REVIEW

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Cancer stem cells as targets for cancer therapy: selected cancers as examples

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Abstract

It is becoming increasingly evident that cancer constitutes a group of diseases involving altered stem-cell maturation/differentiation and the disturbance of regenerative processes. The observed malignant transformation is merely a symptom of normal differentiation processes gone astray rather than the primary event. This review focuses on the role of cancer stem cells (CSCs) in three common but also relatively under-investigated cancers: head and neck, ovarian, and testicular cancer. For didactic purpose, the physiology of stem cells is first introduced using hematopoietic and mesenchymal stem cells as examples. This is followed by a discussion of the (possible) role of CSCs in head and neck, ovarian, and testicular cancer. Aside from basic information about the pathophysiology of these cancers, current research results focused on the discovery of molecular markers specific to these cancers are also discussed. The last part of the review is largely dedicated to signaling pathways active within various normal and CSC types (e.g. Nanog, Nestin, Notch1, Notch2, Oct3 and 4, Wnt). Different elements of these pathways are also discussed in the context of therapeutic opportunities for the development of targeted therapies aimed at CSCs. Finally, alternative targeted anticancer therapies arising from recently identified molecules with cancer-(semi-)selective capabilities (e.g. apoptin, Brevinin-2R) are considered.

Key words: Nanog, Nestin, Notch1, Notch2, Oct4, Wnt.

Abbreviations: BCC – basal cell cancer, BM – bone marrow, BMP – bone morphogenetic protein, CHK2 – checkpoint kinase2, CSC – cancer stem cell, CXCL12 – chemokine (C-X-C-motif) ligand 12, ES – embryonic stem, Flk-1 – fetal liver kinase-1, GCT – germ cell tumor, GPI – guanyl phosphatidylinositol, HH/PTCH – hedgehog/patched pathway, HSC – hematopoietic stem cells, IGCNU – intratubular germ cell neoplasia unclassified, KDR – kinase insert domain-containing receptor, LRP5/6 – low-density lipoprotein receptor-related protein 5/6, MIS – Müllerian inhibiting substance, MSC – mesenchymal stem cell, OGCT – ovarian GCT, OSCC – oral squamous cell carcinoma, PGC – primordial germ cell, ROS – reactive oxygen species, Sca-1 – stem cell antigen-1, SCF-1 – stem cell factor-1, SHH – sonic hedgehog, SMO – smoothened, SP – side population, STAT3 – signal transducer and activator of transcription 3, TGCT – testicular GCTs, VEGFR2 – vascular endothelial growth factor receptor 2, VCAM – vascular cell adhesion molecule, FCS – fetal calf serum.

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INTRODUCTION: ADULT STEM-CELL BIOLOGY, HEMATOPOIETIC AND MESENCHYMAL STEM CELLS AS EXAMPLES

Stem cells are increasingly becoming the focus of various areas of biomedical research. They are pluripotent, clonogenic, and self-renewing, with a plasticity to differentiate into the cell types of the particular tissue in which they reside and often to trans-differentiate [51, 73]. The trans-differentiation potential of stem cells and their capacity for tissue renewal and damage repair bring them into the focus of interest of biotechnologists and clinicians [110, 116].

While the field expands rapidly, so far stem cells are still largely defined by their capacity to (re-)colonize a given tissue niche, or initiate tumor growth (cancer stem cells), as only few molecular markers of stemness exist. Among them, the transcription factor Oct 3/4 (POU5F1/Oct 4) is considered as one of the best indicators of stemness capacity [39]. Its expression in nonmalignant cells is restricted to the pluripotent cells in the embryo and germ cells. Oct 3/4 is also a reliable marker in germ cell tumor diagnostics. It is expressed in the lesions that may initiate gonadoblastoma and carcinoma in situ, as well as in invasive embryonal carcinoma and seminomas (see below).

The same qualities that make stem cells valuable in regenerative medicine, tissue engineering, and biotechnology also carry dangers to the host organism. Mutations affecting their differentiation potential combined with the failure to control their unlimited proliferative capacity may transform them into "cancer stem cells" (CSCs). While this review is dedicated to head and neck, ovarian, and testicular CSCs, we provide below, as an introduction, some basic information about selected normal adult stem-cell populations.

Hematopoietic stem cells

Hematopoietic stem cells (HSCs) are the best-studied adult stem cells thus far and they were the first stem cells used in the clinic, thereby serving as an excellent vehicle for an introduction to stem-cell biology. HSCs are mainly located in the bone marrow (BM) and they are a classical example of multipotent adult stem cells, able to generate all cells of the blood and the immune system [2, 144, 153]. A small percentage of HSCs circulates in and can be isolated from the blood [178]. Lineage-specific differentiation of HSCs is regulated by various cytokines and growth factors which activate several signaling pathways (bone morphogenetic protein (BMP)-, Wnt-, and Notch-pathways) [27]. The sialomucin cell-surface marker CD34 is an important marker for HSCs and some other populations of adult stem cells. CD34 also appears to have a significant role in early hematopoiesis [164]. HSCs are hematopoietic lineage marker negative and thus do not express markers present on progenitor cells committed to a specific differentiation pathway. In mice, HSCs are c-Kit$^+$, Thy-1^{lo}, and stem-cell antigen (Sca)-1$^+$ [77, 171], and a single transplanted cell from this cell population can give rise to life-long hematopoiesis [130, 160]. Similarly, human HSCs are CD34$^+$, Thy-1$^+$ and c-Kit$^+$ [18].

An important lineage marker to exclude for HSCs when harvesting is CD45, a type I transmembrane molecule found on the cell surface of all nucleated hematopoietic cells and progenitors [168]. CD45 has important functions during immunological processes and for differentiation into various hematopoietic cell lineages [136]. For therapeutic intervention, HSCs from BM can be mobilized into the blood stream by administration of granulocyte colony-stimulating factor or granulocyte-macrophage colony-stimulating factor [119]. Human HSCs (CD34$^+$/CD133$^+$/VEGFR3$^+$) can also be isolated from umbilical cord blood. They express the 120-kDa 5-transmembrane-domain glycoprotein and stem-cell surface marker CD133 [111, 170, 181] and can differentiate into mature endothelial cells [134].

The c-Kit receptor belongs to subclass III of the tyrosine kinase receptor family with five immunoglobulin-like extracellular domains [142]. Stem cell factor (SCF) is the ligand for the c-Kit receptor [177] and mediates c-Kit receptor dimerization and activation [142]. C-Kit has an essential function for constitutive hematopoiesis and self-renewal of progenitor cells [126]. Within the human hematopoietic system, 70% of CD34$^+$ cells in BM express c-Kit protein, and this includes lineage-restricted hematopoietic progenitor cells [11, 132] and precursor cells capable of establishing long-term *in vitro* hematopoiesis [154]. The 18-kDa protein Sca-1 (Ly-6A/E) is GPI-anchored to the cell membrane and serves as an early marker for multipotent murine HSCs [161, 172]. Sca-1$^+$ HSCs can be isolated from adult BM, peripheral blood, and spleen [85, 115, 117, 161, 162, 179]. Sca-1 is also present in several non-hematopoietic tissues [173] and has been described in multipotent stem cells in the connective tissue of skeletal muscle [44]. Endothelial precursor cells and HSCs share the markers CD34, Tie-2, and fetal liver kinase (Flk)-1, which suggests a common ancestral stem cell population in the BM [10].

The Flk-1 is a 200- to 230-kDa protein involved in vasculogenesis and, together with CD133, constitutes a marker for endothelial progenitor cells [148]. Murine Flk-1 is also known as vascular endothelial growth factor receptor 2 (VEGFR2), and the kinase insert domain--containing receptor (KDR) is the human homolog. CD34$^+$ BM-derived cells have been demonstrated to differentiate into CD31$^+$ Tie-2 receptor$^+$ endothelial cells, incorporating acetylated LDL and producing nitric oxide in response to VEGF [10, 151]. Human CD34$^+$ KDR$^+$ BM-derived stem cells comprise HSCs [185], endothelial precursors [134], and hemangioblasts [135]. Osteoblasts located in the BM are niche cells mediating quiescence of the HSCs by secreting angiopoietin-1, which binds to the Tie-2 surface receptor on HSCs [9]. The perivascular site serves as another niche for HSCs and reveals local expression of the chemokine CXCL12 [157]. The expres-

sion of both the CXCL12 chemokine receptor CXCR4 and CD44 on HSCs may allow the homing of these cells to different niches [2].

Mesenchymal stem cells

Apart from HSCs, BM also harbors mesenchymal stem cells (MSCs). These cells are negative for the HSC markers CD14 and CD34 and for the HSC exclusion marker CD45. MSCs are present in low numbers in the circulation and in tissues [52]. Only 0.01–0.001% of the mononuclear cells isolated from BM have MSC properties, but their multipotency, proliferative capacity in culture, and ability to recruit to sites of organ injury make them attractive therapeutic targets. STRO1, CD105 (endoglin, gp160), CD73, CD166, CD146 (MCAM, MUC18, A32 antigen, S-endo-1), and SSEA-4 are markers for MSCs and, more recently, this list has been extended to include CD145, CD49a, CD106 (VCAM), and CD90 (Thy-1) [14, 23, 62, 139].

STRO1 is a cell surface antigen on stromal BM cells [155] and serves as a valuable marker for the identification, isolation, and functional characterization of clonogenic human BM stromal cell precursors. $STRO1^+$ MSC can differentiate into multiple mesenchymal lineages, including hematopoiesis-supportive stromal cells, pericytes, adipocytes, myofibroblasts, myocytes, cardiomyocytes, osteoblasts, chondrocytes, and neurons [48, 62, 131, 139, 186]. The MSC surface markers CD146 and CD105 are also expressed on endothelial precursor cells, which possess characteristics similar to those of pericytes [17, 169]. In addition to the expression of the Ca^{2+}-independent cell adhesion molecule CD146, BM and dental pulp MSC express α-smooth muscle actin and their location in the perivascular space may suggest that these MSCs are related to pericytes [152]. CD146 is also expressed in human endothelial cells isolated from cord blood and was shown to be involved in endothelial signaling [8]. MSCs may engraft into various organs and, when injected into the bloodstream, settle at sites of injury [150]. MSCs have been used in various therapies, including the treatment of hemophilia, osteogenesis imperfecta, and cartilage lesions and to accelerate hematopoiesis recovery following chemo/radiotherapy, to prevent scarring after myocardial infarction, and to treat neural lesions following stroke or trauma [75, 150, 176]. Because of the promising therapeutic applications of these multipotent adult stem cells, a number of clinical trials involving MSCs are currently under way (see: http://ncrm.peerlis.com/ncrm/content.aspx?id=17&displaypageid=29, http://ora.ra.cwru.edu/stemcellcenter/research/Clinical%20Trials.htm).

ORAL SQUAMOUS CELL CARCINOMA

In maxillofacial surgery, patients with oral squamous cell carcinoma (OSCC) or basal cell cancer (BCC) of the head and neck represent the majority of oncological clinical cases worldwide. Although CSCs are likely to play a role in the cause of these cancers, there have been no research reports proving the successful isolation of specific CSCs from BCC or OSCC lesions to date. Research reports have used immunohistological studies, investigations on the gene transcript level, and micro-morphological data to demonstrate or refute the CSC hypothesis in these carcinomas. Thus we focus below on recent advances and the ongoing discussion regarding the role of CSCs in the development of head and neck cancers.

OSCC is the sixth most common malignant tumor and its incidence is increasing worldwide. Several models have been proposed to elucidate risk factors, such as smoking and alcoholic beverages. Genetic and epidemiological markers are involved in the generation and progress of OSCC [26, 94]. According to the multi-step carcinogenesis concept, OSCC develops in a series of more or less defined intermediate stages through the accumulation of molecular changes and progression from a pre-cancerous leukoplakia to pre-invasive (erythroplakia) and finally to the invasive cancer disease with lympho-nodal outspread and severe local destruction [122, 137]. The overall five-year survival rate in patients with OSCC is still lower than 50% [13, 45]. According to histopathological grading, OSCC displays variable metastatic potentials, and highly differentiated OSCC (G1) displays slow growth and destruction to adjacent muscle and the jawbone. By contrast, high invasiveness, early spread to regional and cervical lymph nodes, and poor prognosis are hallmarks of poorly differentiated OSCC. OSCC frequently shows local recurrence after the initial surgical or radiological treatment at the primary site and, unfortunately, even after complete resection (R0-treatment) [13, 45]. Despite all attempts, no significant progress has been achieved by combining surgery and radiotherapy, as "traditional" ways of treatment, with chemotherapy (neo-adjuvant strategies). Furthermore, there are no standardized protocols or common therapeutic strategies currently available, and the identification of relevant cellular and molecular markers primarily involved in oral pre-cancerous lesions and OSCC is imperative.

The treatment strategies of OSCC are based on the idea that oral cancer is generated by a population of cells of equal proliferative and aggressive potential [37]. More recent reports suggest that the CSC hypothesis may likely also apply to OSCC [37, 68]. There are various possible scenarios by which OSCC could originate from stem cells: (a) it develops directly from malignant stem cells or (b) a tumorigenic potential is promoted in the supra-basal compartment when differentiating cells deviate from their predetermined differentiation route and transform into malignant intra-epithelial cells. The latter possibility is supported by the existence of the Tis-stage (carcinoma *in situ*) with the presence of typical malignant intraepithelial cells and an intact basal membrane. In Tis-lesions, malignant potential seems to develop in the stratum spinosum, but not in the stratum basale of the squamous epithelium. The dedifferentia-

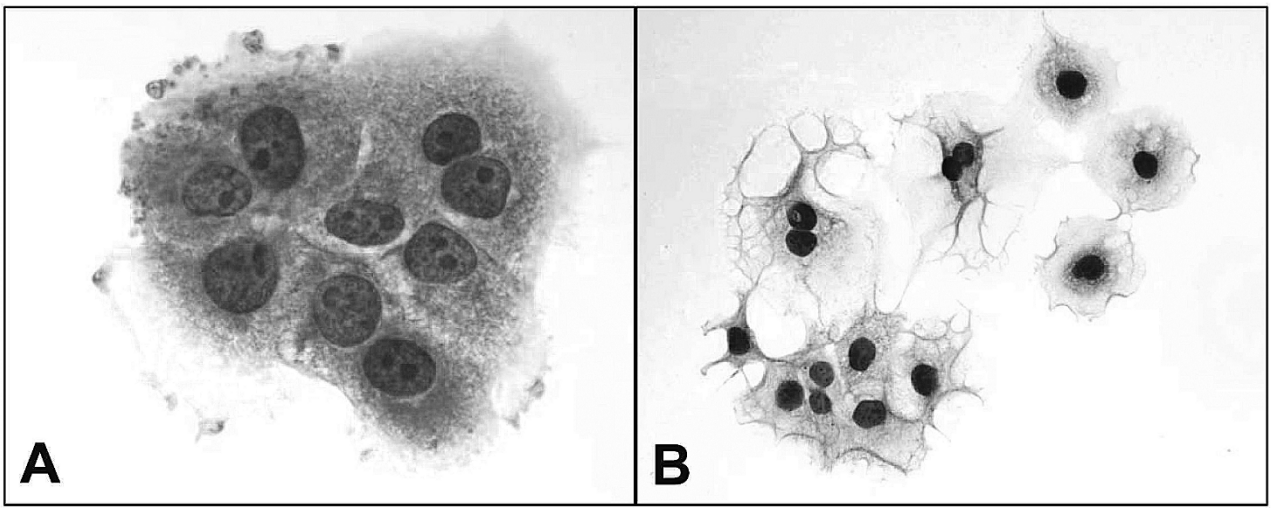

Fig. 1. Possible cancer stem cell populations within Head and Neck cancers. **A** – cell line BHY, initial holoclone formation with typical polygonal cells and dense intercellular contacts (40×). The cells are immunostained for CD97 and show strong cytoplasmatic DAB-brown staining (monoclonal antibody MEM180, 1:100, ABC-method). **B** – BHY cells with spider-like shapes cultured at low density fail to form holoclones. CD97 is only detectable in the perinuclear cytoplasma. Short tandem repeat-analysis (DNA-fingerprint) could prove the identical genetic profile as compared with holoclone cells (20×).

tion cascade in OSCC is typically associated with p53--mutations and loss of DNA-repair mechanisms [25, 175]. In addition, oncogene activation, tumor suppressor gene inactivation (i.e. CD82), and the downregulation of cell-cell contact proteins, such as E-cadherin and β-catenin, were described in OSCC [32, 54, 78, 92, 96, 113, 182]. OSCC is derived from malignant keratinocytes and their corresponding malignant stem cells may be found in the stratum basale of the oral and dermal epithelium, which has been identified in immunohistochemical studies to harbor epithelial stem cells. The highest numbers of stem cells are located adjacent to the dermal papillas and the basal membrane.

Epithelial stem cells express β1-integrin and cytokeratin 15 [102]. Several factors are involved in epidermal cell-cycle control and the regulation of normal differentiation pathways in keratinocyte formation [102]. Notch genes encode large single transmembrane proteins and are crucial for normal epidermal integrity and function. In the skin and oral mucosa, Notch1 and Notch2 signaling pathways induce and control the differentiation of keratinocytes, but their tissue localization suggests specific functions for Notch2, exclusively present in the basal epithelial layer, and Notch1, which is present in all epidermal layers. In skin, Notch1 and Notch2 may act as tumor suppressors and regulate stem-cell maintenance, proliferation, and apoptosis [53, 127, 128].

BCC is the most common form of skin cancer and develops from basal epithelial cells of the skin which become malignant upon prolonged sunlight and/or UV--light exposure. BCC is the result of a long-term process and this carcinoma usually grows much more slowly than squamous cell cancer or malignant melanoma. BCC is believed to be an epidermal stem cell-derived carcinoma of the skin. For BCC, the increase in Sonic hedgehog (SHH) signal transfer and activation of the Wnt pathway were found to be the molecular cause for malignant transformation [128]. This imbalance is strongly connected to Notch deficiency and Notch1 and Notch2 expression are reduced in BCC [128]. Animal experiments showed that deletion of the Notch gene can lead to BCC in mice [123, 140, 167] and UV-light exposure triggered malignant basal cell transformation, especially in Notch1-defient skin areas [128]. BCC has an extremely low metastatic potential and is therefore considered a "semi-malignant" tumor. However, once ulcerated, BCC shows aggressive growth and deep local tissue invasion.

Searching for stem-cell populations

Established OSCC cell lines may contain a small stem-cell fraction with high regenerative potential. When cultured at low densities, OSCC cell lines can generate cell aggregates with morphological and clonal features distinct from those of the rest of the tumor cells. These $CD44^+$ subpopulations vary in their morphological features, display different growth potential for each clonal subtype, and may be considered stem cell-like populations [68]. When cultured at low density in DMEM-F12 plus 10% FCS, fibroblast-shaped BHY--cell lines derived from a highly differentiated OSCC will group into small clusters of cells with an epithelial phenotype and limited mitogenic activity (Mustafa, unpublished data) (Fig. 1). Currently, the source of the CSC population in BHY remains to be determined, this largely due to the lack of stem-cell markers for head and neck cancers. One promising candidate molecule is the EGF-7TM protein CD97. CD97 is highly expressed in BM cells, undifferentiated thyroid carcinoma, and dedifferentiated (G3) OSCC [12, 47, 64, 97, 98, 120]. This surface molecule co-localizes within the basal cell layer

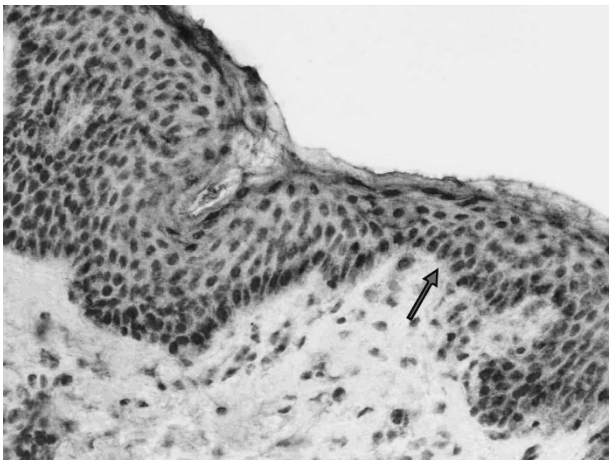

Fig. 2. The presence of tissue committed stem cells within human skin. CD97-immunohistochemistry of a non-pathological human skin sample (MEM180 monoclonal antibody, ABC-method, DAB--labeling). The basal epithelial cells express CD97. CD97 is not detectable in the stratum spinosum and the stratum corneum (20×).

of the oral squamous epithelium and its derivatives (Fig. 2). It is currently not known whether $CD97^+$ β1--integrin-positive cells express other known stem-like markers or possess higher proliferative potential, as would be expected from stem/progenitor cells. The identification of specific stem cell-like markers for OSCC is most definitely challenging and requires the combined approach of high-end cell-separation techniques, such as laser dissection of cells, single-cell Q-RT-PCR, and DNA-fingerprinting [79, 149].

OVARIAN CANCER: PLURIPOTENCY AND PUTATIVE CSCs

Somatic stem cells contribute to normal tissue repair and regeneration, and it has been suggested that multipotent somatic stem cells in the ovary have the potential to regulate surface epithelium repair after ovulatory rupture. Furthermore, preliminary observations indicate the existence of proliferative germ cells sustaining oocyte- and folliculogenesis in the post-natal ovary in the mouse [82], and the surface epithelium of adult women is a source of oocyte and granulosa cells *in vitro* [87], further indicating that the ovarian tunica albuginea contains stem cells. Ovarian somatic stem cells within this epithelial layer are expected to divide asymmetrically, yielding both a daughter cell that proceeds to terminal differentiation and an undifferentiated self-copy. Repeated asymmetric self-renewal sets the stage for somatic stem cells and their immediate progenitors to accrue mutations over time, which ultimately can lead to their transformation into CSCs and malignant progression. Inflammatory mediators and reactive oxidants generated during the ovulatory process and healing of the ovulatory wound [121] may be important contributors to the carcinogenic dedifferentiation of ovarian surface epithelial cells inducing DNA strand breaks and oxidative base (8-oxoguanine) damage [118]. A genetically altered stem or progenitor cell with damaged DNA, but not committed to death, could therefore give rise to a transformed phenotype that is propagated upon healing of the ovulatory wound.

Epithelial ovarian cancer is an extremely aggressive disease associated with a lack of early symptoms, rapid progression to peritoneal metastases, and poor prognosis. An initial improvement is observed when surgical removal of ovarian cancer tissues is followed by chemotherapy. Drug-resistant tumors, however, will recur in the vast majority of patients, leading to a five--year survival rate of less than 30% [29, 80].

As was recently demonstrated for breast and prostate cancer as well as other malignancies, including ovarian cancer, CSCs may not only survive chemotherapy, but chemotherapy may actively select for chemoresistant, highly aggressive CSCs [183]. Contributing factors are the preferential expression of chemoresistance molecules from the ATP-binding cassette (ABC) transporter family, such as the multidrug-resistance gene 1 and the breast cancer-resistance protein 1, which contribute to chemoresistance by cellular clearance of lipophilic drugs [61]. High expression of ABC-G2 has been observed in the side population (SP), which constitutes a cell population of undifferentiated cells [42, 95] that efficiently exclude Hoechst 33342, representing an enriched source of stem cells [31]. Cancer cells belonging to the SP are immature, poorly differentiated, and highly tumorigenic. Gene expression profiles indicate that cells in the SP-gated fraction express high levels of stem-cell markers and low levels of differentiation markers [66]. Recently, an SP has been identified in two distinct mouse ovarian cancer cell lines as well as in human ovarian cell lines and primary ascites tumor cells [165]. These SP cells show growth characteristics similar to CSCs [71] and are capable of self-renewal and asymmetric division in culture. *In vivo*, ovarian SP cells have the potential to initiate tumor growth more quickly and at lower cell numbers than non-SP ovarian cancer cells [165]. Moreover, cell clones with sustained clonogenic potential *in vitro* and tumorgenicity *in vivo*, even after serial transplantation in mice, have been identified in ovarian cancer [16]. The possible multipotency of such clones was further supported by the expression of three "stemness" markers: Oct4, Nanog, and Nestin [16]. More recently, Ferrandina et al. [50] identified the expression of CD133 antigen, considered to be a marker of undifferentiated cells, in a cellular subpopulation in a large variety of ovarian tissues. $CD133^+$ ovarian tumor cells exhibited higher clonogenic and more extensive proliferative potential than $CD133^-$ cells, which is similar to previous reports for other human malignancies [36, 156].

Due to the preferential expression of chemoresistance molecules, it is believed that CSCs may survive after chemotherapy and therefore induce relapse in several patients. It therefore seems intuitive that only the eradication of CSCs can lead to an effective cancer ther-

apy. Recent and preliminary studies have suggested Müllerian inhibiting substance (MIS) as a possible adjuvant to classical ovarian cancer chemotherapeutic regimes in targeting putative CSCs. In the embryonic urogenital ridge, an intact MIS signaling pathway is required for MIS responsiveness [184]. Similarly, a variety of ovarian CSCs, both SP and non-SP, possess functional receptors for MIS. Treatment with MIS is able to inhibit cell proliferation in both SP and non-SP cells, whereas classic chemotherapies inhibit mainly non-SP cells. Thus MIS is able to actively suppress growth of the chemoresistant SP subpopulation [165].

Ovarian cancers come in different forms. Besides surface epithelium-derived ovarian cancer, other categories of ovarian tumors are represented by sex-cord and germ-cell tumors (GCTs). GCTs comprise a heterogeneous group of neoplasms predominantly found along the midline of the body, on the migration route of the primordial germ cells (PGCs) during embryogenesis from the yolk sac to the genital ridge [7]. Different histopathological entities can be distinguished: (a) teratomas and yolk sac tumors, (b) seminomas/dysgerminomas/germinomas of the testis and ovary, mediastinum, and midline of the brain, (c) dermoid cysts of the ovary, and (d) spermatocytic seminomas of elderly testis. Of these, tumors of types a, c, and d are composed of differentiated tissues, whereas type b GCTs demonstrate pluripotent potential. A close resemblance between testicular GCTs and embryonic stem (ES) cells has been shown [4]. Specific expression of Oct 3/4 has been related to the pluripotent capacity of testicular GCTs, suggesting their origin from transformed pluripotent progenitor cells, such as PGCs [100]. Oct 3/4 is critical for the self-renewal of ES cells [124] and, under physiological conditions, Oct 3/4 transcription factor is expressed exclusively in pluripotent ES cells, PGCs, oogonia, and gonocytes [60, 65, 147] and its expression is downregulated during differentiation [138]. Many similarities exist between testicular and ovarian GCTs, including morphological resemblance and a similar pattern of chromosomal alterations [143]. The pathobiology of testicular seminomas is discussed in detail in the next paragraph. Two studies recently demonstrated that ovarian dysgerminomas are characterized by strong positivity for Oct 3/4 [34, 100], which is similar to testicular seminomas. Hoei-Hansen et al. [72] investigated the expression of additional stem cell-related markers of pluripotency in human dysgerminomas. In addition to Oct 3/4, the authors identified the expression of c-Kit, also known as a tyrosine kinase receptor for SCF, Nanog, and AP-2γ. The c-Kit/SCF system has particular importance in the origin of ovarian GCTs (OGCTs) due to its role in PGC proliferation and survival in the developing human gonad [114]. In particular, it has been suggested that the high expression of c-Kit in dysgerminomas is due to spontaneous gene mutations occurring before oocytes enter meiosis, leading to increased survival and proliferation of undifferentiated germ cells which are committed to pluripotency [72].

Malignant OGCTs have a median onset age of 18 years and represent approximately 3% of all ovarian cancers [125]. The fact that OGCTs normally affect women in their reproductive years implies the importance of optimal therapy to maximize the percentage of patients in whom ovarian function can be conserved. The immunohistochemical detection of stem cell-related markers offers a promising tool in the accurate diagnosis and evaluation of OGCTs, facilitating the diagnosis of pre-malignant germ-cell lesions and offering a choice of prompt and targeted therapy.

HUMAN TESTICULAR GCTs

Testicular GCTs (TGCTs) are the most common malignant tumors in the male Caucasian population between the second to fourth decade of life [1, 109]. As first described by Skakkebaek [158, 159], intratubular germ-cell neoplasia unclassified (IGCNU, also known as carcinoma *in situ*) represents the common precursor lesion of all TGCTs except spermatocytic seminomas. According to the current hypothesis, delayed or compromised maturation of fetal germ cells, i.e. PGCs/gonocytes, leads to the persistence of immature germ cells which subsequently may undergo malignant transformation [129]. The precise nature of transformation of these premature germ cells to IGCNU is not known. Recent studies, however, identified specific differentiation stages of fetal germ cells, which indicates that the process of normal maturation is likely to be interrupted at a distinct period of fetal development prior to neoplastic transformation [133]. Once established, IGCNU will always progress to TGCTs and exhibit a wide spectrum of histological subtypes, including seminoma, embryonal carcinoma, teratoma, choriocarcinoma, and tumors of the yolk sac. Among these, embryonal carcinoma is a malignant pluripotent counterpart of ES cells and is able to differentiate into all three germ lineages.

Previous studies showed a substantial overlap in the expression patterns of embryonal carcinomas and ES cells [5, 163]. Despite differential histology, seminomas and IGCNU share a high overlap of gene expression with ES cells [4, 20, 21]. Pluripotency genes such as Oct 3/4 and Nanog are highly expressed in IGCNU, seminomas, and embryonal carcinomas, but not in other histological types of TGCTs. The entirely pluripotent characteristic of embryonal carcinomas, however, might be established by SOX2, which is highly expressed in embryonal carcinomas but missing in IGCNU and seminomas (Fig. 3). The transcription factor SOX2 is essential for maintaining the pluripotent phenotype in ES cells and is a partner of Oct 3/4 in regulating several ES cell-specific genes [145]. *In vitro* experiments with ES cell nuclear extracts demonstrated that Oct 3/4 and SOX2 interact specifically and bind to a composite regulatory element. Activation of this element maintains Oct 3/4 and SOX2 expression in pluripotent cells [35].

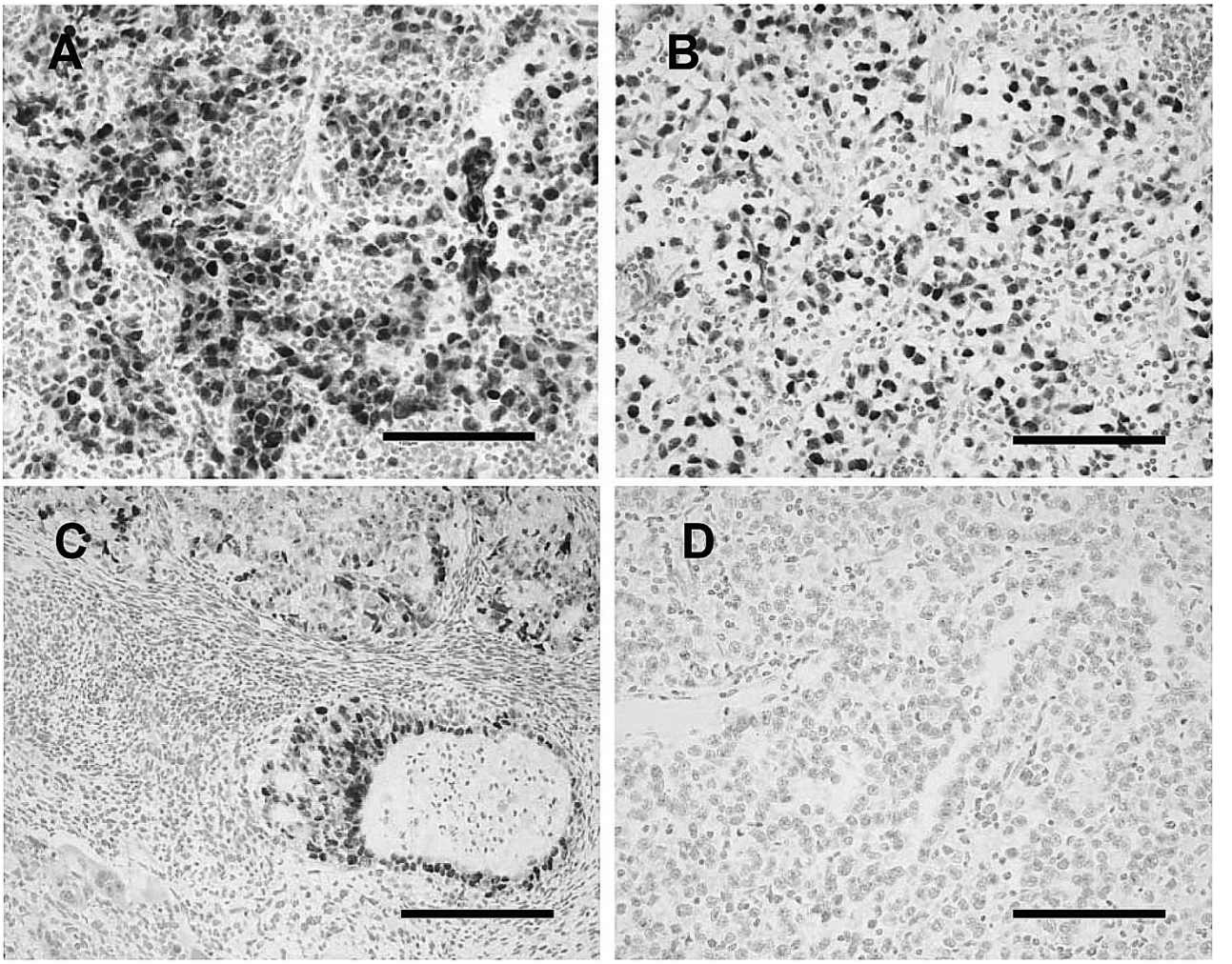

Fig. 3. Stem cell markers expressed in both embryonal carcinomas and seminomas. Pluripotency regulators POU5F1/ Oct 3/4 and NANOG are expressed both in embryonal carcinomas and seminomas as shown here by immunohistochemical staining on a seminoma sample (**A** – POU5F1, **B** – NANOG). In contrast, pluripotency regulator SOX2 is differentially expressed in embryonal carcinoma only (**C** – embryonal carcinoma component of a TGCT with a mixed histology expressing SOX2, **D** – compare a completely negative staining in a seminoma). Bar=100 μm.

Thus the SOX2-Oct 3/4 interaction may directly assist in reprogramming or serve as a driving factor leading to the reprogramming of common undifferentiated neoplastic germ cells to become embryonal carcinoma. Several other candidate genes which may contribute to the maintenance of pluripotency have recently been detected by genome-wide arrays and validated by immunohistochemistry, e.g. CENPA and PODXL [20]. Their role in TGCTs should be elucidated by functional studies in the future. In contrast to embryonal carcinomas, seminomas express many genes associated with normal spermatogenesis [21].

ES cells and TGCTs not only exhibit similar gene expressions, but also display similar epigenetic DNA--modifications. Compared with normal somatic cells, ES cells show a very low mutation rate (10^{-6} in ES cells and 10^{-4} in somatic cells) [30]. Similarly, TGCTs exhibit a very low mutation rate compared with somatic malignancies [22]. The receptor tyrosine kinase c-Kit represents the only exception from this general finding. Several studies have shown that different gain-of-function c-Kit mutations are often present in bilateral, but not in unilateral GCTs [19, 99]. These differences are likely due to a very early formation of the mutations, probably during embryonal development prior to testis formation. Most TGCTs are highly sensitive to DNA damaging agents, most likely because of the embryonal characteristics of these tumors. ES cells are equally sensitive to irradiation and chemotherapy and the molecular basis for this sensitivity is the cytoplasmic expression of the checkpoint kinase CHK2, which leads to an accelerated transition from G1 to S phase. It is through this mechanism that only intact cells survive, while the damaged cells become apoptotic [74]. Furthermore, all invasive TGCTs show a consistent gain of the short arm of chromosome 12, as found in ES cells upon extensive *in vitro* culturing [43]. In contrast to many other malignancies, TGCTs have a wild-type p53. A specific cluster of miRNAs which are also expressed in ES cells prevent p53-dependent cellular senescence upon oncogenic stress [174].

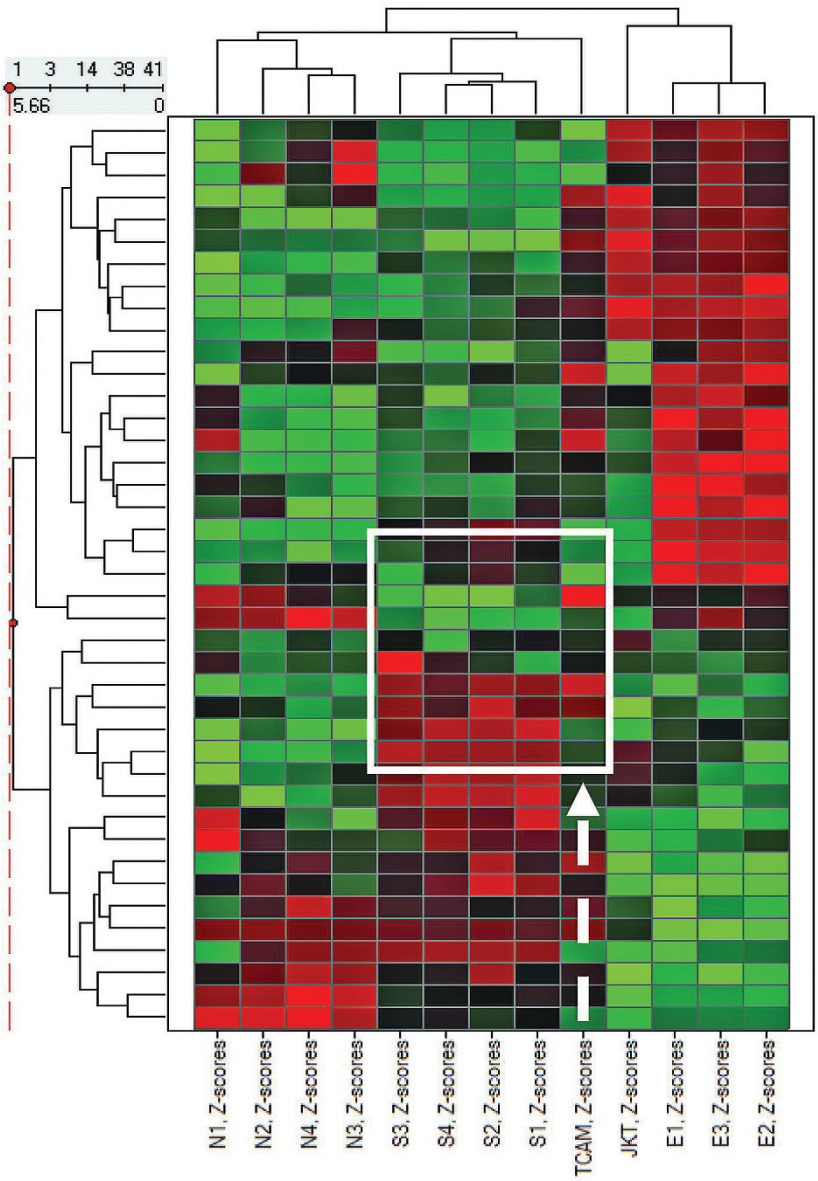

Fig. 4. Whole genome expression analysis and comparison between normal testicular tissues, seminoma, embryonal carcinoma, and the relevant cell line models. Whole genome expression analysis of normal testicular tissues (N1-N4), seminoma (S1-S4), embryonal carcinoma (E1-E3) and the cell lines JKT1 and TCam-2 shows that JKT1 clusters with seminoma. The seminoma-like nature was confirmed using reverse transcription/polymerase chain reaction (see main text for details).

While there are no animal models for seminoma to date, two groups have been successful in generating cell lines from seminoma patients [88, 112] which are now being used to study seminomas. These cell lines are JKT-1 [70, 81, 90, 146] and TCam-2 [59, 89]. Gene expression and immunohistochemical profiling have shown that only the TCam-2 cell line is representative for seminoma (Fig. 4) [40, 41, 46]. Both cell lines are essential for future studies on the molecular mechanisms of reprogramming and pluripotency in TGCTs and seminomas.

TARGETING SIGNALING PATHWAYS ACTIVE IN CSCS DURING CANCER THERAPY

Neoplastic germ cells retain their embryonic features while the normal maturation process of spermatogenesis causes the downregulation of embryonic gene expression and pluripotency in gametes. Therefore, TGCTs represent embryonic cancers found in adults. Both the seminomas and non-seminomas have their specific populations of stem-cell representatives derived from PGCs/gonocytes and from ES cells, respectively. In the future, the potential and functional process of reprogramming the precursor neoplasia IGCNU to different histological types of GCTs will provide potentially new and exciting avenues for the treatment of TGCTs. Recent progress in novel global screening technologies such as genomics, proteomics, and, in the future, metabolomics as well as combinatorial approaches will provide targets and tools for such reprogramming strategies [3, 6, 91].

CSCs have recently become an attractive target for novel anticancer therapies and several biochemical and physiological factors have emerged as potential targets for drug development. Although a multitude of potential therapeutic approaches have been identified, many can be broadly classified as either differentiation or elimination therapy in which CSCs are coaxed to differentiate or are themselves eliminated, respectively [15, 108, 187]. Molecules that have been implicated in playing a role in CSC signaling spanning several tissue types, and may subsequently serve as potential targets for the development of novel therapies, include CD133 [166] and components of the hedgehog/patched (HH/PTCH) [101], Wnt [84], and Notch signaling cascades [84, 108] as well as signal transducer and activator of transcription 3 (STAT3) [58] and telomerase [67]. Regulating various components of the CSC microenvironment and niche, such as the intracellular reactive oxygen species (ROS) levels [166] and tumor vasculature [166, 180], have also been suggested as a potential means of therapy. For a number of cancers, immunotherapeutic approaches, either by targeting antigens over-expressed by cancer cells or, in rare cases, by the induction of the "graft-versus-disease" response, have been successful [24, 83, 93, 141].

Investigators recently demonstrated that $CD133^+$ cells in human brain cancers possess both self-renewal

and differentiation properties as well as the ability to initiate tumor growth *in vivo*, whereas $CD133^-$ cells do not. These human $CD133^+$ brain CSCs, isolated from fresh tumor specimens, are capable of escaping the fatal damage induced by ionizing radiation via preferential activation of DNA repair checkpoints through phosphorylation of the cell cycle checkpoint proteins CHK1 and CHK2. Conversely, it was also shown that two pharmacological inhibitors of CHK1 and CHK2 can reduce the radioresistance of $CD133^+$ cells by disrupting the otherwise efficient DNA repair mechanisms. Another study revealed that BMPs, which normally function as soluble factors that induce the differentiation of neural precursor cells into mature astrocytes, can also induce $CD133^+$ brain CSCs to differentiate, which in turn significantly impedes their original tumor-initiating capacity [166]. This implies that forced differentiation of $CD133^+$ brain CSCs via therapeutically induced over-expression of BMPs may serve as an effective means of sensitizing the tumor population to conventional chemotherapy and reducing the rate of recurrence. Alternatively, it is also implied that the cancer-selective inhibition of CD133 in brain CSCs can also sensitize them to radiotherapy and reduce their self-renewal and differentiation capabilities.

Several pathways play a role in the growth and maintenance of normal stem cells in several tissue types, among which is the HH/PTCH pathway. Mammalian *HH* genes, which include the homologues *IHH*, *DHH*, and *SHH*, are highly expressed in small-cell lung, prostate, breast, gastric, and pancreatic cancer cell lines. In the HH/PTCH pathway, mutational inactivation of PTCH, which serves as the receptor for HH, leads to the constitutive activation of smoothened (SMO), which is a G-protein-coupled receptor family protein that is regulated by PTCH. Deregulation of the HH/PTCH pathway has been proposed to be a precursor for stem cell activation in cancers. Cyclopamine, which is a steroid-like compound that binds to and inactivates SMO, inhibits the growth of cells with activated HH signaling. This was shown in mice treated with cyclopamine in which the PC-3 and DU-145 human prostate cancer cell lines were grown as xenografts and eradicated after 21 days. It was recently shown that vitamin D_3 functions as an essential signaling molecule between PTCH and SMO in that it is normally secreted by PTCH and inhibits SMO on adjacent cells and the one from which it was secreted. Cyclopamine may function in a similar manner as it competes with vitamin D_3 for binding on SMO. This suggests that vitamin D_3 and its steroidal derivatives can be used as anticancer compounds in CSCs with active HH signaling. Cyclopamine treatment also significantly down-regulated the expression of *GLI2*, *GLI3*, *PTCH*, *SUFU*, and *SHH*, all of which are components of the HH/PTCH pathway, suggesting that the relative mRNA or protein expression levels of these genes can be utilized as a scale in evaluating the effectiveness of targeted inhibitors of human CSCs [101].

Other signaling cascades involved in the self-renewal and differentiation of normal stem cells are the Notch and canonical Wnt pathways, the former playing an especially important role in neuronal stem cells. Synergy between the canonical Wnt and Notch pathways is involved in inhibiting terminal differentiation of intestinal epithelial cells and both are necessary for the self-renewal of HSCs. In the canonical Wnt pathway, extracellular Wnt proteins bind to the receptor of the Frizzled (FZD) family. This event, coupled with the activity of the LRP5 and LRP6 coreceptors, activates a pathway leading to the inhibition of proteolysis and subsequent nuclear accumulation of β-catenin, which in turn activates the transcription of *FGF20*, *DKK1*, *WISP1*, *Myc*, and *CCND1* through an interaction with T-cell factor/lymphoid enhancer factor proteins. It is thought that deregulation of this pathway induces the transformation of normal stem cells into CSCs.

PKF118-310 and ZTM000990 are small-molecule leads targeted to the canonical Wnt signaling pathway, whereas anti-Wnt1 and anti-Wnt2 are monoclonal antibodies that have demonstrated anticancer effects *in vitro* [84]. Notch ligand binding to Notch family receptors results in the release of the Notch intracellular domain into the cytoplasm via proteolysis. The intracellular domain in turn binds to HLH and induces the transcriptional activation of several downstream target genes [108]. Aberrant activity of the Notch pathway is an early event in a pre-invasive breast cancer, ductal carcinoma *in situ*. Over-expression of the Notch1 intracellular domain in ductal carcinoma *in situ* was also predicted to result in a reduced time of recurrence, five years after surgery [49]. Over-expression of the active form of Notch1 in the human lung adenocarcinoma cell line A549 resulted in an inhibition of tumor-initiating and colony-forming properties when implanted in nude mice and grown in methylcellulose medium, respectively [38]. This implies that therapies targeting the Notch pathway, such as agents that induce an over-expression of Notch1, may function as a novel means of elimination therapy in human CSCs.

STAT3 plays a role in several processes, including cell survival, proliferation, differentiation, oncogenesis, metastasis, immune invasion, and angiogenesis, under both physiological and pathological conditions [33, 76]. It was recently shown that a small subpopulation of human self-renewing bone sarcoma cells have the ability to form spherical suspended clonal colonies known as "sarcospheres" under serum-starved and anchorage-independent conditions. These sarcospheres expressed activated *STAT3* as well as *Nanog*, Oct3, and Oct4, the latter three of which are markers of pluripotent ES cells. It was shown that as the sarcospheres grew larger and the total number of cells contained therein increased, they adopted an increasingly heterogeneous genotype with a lower percentage of the cells expressing the ES cell markers [58]. The ability of STAT3, in conjunction with Nanog, Oct3, and Oct4, to maintain the "stemness" of the bone sarcoma sarcospheres coupled with its extensively studied over-expression in several human cancers makes it an increasingly attractive and important target for therapy against both bulk tumor cells and CSCs.

A target that may result in increased tumor-selective toxicity to CSCs is telomerase. In most cells, the telomere (chromosome end-sequence) shortens as they divide. The length of the telomeres is preserved by an enzyme called telomerase (RNA template-dependent DNA-synthase), which adds relatively short repeats of nucleotides to chromosome ends. Normal stem cells require telomerase in order to prevent a shortening of their telomeres and replicative senescence (loss of telomere length reserve), although it has been shown that the relatively long telomeres of normal stem cells allows them to temporarily proliferate in the absence of telomerase. It has also been suggested that the presence of telomerase and its subsequent activity in most malignancies is an absolute requirement for growth and maintenance as a means of compensating for the characteristically short telomeres in cancer cells [67, 86]. Taken together, this implies that the inhibition of telomerase may not only represent a potent therapeutic approach against CSCs, but one that offers a reasonable degree of tumor specificity and differential sensitivity due to the differences in telomere length between normal stem cells and CSCs. GRN163L, a direct inhibitor of the RNA template region of human telomerase (hTR), is a 13-mer oligonucleotide lipid conjugate that is currently in clinical trials for patients with chronic lymphocytic leukemia, multiple myeloma, solid tumors, and non-small-cell lung cancer [67]. It is also possible that GRN163L will play a role in eliminating CSCs with active telomerase and reduce the rate of recurrence.

Alternative to the aforementioned biochemical targets for novel therapeutic development against CSCs there are also a number of physiological targets. Among these are the intracellular ROS levels [166] as well as the tumor vasculature [166, 180] that nourishes and supplies CSCs. ROS play a role in tumor initiation in several animal models and humans. Among other factors, superoxide dismutases dependent on bivalent cations play an important role in neutralizing the more active forms of ROS; thus the removal of some bivalent cations kills cancer cells [69]. Although little is known of how ROS status directly relates to CSCs, it is has been suggested that the cellular redox state plays a role in the balance between self-renewal and the differentiation potential of stem cells. It was shown that extracellular growth factors that promote self-renewal and differentiation caused progenitor cells to be more reduced and oxidized, respectively [166]. If more conclusive studies are done that link ROS status with CSCs, this may provide an exploitable target for therapy. Since CSCs exist among a heterogeneous bulk tumor population supplied by a tumor vasculature, it is logical to assume that inhibition of such vessels could also terminate the nutrient supply to CSCs and reduce their resistance to radio- and chemotherapy [180]. Coupling angiogenesis inhibitors with targeted inhibitors of CD133, STAT3, and hTR or the HH/PTCH, Wnt, and Notch pathways may provide a novel and effective means of significantly improving cancer therapy and reducing remission across many malignant tissue types.

Aside from therapeutic approaches that focus on certain known pathways, other novel ways to attack cancer have recently been proposed as well. An interesting example is proteins that either selectively (e.g. apoptin) [28, 106] or semi-selectively (e.g. Brevinin-2R, S100A8/A9) [55, 56, 63] kill cancer cells by mechanism(s) yet to be fully defined. In the case of apoptin, however, it seems that its selectivity is dependent upon the utilization of the very pathways involved in cell proliferation, such as PI3-K/Akt/CDK2, and their targeting (or "hijacking") to induce cell death, likely via a mitotic catastrophe-related mechanism [104, 105, 107]. Interestingly, opposing processes such as cell survival, cell death, and cell proliferation seem to be tightly interconnected in the cell, and some stimuli may have opposite effects depending on the quantity or duration [57, 103].

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