach to personalize cancer medicine by assessing a patient's prognosis and response to chemotherapy. E.g. Dittrich *et al.* (44) demonstrated that clonogenic growth in the HTCA is an independent prognostic factor for worse outcome in patients with ovarian cancer and most likely reflects the aggressiveness of tumors with a higher cancer stem cell fraction. Unlike the culture and sensitivity assays for antimicrobial drugs, the HTCA was found to be a better predictor for clinical resistance rather than sensitivity (41, 45) (Table I). Hamburger *et al.* performed the first HTCA chemosensitivity studies comparing *in vitro* drug activity to clinical response and found a correct prediction for drug resistance in ovarian carcinoma and myeloma of 100%, whereas the predictive value for drug sensitivity was only 89% (Table I). Similarly, when Fiebig *et al.* compared the response of a cohort of 66 assorted patient tumors in the HTCA *in vitro* to the clinical response, 62% of the comparisons for drug sensitivity, and 92% of the comparisons for drug resistance were accurate (Table I).

The first randomized clinical trial using the HTCA as a guidance for treatment decisions was published by Von Hoff *et al.* in 1983 (46). There were a total of 470 patients with 27 types of advanced metastatic cancers. The overall response rate in the assay-guided therapy was 25%, compared to 14% in the empiric treatment group ($p=0.0005$) (Table I) (46, 47). Subsequently, a second randomized clinical trial with 211 ovarian cancer patients showed that the response rate (22 %) was significantly higher in assay-guided therapy ($p=0.03$) compared to the empiric therapy group (3%) (47, 48). However, selecting chemotherapy for individual patients based on the *in vitro* drug sensitivity testing is not currently recommended outside of the clinical trial setting (41). Indirectly, these data demonstrate that a fraction of extremely therapy-resistant cancer cells exists, which appears to be at least partially composed of cancer stem cells.

Cancer Stem Cell-Directed Therapies

There are several mechanisms of drug resistance in cancer stem cells. Cancer stem cells primarily exist in G_0 phase and thus are resistant to cell cycle specific drugs such as 5-Fluouracil (5-FU) (49). Cancer stem cells also have a

Table I. *Predictive value of the HTCA in patient sensitivity testing.*

Study	Type	Cases	Tumor Type	Predictive Value	
				Sensitivity	Resistance
Salmon *et al.* (47) 1978	NR Retro/Pro	32	Multiple myeloma and ovarian cancers	91.67%	100%
Von Hoff *et al.* (46) 1983	NR/ Pro	1,145	Multiple tumor types: both hematologic malignancies and solid tumors	25%	NE
Von Hoff *et al.* (48) 1991	NR/ Pro	211	Ovarian cancer	28%	NE
Fiebig *et al.* (45) 2004	NR	66	Multiple tumor types: breast, lung, ovary, sarcoma, stomach, testicular, CNS, colon, head and neck, kidney, melanoma, esophagus, and pancreatic cancers	62%	92%

NR = Non randomized clinical trial, Pro = Prospective clinical trial, Retro = Retrospective clinical trial, NE = Not evaluated.

remarkable capacity for repair of DNA damage such as that caused by alkylating agents (50). Most importantly, cancer stem cells exhibit a high expression of the ATP-binding cassette (ABC) transporters especially BCRP (breast cancer resistance protein or ABCG-2) and MDR1 (multi-drug resistance-1, ABCB1, or P-glycoprotein) as part of their self-protection capabilities. Amongst the substrates of these transporters are anthracyclines, vinca alkaloids and other natural product derivatives (51). This mechanism of drug resistance has significant potential for development of stem cell-directed treatment regimens. Several ABC transporter inhibitors are currently under clinical investigation (Table II). Phase I/II clinical trials include *e.g.* combining of the novel potent P-glycoprotein inhibitor, Zosuquidar, with chemotherapy. It will be interesting to evaluate whether the combination of conventional cytotoxic debulking therapy and the novel strategy of resistance reversal can lead to a better outcome (52).

In addition, cancer stem cells also express higher levels of antiapoptotic proteins, especially the Bcl-2 family members. Oblimersen, a Bcl-2 antisense oligonucleotide, is also currently been studied in multiple cancers (53, 54) (Table II).

Targeting self-renewal pathways of cancer stem cells such as Notch, Wnt, and Hedgehog signaling is a third avenue under investigation. While these essential pathways are shared by normal stem cells, multiple studies in animal models have shown that interdicting these self-renewal pathways may selectively target cancer stem cells (18). Inhibitors of the Hedgehog pathway, such as cyclopamine (Table II), were identified and have a therapeutic window with remarkable activity against a wide range of human cancer cell lines and tumor xenografts including ovarian cancer (55), medulloblastoma (56), gastrointestinal neuroendocrine carcinomas (57), and prostate cancer (19). Similarly, gamma secretase inhibitors such as LY-411,575, which block Notch activation, were found to have activity in Kaposi's sarcoma (58), breast cancers (59) and melanoma (60).

Another possibility to target the self-renewal capacity of cancer stem cells is to force them into differentiation (61). Retinoic acid can induce differentiation in embryonic stem cells (62). All-trans retinoic acid (ATRA) has been used with great success in combination chemotherapy in acute promyelocytic leukemia (APL), a disease with a cure rate of more than 70%. This suggests that cancer stem cells are being successfully eliminated in this particular disease (63-65). Arsenic trioxide, an agent that may function through telomere targeting (described below), has recently been demonstrated to further improve the rate of cure in patients with APL (66, 67). Histone deacetylases (HDACs) regulate gene transcriptional activity. HDAC inhibitors can be used for epigenetic reprogramming of silenced genes and thus induce cell differentiation (Table II). The HDAC inhibitor, vorinostat (SAHA), has been found to have activity in multiple malignancies including leukemia (68), lymphoma (69), thyroid cancer (70), myeloma (71), and hepatocellular carcinoma (72). Thus, SAHA might have the potential for targeting CSCs and prove a good combination partner for debulking agents.

The stem cell niche (Figure 1), although not well defined at present, must also be considered as a CSC target. The nice is a specialized cellular environment that provides stem cells with the support needed for self-renewal (73). Vascular endothelial growth factor (VEGF) has recently been validated as a target for anti-cancer therapeutics with demonstrated improvement in outcomes in colon (74), lung (75) and breast cancer (76, 77) when bevacizumab (anti-VEGF antibody) has been combined with cytotoxic chemotherapy (Table II). Initially, the driving hypothesis for this approach was that tumors could not grow beyond a certain size without generating a new blood supply and therefore, inhibition of the blood supply would obliterate the malignancy, resulting in "normalization" of blood supply (78). More recently, a stem cell-related hypothesis has been proposed that would better explain the efficacy of bevacizumab. It has become

Table II. *Drugs with cancer stem cell targets.*

Drugs	Specific Targets	References
Zosuquidar	P-glycoprotein	Sandler *et al.*, 2004 (52)
Oblimersen	Bcl-2 antisense oligonucleotide	Bedikian AY *et al.*, 2006 (53) Badros AZ *et al.*, 2005 (54)
Cyclopamine	Hedgehog pathway	Chen X *et al.*, 2006 (55) Romer JT *et al.*, 2004 (56) Shida T *et al.*, 2006 (57) Karhadkar SS *et al.*, 2004 (19)
LY-411,575 Gamma secretase inhibitor	Notch pathway	Pece S *et al.*, 2004 (59) Nickoloff BJ *et al.*, 2005 (60) Lan K *et al.*, 2006 (58)
Retinoic acid (ATRA)	Differentiation	Sanz MA *et al.*, 2000 (63) Sanz MA *et al.*, 2004 (64) Sanz MA *et al.*, 1999 (65)
HDAC inhbitors *e.g.* SAHA	Differentiation Epigenetic reprograming	Jones LK *et al.*, 2005 (68) O'Connor OA *et al.*, 2005 (69) Luong QT *et al.*, 2006 (70) Yaccoby S *et al.*, 2006 (71) Ocker M *et al.*, 2005 (72)
Bevacizumab (Avastin)	Stem cell niche	Yang ZJ *et al.*, 2007 (79)
RHPS4	Telomerase (hTERT)	Cookson *et al.*, 2005 (85)
Cisplatin	Telomerase	Burger *et al.*, 1999 (87)
GRN163L	Telomerase (hTERC)	Dikmen *et al.*, 2005 (91)

ATRA (All-trans retinoic acid); HDAC (histone deacetylase); hTERT (human telomerase reverse transcriptase); hTERC (human telomerase RNA component).

apparent that cancer stem cells live in vascularized lacunae and that anti-VEGF therapy may destroy these niches and consequently improve outcome (79).

Finally, limitless proliferation is a key feature that defined stem cells and is accomplished by activation of the enzyme telomerase. Telomerase prevents telomere-associated replicative senescence and DNA-damage by adding telomeric repeat sequences at chromosomal ends. Over-expression of the catalytic subunit of telomerase, hTERT, has been found to promote stem cell mobilization, whereas short telomeres have been reported to cause stem cell failure (80, 81). Although telomerase is present in embryonic tissues, germ cells, adult stem cells, and tumor cells, a therapeutic window for targeting cancer might exist. CSCs may have shorter telomeres than normal stem cells and be more susceptible to loss of telomerase/telomere uncapping, thereby providing a promising therapeutic target which is discussed in more detail below (35).

Telomerase Inhibition and Cancer Stem Cell Targeting

When telomerase emerged as a cancer inhibitory target, our laboratory was the first to note that the standard agent cisplatin (CDDP) has telomerase inhibitory properties and that these effects might contribute to the curability of testicular germ cell tumors (TGCT) by CDDP (82-84). The fact that telomerase modulation is associated with the eradication of TGCTs, which contain undifferentiated pluripotent stem cells, supports telomeres and telomerase as cancer stem cell targets (Table II). We have recently created a unique pair of stable isogenic breast cancer cell lines, MCF-7 cells and mutant MCF-7 hTERT-expressing clones (85). In mutant hTERT MCF-7 cells, telomerase activity was drastically reduced and telomere length shortened to a critical size of 1.9Kb after 120 population doublings. In the HTCA, these MCF-7 clones were less clonogenic and formed much smaller colonies compared to control cells, demonstrating a function for telomerase in

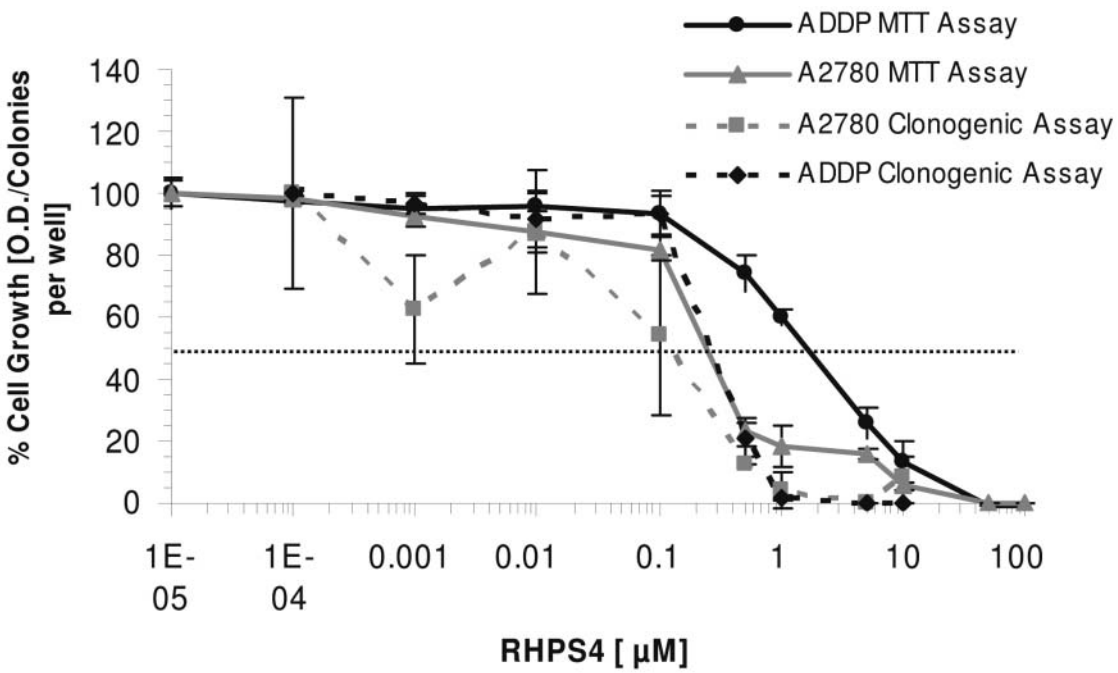

Figure 3. *Dose-effect curves for the treatment of A2780 and ADDP ovarian cancer cells with the telomere targeting agent RHPS4 in the MTT and HTCA assays. The proliferation of bulk tumor cells was analyzed by MTT assay (solid lines), and the proliferation of cancer stem cells by the HTCA (dashed lines). Effects are depicted as % of control growth (vehicle treated control = 100%). The horizontal punctuate line indicates 50% of cell growth inhibition and was used to delineate inhibitory concentrations 50% (IC_{50}). HTCA IC_{50} A2780 = 0.1 µM, ADDP = 0.25 µM; MTT IC_{50} A2780 = 0.25 µM, ADDP = 2.0 µM.*

clonogenic growth. Similar to genetic telomerase inhibition by mutation, the telomere targeting agent RHPS4, which specifically inhibits telomerase by displacing hTERT from the telomeres (85) was significantly more active in cell populations that grow in the HTCA than bulk MCF-7 cells assayed by using the methyltetrazolium (MTT) proliferation test. This observation was made in many other tumor cell lines including the epithelial ovarian cancer lines A2780 and ADDP for which data are depicted in Figure 3. ADDP is a cisplatin resistant subclone of A2780 (resistance factor 10x) (86) with up-regulated telomerase activity levels and elongated telomeres (87). This translates into a 25-times higher plating efficiency in the HTCA compared to parental A2780 cells, suggesting an expansion in cancer stem cells in the ADDP line. We further analyzed these cell lines with the side population (SP) method. The SP is enriched with stem/primitive progenitor cells and has the distinct capacity to efflux Hoechst 33342 dye due to the presence of ABC transporters (88-90). This leads to a unique appearance of SP cells by fluorescence-activated cell sorting (FACS). While the original epithelial ovarian cancer cell line A2780 exhibits no measurable SP phenotype, SP was detected in ADDP, a cisplatin resistant subclone, at a mean percentage of 0.03% (data not shown). Notably, while the telomere targeting agent RHPS4 is largely cross-resistant with cisplatin in effecting bulk tumor cell growth (resistance factor ~8, Figure 3), both A2780 and ADDP cells were equally sensitive to RHPS4 when grown in the HTCA (Figure 3). These data indicate that RHPS4 might be able to target cancer stem cells and overcome their resistance to standard cytotoxic agents (Table II). RHPS4 is currently undergoing preclinical toxicology evaluation and is scheduled for entry into phase I clinical trials. The first telomerase inhibitor in clinical development is GRN163L, a 13-mer antisense oligonucleotide N3' → P5' thio-phosphoramidate targeting the telomerase RNA component, which has also shown activity against clonogenic tumor cell growth (35, 91). Both, RHPS4 and GRN163L have potential as combination partners with cytotoxic debulking agents (Figures 1, 2). Moreover, novel strategies utilizing cisplatin-based regimens should be considered with the new insight that they target telomerase and thus inhibit cancer stem cell growth.

Future Perspectives and Importance of Cancer Stem Cell Therapies

More than three decades ago, the culturing of cancer stem cells from patient tumors and evaluating their response to anticancer drugs was a first attempt toward individualized cancer therapy (25, 41). The approach was unsuccessful because of the intrinsic resistance of cancer stem cells to

cytotoxic agents, the only available drugs at that time. Now, with a better understanding of the underlying molecular mechanisms and biology, we are able to rationally attack cancer stem cells (Figure 1). Most interestingly, combination chemotherapy regimens, that can cure tumors such as testicular cancers and APL, contain agents that in retrospect target crucial stem cell pathways, namely self-renewal and limitless proliferation. As summarized in Table II, multiple new compounds that target key cancer stem cell pathways have evolved from anticancer drug development efforts. However, clinical trials must be designed to examine cancer stem cell markers and effects of new agents on this rare cell population. For this purpose the HTCA should be revisited as a prognostic factor (44). We must further understand that an ultimate cure for cancer may require a multi-step approach. Initially, differentiated cancer cells that make up the bulk of the tumor could be treated with conventional chemotherapy, radiation, or surgery (Figures 1, 2). These strategies will not only alleviate patients' symptoms caused by the bulk tumor mass, but they may also mobilize cancer stem cells from their niches and render them more susceptible to therapy. Subsequently, when tumor burden is low, cancer stem cell-directed treatments should be initiated. It is possible that these treatments may require long-term administration in order to prevent relapses and recurrences (Figure 2). However, it is conceivable that short term administration of these agents may be beneficial as has been demonstrated with cisplatin based therapy in germ cell tumors.

Finally, individualized cancer therapies that are highly specific and effective will require the recognition of tumor cell heterogeneity and follow a multifaceted approach. Preclinical testing will be needed to optimize strategies and novel clinical trial designs must be implemented that will test the efficacy of this approach.

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References

1 Huff CA, Matsui W, Smith BD *et al*: The paradox of response and survival in cancer therapeutics. Blood *107*: 431-434, 2006.
2 McCormick F: New-age drug meets resistance. Nature *412*: 281-282, 2001.
3 Al-Hajj M, Becker MW, Wicha M *et al*: Therapeutic implications of cancer stem cells. Curr Opin Genet Dev *14*: 43-47, 2004.
4 Jordan CT: Targeting the most critical cells: approaching leukemia therapy as a problem in stem cell biology. Nat Clin Pract Oncol *2*: 224-225, 2005.
5 Abbott A: Cancer: the root of the problem. Nature *442*: 742-743, 2006.
6 Wikipedia tfe: Ernest McCulloch, 2006.
7 Becker AJ, Mc CE and Till JE: Cytological demonstration of the clonal nature of spleen colonies derived from transplanted mouse marrow cells. Nature *197*: 452-454, 1963.
8 Reya T, Morrison SJ, Clarke MF *et al*: Stem cells, cancer, and cancer stem cells. Nature *414*: 105-111, 2001.
9 Clevers H: Stem cells, asymmetric division and cancer. Nat Genet *37*: 1027-1028, 2005.
10 Caussinus E and Gonzalez C: Induction of tumor growth by altered stem-cell asymmetric division in Drosophila melanogaster. Nat Genet *37*: 1125-1129, 2005.
11 Scharenberg CW, Harkey MA and Torok-Storb B: The ABCG2 transporter is an efficient Hoechst 33342 efflux pump and is preferentially expressed by immature human hematopoietic progenitors. Blood *99*: 507-512, 2002.
12 Bruce WR and Van Der Gaag H: A quantitative assay for the number of murine lymphoma cells capable of proliferation *in vivo*. Nature *199*: 79-80, 1963.
13 Park CH, Bergsagel DE and McCulloch EA: Mouse myeloma tumor stem cells: a primary cell culture assay. J Natl Cancer Inst *46*: 411-422, 1971.
14 Bonnet D and Dick JE: Human acute myeloid leukemia is organized as a hierarchy that originates from a primitive hematopoietic cell. Nat Med *3*: 730-737, 1997.
15 Hope KJ, Jin L and Dick JE: Acute myeloid leukemia originates from a hierarchy of leukemic stem cell classes that differ in self-renewal capacity. Nat Immunol *5*: 738-743, 2004.
16 Liu S, Dontu G and Wicha MS: Mammary stem cells, self-renewal pathways, and carcinogenesis. Breast Cancer Res *7*: 86-95, 2005.
17 Dontu G, Jackson KW, McNicholas E *et al*: Role of Notch signaling in cell-fate determination of human mammary stem/progenitor cells. Breast Cancer Res *6*: R605-615, 2004.
18 Wicha MS, Liu S and Dontu G: Cancer stem cells: an old idea – a paradigm shift. Cancer Res *66*: 1883-1890; discussion 1895-1896, 2006.
19 Karhadkar SS, Bova GS, Abdallah N *et al*: Hedgehog signalling in prostate regeneration, neoplasia and metastasis. Nature *431*: 707-712, 2004.
20 Gailani MR, Stahle-Backdahl M, Leffell DJ *et al*: The role of the human homologue of Drosophila patched in sporadic basal cell carcinomas. Nat Genet *14*: 78-81, 1996.
21 Dievart A, Beaulieu N and Jolicoeur P: Involvement of Notch1 in the development of mouse mammary tumors. Oncogene *18*: 5973-5981, 1999.
22 Olsen CL, Hsu PP, Glienke J *et al*: Hedgehog-interacting protein is highly expressed in endothelial cells but down-regulated during angiogenesis and in several human tumors. BMC Cancer *4*: 43, 2004.
23 Fidler IJ and Hart IR: Biological diversity in metastatic neoplasms: origins and implications. Science *217*: 998-1003, 1982.
24 Heppner GH: Tumor heterogeneity. Cancer Res *44*: 2259-2265, 1984.
25 Hamburger AW and Salmon SE: Primary bioassay of human tumor stem cells. Science *197*: 461-463, 1977.
26 Al-Hajj M, Wicha MS, Benito-Hernandez A *et al*: Prospective identification of tumorigenic breast cancer cells. Proc Natl Acad Sci USA *100*: 3983-3988, 2003.
27 Singh SK, Hawkins C, Clarke ID *et al*: Identification of human brain tumour initiating cells. Nature *432*: 396-401, 2004.

28 Singh SK, Clarke ID, Hide T *et al*: Cancer stem cells in nervous system tumors. Oncogene *23*: 7267-7273, 2004.
29 Matsui W, Huff CA, Wang Q *et al*: Characterization of clonogenic multiple myeloma cells. Blood *103*: 2332-2336, 2004.
30 Collins AT, Berry PA, Hyde C *et al*: Prospective identification of tumorigenic prostate cancer stem cells. Cancer Res *65*: 10946-10951, 2005.
31 Kim CF, Jackson EL, Woolfenden AE *et al*: Identification of bronchioalveolar stem cells in normal lung and lung cancer. Cell *121*: 823-835, 2005.
32 Prince ME, Sivanandan R, Kaczorowski A *et al*: Identification of a subpopulation of cells with cancer stem cell properties in head and neck squamous cell carcinoma. Proc Natl Acad Sci USA *104*: 973-978, 2007.
33 Li C, Heidt DG, Dalerba P *et al*: Identification of pancreatic cancer stem cells. Cancer Res *67*: 1030-1037, 2007.
34 O'Brien CA, Pollett A, Gallinger S *et al*: A human colon cancer cell capable of initiating tumour growth in immunodeficient mice. Nature *445*: 106-110, 2007.
35 Burger AM: Highlights in experimental therapeutics. Cancer Lett *245*: 11-21, 2007.
36 Angstreich GR, Matsui W, Huff CA *et al*: Effects of imatinib and interferon on primitive chronic myeloid leukaemia progenitors. Br J Haematol *130*: 373-381, 2005.
37 NCCN: Chronic myelogenous leukemia clinical practice guidelines in oncology. Journal of the National Comprehensive Cancer Network *1*: 482-500, 2003.
38 O'Brien SG, Guilhot F, Larson RA *et al*: Imatinib compared with interferon and low-dose cytarabine for newly diagnosed chronic-phase chronic myeloid leukemia. N Engl J Med *348*: 994-1004, 2003.
39 Cortes J, O'Brien S and Kantarjian H: Discontinuation of imatinib therapy after achieving a molecular response. Blood *104*: 2204-2205, 2004.
40 Mauro MJ DB, Kuyl J, Kurilik G and Maziarz RT: Increasing levels of detectable leukemia in imatinib treated CML patients with previously undetectable or very low levels of BCR-ABL. Proc ASCO *22*: 569, 2003.
41 Salmon SE, Hamburger AW, Soehnlen B *et al*: Quantitation of differential sensitivity of human-tumor stem cells to anticancer drugs. N Engl J Med *298*: 1321-1327, 1978.
42 Schrag D, Garewal HS, Burstein HJ *et al*: American Society of Clinical Oncology Technology Assessment: chemotherapy sensitivity and resistance assays. J Clin Oncol *22*: 3631-3638, 2004.
43 Buick RN and MacKillop WJ: Measurement of self-renewal in culture of clonogenic cells from human ovarian carcinoma. Br J Cancer *44*: 349-355, 1981.
44 Dittrich C, Dittrich E, Sevelda P *et al*: Clonogenic growth *in vitro*: an independent biologic prognostic factor in ovarian carcinoma. J Clin Oncol *9*: 381-388, 1991.
45 Fiebig HH, Maier A and Burger AM: Clonogenic assay with established human tumour xenografts: correlation of *in vitro* to *in vivo* activity as a basis for anticancer drug discovery. Eur J Cancer *40*: 802-820, 2004.
46 Von Hoff DD, Clark GM, Stogdill BJ *et al*: Prospective clinical trial of a human tumor cloning system. Cancer Res *43*: 1926-1931, 1983.
47 Samson DJ, Seidenfeld J, Ziegler K *et al*: Chemotherapy sensitivity and resistance assays: a systematic review. J Clin Oncol *22*: 3618-3630, 2004.
48 Von Hoff DD, Kronmal R, Salmon SE *et al*: A Southwest Oncology Group study on the use of a human tumor cloning assay for predicting response in patients with ovarian cancer. Cancer *67*: 20-27, 1991.
49 Venezia TA, Merchant AA, Ramos CA *et al*: Molecular signatures of proliferation and quiescence in hematopoietic stem cells. PLoS Biol *2*: e301, 2004.
50 Bao S, Wu Q, McLendon RE *et al*: Glioma stem cells promote radioresistance by preferential activation of the DNA damage response. Nature *444*: 756-760, 2006.
51 Donnenberg VS and Donnenberg AD: Multiple drug resistance in cancer revisited: the cancer stem cell hypothesis. J Clin Pharmacol *45*: 872-877, 2005.
52 Sandler A, Gordon M, De Alwis DP *et al*: A Phase I trial of a potent P-glycoprotein inhibitor, zosuquidar trihydrochloride (LY335979), administered intravenously in combination with doxorubicin in patients with advanced malignancy. Clin Cancer Res *10*: 3265-3272, 2004.
53 Bedikian AY, Millward M, Pehamberger H *et al*: Bcl-2 antisense (oblimersen sodium) plus dacarbazine in patients with advanced melanoma: the Oblimersen Melanoma Study Group. J Clin Oncol *24*: 4738-4745, 2006.
54 Badros AZ, Goloubeva O, Rapoport AP *et al*: Phase II study of G3139, a Bcl-2 antisense oligonucleotide, in combination with dexamethasone and thalidomide in relapsed multiple myeloma patients. J Clin Oncol *23*: 4089-4099, 2005.
55 Chen X, Horiuchi A, Kikuchi N *et al*: Hedgehog signal pathway is activated in ovarian carcinomas, correlating with cell proliferation: It's inhibition leads to growth suppression and apoptosis. Cancer Sci *98*: 68-76, 2007.
56 Romer JT, Kimura H, Magdaleno S *et al*: Suppression of the Shh pathway using a small molecule inhibitor eliminates medulloblastoma in Ptc1(+/–)p53(–/–) mice. Cancer Cell *6*: 229-240, 2004.
57 Shida T, Furuya M, Nikaido T *et al*: Sonic Hedgehog-Gli1 Signaling Pathway Might Become an Effective Therapeutic Target in Gastrointestinal Neuroendocrine Carcinomas. Cancer Biol Ther *5*: 1530-1538, 2006.
58 Lan K, Choudhuri T, Murakami M *et al*: Intracellular activated Notch1 is critical for proliferation of Kaposi's sarcoma-associated herpesvirus-associated B-lymphoma cell lines *in vitro*. J Virol *80*: 6411-6419, 2006.
59 Pece S, Serresi M, Santolini E *et al*: Loss of negative regulation by Numb over Notch is relevant to human breast carcinogenesis. J Cell Biol *167*: 215-221, 2004.
60 Nickoloff BJ, Hendrix MJ, Pollock PM *et al*: Notch and NOXA-related pathways in melanoma cells. J Investig Dermatol Symp Proc *10*: 95-104, 2005.
61 Massard C, Deutsch E and Soria JC: Tumour stem cell-targeted treatment: elimination or differentiation. Ann Oncol *17*: 1620-1624, 2006.
62 Motohashi T, Aoki H, Chiba K *et al*: Multipotent cell fate of neural crest-like cells derived from embryonic stem cells. Stem Cells *25*: 402-410, 2007.
63 Sanz MA, Lo Coco F, Martin G *et al*: Definition of relapse risk and role of nonanthracycline drugs for consolidation in patients with acute promyelocytic leukemia: a joint study of the PETHEMA and GIMEMA cooperative groups. Blood *96*: 1247-1253, 2000.

64 Sanz MA, Martin G, Gonzalez M *et al*: Risk-adapted treatment of acute promyelocytic leukemia with all-trans-retinoic acid and anthracycline monochemotherapy: a multicenter study by the PETHEMA group. Blood *103*: 1237-1243, 2004.

65 Sanz MA, Martin G, Rayon C *et al*: A modified AIDA protocol with anthracycline-based consolidation results in high antileukemic efficacy and reduced toxicity in newly diagnosed PML/RARalpha-positive acute promyelocytic leukemia. PETHEMA group. Blood *94*: 3015-3021, 1999.

66 Mathews V, George B, Lakshmi KM *et al*: Single-agent arsenic trioxide in the treatment of newly diagnosed acute promyelocytic leukemia: durable remissions with minimal toxicity. Blood *107*: 2627-2632, 2006.

67 Estey E, Garcia-Manero G, Ferrajoli A *et al*: Use of all-trans retinoic acid plus arsenic trioxide as an alternative to chemotherapy in untreated acute promyelocytic leukemia. Blood *107*: 3469-3473, 2006.

68 Jones LK and Saha V: Philadelphia positive acute lymphoblastic leukaemia of childhood. Br J Haematol *130*: 489-500, 2005.

69 O'Connor OA: Developing new drugs for the treatment of lymphoma. Eur J Haematol 66 (*Suppl)*: 150-158, 2005.

70 Luong QT, O'Kelly J, Braunstein GD *et al*: Antitumor activity of suberoylanilide hydroxamic acid against thyroid cancer cell lines *in vitro* and *in vivo*. Clin Cancer Res *12*: 5570-5577, 2006.

71 Yaccoby S, Wezeman MJ, Zangari M *et al*: Inhibitory effects of osteoblasts and increased bone formation on myeloma in novel culture systems and a myelomatous mouse model. Haematologica *91*: 192-199, 2006.

72 Ocker M, Alajati A, Ganslmayer M *et al*: The histone-deacetylase inhibitor SAHA potentiates proapoptotic effects of 5-fluorouracil and irinotecan in hepatoma cells. J Cancer Res Clin Oncol *131*: 385-394, 2005.

73 Xie T and Spradling AC: A niche maintaining germ line stem cells in the Drosophila ovary. Science *290*: 328-330, 2000.

74 Hurwitz H, Fehrenbacher L, Novotny W *et al*: Bevacizumab plus irinotecan, fluorouracil, and leucovorin for metastatic colorectal cancer. N Engl J Med *350*: 2335-2342, 2004.

75 Sandler A, Gray R, Perry MC *et al*: Paclitaxel-carboplatin alone or with bevacizumab for non-small-cell lung cancer. N Engl J Med *355*: 2542-2550, 2006.

76 Wedam SB, Low JA, Yang SX *et al*: Antiangiogenic and antitumor effects of bevacizumab in patients with inflammatory and locally advanced breast cancer. J Clin Oncol *24*: 769-777, 2006.

77 Miller KD, Chap LI, Holmes FA *et al*: Randomized phase III trial of capecitabine compared with bevacizumab plus capecitabine in patients with previously treated metastatic breast cancer. J Clin Oncol *23*: 792-799, 2005.

78 Jain RK: Normalization of tumor vasculature: an emerging concept in antiangiogenic therapy. Science *307*: 58-62, 2005.

79 Yang ZJ and Wechsler-Reya RJ: Hit 'em where they live: targeting the cancer stem cell niche. Cancer Cell *11*: 3-5, 2007.

80 Sarin KY, Cheung P, Gilison D *et al*: Conditional telomerase induction causes proliferation of hair follicle stem cells. Nature *436*: 1048-1052, 2005.

81 Hao LY, Armanios M, Strong MA *et al*: Short telomeres, even in the presence of telomerase, limit tissue renewal capacity. Cell *123*: 1121-1131, 2005.

82 Burger AM: Telomerase in cancer diagnosis and therapy. BioDrugs *12*: 413-422, 1999.

83 Burger AM, Double JA and Newell DR: Inhibition of telomerase activity by cisplatin in human testicular cancer cells. Eur J Cancer *33*: 638-644, 1997.

84 Schrader M, Burger AM, Muller M *et al*: Quantification of human telomerase reverse transcriptase mRNA in testicular germ cell tumors by quantitative fluorescence real-time RT-PCR. Oncol Rep *9*: 1097-1105, 2002.

85 Cookson JC, Dai F, Smith V *et al*: Pharmacodynamics of the G-quadruplex-stabilizing telomerase inhibitor 3,11-difluoro-6,8,13-trimethyl-8H-quino[4,3,2-kl]acridinium methosulfate (RHPS4) *in vitro*: activity in human tumor cells correlates with telomere length and can be enhanced, or antagonized, with cytotoxic agents. Mol Pharmacol *68*: 1551-1558, 2005.

86 Aird RE, Cummings J, Ritchie AA *et al*: *In vitro* and *in vivo* activity and cross resistance profiles of novel ruthenium (II) organometallic arene complexes in human ovarian cancer. Br J Cancer *86*: 1652-1657, 2002.

87 Burger AMaHP: Telomerase in germ cell tumours: inhibition of telomerase activity after cisplatin-based therapy. Germ Cell Tumours *IV*: 73-80, 1998.

88 Lin KK and Goodell MA: Purification of hematopoietic stem cells using the side population. Methods Enzymol *420*: 255-264, 2006.

89 Hirschmann-Jax C, Foster AE, Wulf GG *et al*: A distinct "side population" of cells with high drug efflux capacity in human tumor cells. Proc Natl Acad Sci USA 101: 14228-14233, 2004.

90 Goodell MA, McKinney-Freeman S and Camargo FD: Isolation and characterization of side population cells. Methods Mol Biol *290*: 343-352, 2005.

91 Dikmen ZG, Gellert GC, Jackson S *et al*: *In vivo* inhibition of lung cancer by GRN163L: a novel human telomerase inhibitor. Cancer Res *65*: 7866-7873, 2005.

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