Cancer stem cell markers in common cancers – therapeutic implications

Thomas Klonisch¹, Emilia Wiechec², Sabine Hombach-Klonisch¹,
Sudharsana R. Ande^{3,4}, Sebastian Wesselborg⁵, Klaus Schulze-Osthoff^{6*} and
Marek Los^{1,3,4,7*}

¹ Department of Human Anatomy and Cell Science, University of Manitoba, Winnipeg, R3E 0W3, MB, Canada

² Institute of Human Genetics, University of Aarhus, DK-8000 Aarhus, Denmark

³ Manitoba Institute of Cell Biology, CancerCare Manitoba, Winnipeg, R3E 0V9, MB, Canada

⁴ Department of Biochemistry and Medical Genetics, University of Manitoba, Winnipeg, R3E 0W3, MB, Canada

⁵ Department of Internal Medicine I, University of Tübingen, D-72076 Tübingen, Germany

⁶ Interfaculty Institute for Biochemistry, University of Tübingen, D-72076 Tübingen, Germany

⁷ Current address: BioApplications Enterprises, Winnipeg, R2V 2N6, MB, Canada

Rapid advances in the cancer stem cell (CSC) field have provided cause for optimism for the development of more reliable cancer therapies in the future. Strategies aimed at efficient targeting of CSCs are becoming important for monitoring the progress of cancer therapy and for evaluating new therapeutic approaches. Here, we characterize and compare the specific markers that have been found to be present on stem cells, cancer cells and CSCs in selected tissues (colon, breast, liver, pancreas and prostate). We then discuss future directions of this intriguing new research field in the context of new diagnostic and therapeutic opportunities.

Introduction – the biology of stem cells and their surface markers

The ability of adult stem cells to be long-lived and capable of self-renewal and multi-lineage differentiation makes these cells unique and important in normal physiology and pathological conditions [1]. Their differentiation potential and capacity for tissue renewal and damage repair make stem cells valuable for regenerative medicine, tissue engineering and biotechnology applications [2]. However, the same qualities might also pose a serious threat to the host organism (Box 1). If stem cell differentiation potential becomes impaired and their proliferative capacity becomes uncontrolled, these mutated, potentially tumorigenic, self-renewable stem cells have the potential to cause cancer. Although this is still the subject of considerable debate, it is possible that the development of cancer stem cells (CSCs) might involve at least two of the following events: (i) a change in the microenvironment of the stem cell niche within a tissue; (ii) alterations in cellular metabolism, cell cycle control and/or progression and signaling pathways as a result of mutations and epigenetic changes; and (iii) amplification of cell populations with an altered molecular phenotype that give rise to heterogeneous primary tumors and metastases.

In recent years, CSCs have been recognized as important components in carcinogenesis, and they could form the

basis of many (if not all) tumor types. CSCs have been isolated from cancers of the breast, brain, blood (leukemia), skin (melanoma), head and neck, thyroid, cervix, lung, organs of the gastrointestinal and reproductive tract and retina [2]. The identification of CSC-specific marker sets (Table 1) and the targeted therapeutic destruction of CSCs remain a challenge. Current anticancer therapies mostly fail to eradicate CSC clones and instead favor expansion of the CSC pool and/or select for resistant CSC clones, thus leading to a fatal outcome of the disease.

Here we summarize the current knowledge of CSCs in some of the most frequent human cancers: pancreatic, hepatocellular, colorectal, breast and prostate carcinomas. We discuss their various markers and provide insights into new therapeutic approaches for more specific targeting and eradication of CSCs.

Pancreas, liver and colorectal stem/progenitor cells and CSCs

Pancreas

The pancreas consists of an exocrine part (acini and ducts), which secretes digestive enzymes, and pancreatic islets of Langerhans composed of α , β , δ and PP endocrine cells. Loss of functional pancreatic islet cells results in type I diabetes with deregulated blood glucose homeostasis, and this has been a major incentive for identifying pancreatic stem cells, which, if properly programmed, can act as a renewable source for novel β -cell transplantation. A novel treatment regime now allows human embryonic stem cells (ESCs) to be programmed to undergo pancreatic development and generate hormone-expressing cells *in vitro* [3]. Potential candidates for adult pancreatic stem cells include pancreatic ductal cells and cells positive for neurogenin 3 (Ngn3) and pancreatic duodenal homeobox factor 1 (Pdx1). Pluripotent ductal epithelial stem cells were shown to generate islets composed of α , β and δ cells during long-term culture [4]. Nestin-positive, hormone-negative mesenchymal cells were also described as endocrine precursor cells within the pancreatic islets and ducts [5], but these cells do not contribute to insulin-producing cells in the human pancreas [6]. Instead, endocrine progenitor

Corresponding author: Los, M. (mjelos@gmail.com)

* Both authors share senior authorship.

Box 1. Cancer stem cells (CSCs)

Tissue stem cells form a small population of cells (usually much less than 1% within a given tissue), have self-renewing potential and display significant plasticity. The capacity of stem cells for tissue renewal and damage repair has great potential applications for biotechnology and medicine. Unfortunately, the same qualities that make stem cells so valuable for regenerative medicine, tissue engineering and biotechnology, can pose a serious threat to the host organism if their differentiation potential becomes uncontrolled. Mutations within regulatory pathways can impair stem cell functions so that their high proliferative potential, and frequently their migratory capabilities as well, make these mutated stem cells tumorigenic. In addition, mutations in normal differentiated cells can also produce cancer, and the precise origin (stem cells or differentiated cells) of cancerous cells remains unclear. In either case, these tumorigenic stem/progenitor cell populations are collectively named CSCs.

cells were shown to express the transcription factors Ngn3 (and Isl1 and NeuroD), whereas cells positive for Pdx1 contributed to exocrine, endocrine and ductal tissue in the pancreas [7].

Pancreatic adenocarcinoma is the fourth most common cause of cancer death in the USA, displays extensive local destruction and early metastasis and has the worst prognosis of all human tumors (3% five-year survival). Pdx1 has been detected in pancreatic cancer [8], but no association with pancreatic CSCs has been reported for any of the factors involved in pancreatogenesis. Pancreatic CSCs represent less than 1% of all pancreatic cancer cells and express the surface markers CD44⁺, CD24⁺ and epithelial-specific antigen (ESA)⁺. When injected into non-obese diabetic/severe combined immunodeficiency (NOD/SCID) mice, these CSCs, but not cancer cells devoid of these surface markers, self-renewed, produced differentiated progeny, had a 100-fold increased tumorigenic potential, generated a tumor morphology similar to the patient's primary cancer and maintained their surface marker phenotype after repeated passages as xenographs. The CD44⁺, CD24⁺, ESA⁺ CSCs showed a strong transcriptional upregulation of sonic hedgehog (SHH) and the polycomb group (PCG) gene family member BMI-1 [9]. Pancreatic adeno-

carcinoma also contains 1–3% of CD133⁺ cancer cells, some of which also show high expression of CXC chemokine receptor 4 (CXCR4). These CD133⁺, CXCR4⁺ cells, but not CD133⁺, CXCR4[−] cells, were able to metastasize, and abrogation of signaling by CXCR4 similarly blocked tissue invasion [10]. Thus, there might be more than one type of CSC in pancreatic adenocarcinoma.

Liver

The liver has high regenerative potential, and hepatic small oval progenitor cells around the peripheral branches of the bile ducts, the canals of Hering, can differentiate into biliary epithelial cells and hepatocytes [11]. These oval liver progenitor cells share molecular markers with adult hepatocytes (albumin, cytokeratin 7 [CK7], CK19, oval cell markers OV6, A6, OV1, chromogranin-A, NCAM [neural cell adhesion molecule, connexin-43]) and fetal hepatocytes (α -fetoprotein) (Table 1) [11,12]. They are also positive for more common stem cell markers such as CD34⁺, Thy-1⁺, c-Kit⁺ and Flt-3⁺ (FMS-like tyrosine kinase 3) [5], and thus it currently remains unclear whether these stem cells are derived from the bone marrow and just migrate to this periportal niche or whether they represent true resident liver stem/progenitor cells. Binding of stromal-derived factor 1 α (SDF-1 α) to its surface receptor CXCR4 activates oval hepatic cells [13]. Novel antigen profiles emerge with CD117⁺, CD133⁺ hepatic precursors in regenerating liver tissue [14] and with a resident CD45[−], CD90⁺ subpopulation of tumor cells in hepatocellular carcinoma (HCC), both of which might qualify as hepatic CSCs [15].

HCC is highly prevalent in Africa and Asia, has a high mortality and does not respond well to conventional therapy [16]. The CD90⁺ cells are not present in the normal liver and, when injected into immunodeficient mice, create tumors repeatedly. In human HCC and HCC cell lines, specifically CD133⁺ cells, not CD133[−] cells, had the ability to self-renew, create differentiated progenies and form tumors [17]. This coincided with the expression of genes associated with stem/progenitor status, such as β -catenin, NOTCH, BMI and OCT3/4. When compared to CD133[−] cells, the CD133⁺ cells isolated from the HCC cell lines

Table 1. Markers frequently used to identify adult stem cells within the prostate, breast and intestine

Marker	Other names	Characteristics	Refs
CD24	Heat-stable antigen	Found on breast CSCs; cell surface proteins on human mammary repopulating units in mice	[55]
CD29	Integrin β 1	Found on breast CSCs; cell surface proteins on rodent mammary repopulating units in mice	[36]
ESA	Epithelial-specific antigen	Found on breast and pancreatic CSCs; cell-adhesion molecule; expression level is elevated during tumorigenesis	[50]
CD44		Found on breast and prostate CSCs; multistructural and multifunctional surface glycoprotein, also implicated in inflammation	[89,101]
CD49f	(Integrin α 6)	Found on prostate CSCs; coordination between cytoskeleton and adhesion to extracellular matrix	[76,77]
CD133	Prominin-1	Found on prostate and breast CSCs; five transmembrane domain cell-surface glycoprotein that localizes to membrane protrusions; also a marker for neuroepithelial, hematopoietic, breast and endothelial progenitor cells	[57,72,88]
P63		Found on prostate CSCs; p53 homolog, highly expressed in embryonic ectoderm and in the nuclei of basal regenerative cells of many epithelial tissues in the adult including skin, breast myoepithelium, oral epithelium, prostate and urothelia; in prostate: basal prostate cell marker	[68,92]
Stem cell antigen	Sca1, Ly-6A/E	Found on prostate and breast CSCs; stem cell marker; found also on muscle, hematopoietic and breast stem cells	[75]
NCAM, CD34, Thy-1, c-Kit, Flt-3		Found on intestinal CSCs; marker for liver and pancreas stem cells	[12]

showed higher expression of CD44 and CD34, but both CD133 subpopulations displayed similar expression for CD29, CD49f (integrin $\alpha 6$), CD90 and CD117 [17].

Colorectal tissue

Intestinal stem cells generate $>10^{10}$ new cells daily, which differentiate along a vertical axis within the human gut. In the colorectum, stem cells reside at the bottom of epithelial crypts, and dysregulation of the AKT/PKB, Wnt and/or bone morphogenic protein (BMP) signaling pathways disturbs intestinal stem cell self-renewal [18]. Mushashi-1, a marker for neuronal stem cells, has been proposed as a potential stem cell marker in the gastrointestinal tract and colon epithelial crypt cells [19]. Colorectal cancer is the third most frequent cancer and the second leading cause of cancer-related death in the Western world [20]. Current anticancer therapies fail to effectively target colorectal cancer cells. Colorectal carcinoma develops as a result of successive mutations during clonal expansion of a single stem cell located at the bottom of the colorectal crypt. These mutated stem cells display enhanced proliferative potential, gain independence of extrinsic growth control signals and claim autonomous control over all metabolic activities [21]. Hereditary non-polyposis colorectal cancer involves mutations of DNA mismatch repair genes, the *adenomatous polyposis coli* (APC) gene, β -catenin, K-RAS, SMAD4 and the P53 gene [22]. There are at least six distinct inherited polyposis syndromes that are considered precancerous lesions because they frequently convert into colon cancer. Mutations in the APC gene, a critical component in WNT signaling pathway, cause familial adenomatous polyposis syndrome (FAP syndrome) [23]. Juvenile polyposis syndrome is the result of mutations in the *bone morphogenic protein receptor 1A* (BMPR-1A) and *transforming growth factor-1 β* (TGF-1 β) family signaling mediators SMAD4 and *endoglin*, and this precancerous condition is estimated to cause colorectal cancer in 9% to 50% of cases [24]. Alterations in receptor tyrosine kinase signaling pathways and mutations in RAS GTPase can also contribute to inherited polyposis syndrome [25]. Mutations in *phosphatase and tensin homolog deleted on chromosome 10* (PTEN), a phosphatase that antagonizes PI3-kinase-mediated activation of the AKT pathway, cause Cowden syndrome, which results in tumors in various organs, including the gastrointestinal tract, central nervous system, skin, breast and thyroid gland [26]. Colorectal CSCs express CD133⁺, ESA⁺ (EpCAM⁺) but are devoid of differentiation markers such as CK20 [27]. CD133⁺ colorectal CSCs grow exponentially *in vitro* as undifferentiated spheres under serum-free conditions and generate tumors in NOD/SCID mice with morphological and antigenic profiles similar to their tumor of origin [27]. When single-cell suspensions isolated from solid colorectal cancer tissues were tested for tumorigenicity in NOD/SCID mice, only a small population of cells had the capacity to induce tumors, and these cells were positive for ESA and CD44 [28]. These ESA^{High}, CD44⁺ colon cells were also positive for CD49f and aldehyde dehydrogenase enzymatic activity and showed enhanced tumorigenicity [27]. CD166 is another differential marker for colorectal CSCs [28]. Thus, CD133, CD166, CD44, CD49f and ESA are part of a hierarchical

signature for colorectal (cancer) stem cells and can be used for the enrichment of colorectal CSC cellular subsets within the CD133-expressing CSC population [28]. CD133⁺ colon CSCs produce interleukin-4 (IL-4), which is likely to act via CXCR4 to enhance their survival and autocrine growth, and this has therapeutic implications [29].

Breast stem/progenitor cells and CSCs

Breast cancer is the major cause of cancer-related death among women worldwide, and more than 40 000 breast cancer fatalities occur annually in the USA alone. Breast CSCs originate from mammary multipotent stem cells as a consequence of genetic defects caused by damaging agents (e.g. radiation) that affect pathways governing self-renewal and differentiation (Figure 1).

The adult human mammary gland is composed of a series of branched ducts and lobulo-alveolar units embedded in fatty tissue and is composed of three cell lineages: (i) myoepithelial cells, which form the basal layer of ducts and alveoli; (ii) ductal epithelial cells; and (iii) alveolar epithelial cells, which line the alveoli and are capable of milk protein production. Myoepithelial cells express the α -form of smooth muscle actin (SMA), common acute lymphoblastic leukemia antigen (CALLA, also known as CD10) and CK14, whereas luminal epithelial cells express MUC-1, CK8, CK18 and CK19. ESA is present on all epithelial breast cells. The existence of morphologically distinct suprabasal small light cells in rodents [30] and basal clear cells in the human and bovine mammary gland [31,32] pointed towards candidate stem cell populations in the breast. In the human breast epithelium, a stem/progenitor cell hierarchy might exist that is important for normal mammary organogenesis and function and that might be relevant for breast carcinogenesis. The hierarchy consists of: (i) luminal progenitor cells (MUC-1⁺, CALLA⁻, ESA⁺) also positive for CK8/18⁺, CK9⁺ and CK19⁺; (ii) distinct bilineage progenitors (MUC-1^{low}, CALLA^{low/-}, ESA⁺) capable of differentiating into both luminal and myoepithelial cells; (iii) myoepithelial-restricted progenitors (MUC-1⁻, CALLA⁺, ESA⁻, CK14⁺, CD44v6⁺); and (iv) more primitive progenitor cells giving rise to both luminal and myoepithelial cell types (MUC-1⁻, CALLA⁻, ESA⁻) [33,34].

Similarly, the murine mammary gland was shown to contain stem cells [35]. In the murine system, a single mammary stem cell from a CD29^{hi}, CD24⁺, Lin⁻ [36] or CD49^{thi}, CD24⁺ population [37] is able to reconstitute a complete mammary gland *in vivo*. The CD29^{hi}, CD24⁺, Lin⁻ cell fraction was enriched in CD49f SCA-1^{low} (stem cell antigen-1) and ABCG2^{low} (ATP-binding cassette transmembrane pump, isotype G2; also known as breast cancer resistance protein [BCRP] and mitoxantrone-resistance protein [MRP]) [36]. Luminal progenitor cells capable of lactogenic differentiation were of the phenotype CD29^{low}, CD24⁺, Lin⁻, CK18⁺, whereas CK14 was mainly expressed in the CD29^{hi}, CD24⁺, Lin⁻ population and is devoid of estrogen receptor (ER⁻). Myoepithelial progenitors were defined as CD49^{low}, CD24^{med}, Lin⁻ [37]. The murine mammary stem cell populations CD29^{hi}, CD24⁺, Lin⁻ and CD49^{thi}, CD24⁺ did not express ER or PR (progester-

Figure 1. Implication of stem cells in breast development and breast tumorigenesis. Normal breast stem cells are characterized by their ability to self-renew and differentiate, which leads to formation of a normal, functional mammary gland. Breast cancer stem cells originate from breast stem/progenitor cells that have undergone genetic alterations but have retained their ability to self-renew and differentiate. Breast cancer tissue, like other tissues from other tumors, contains a heterogeneous population of breast cancer cells. A small tumorigenic cancer stem/progenitor cell population has the ability to self-replicate, whereas the large majority of differentiated cancer cells forming the bulk of the breast tumor have limited proliferation potential and are weakly or non-tumorigenic when injected into nude mice.

one receptor) but progenies thereof were ER⁺ and PR⁺. The luminal epithelial cells with the phenotype SCA-1⁺, CD24^{hi}, CD133⁺ were ER⁺, PR⁺ and prolactin receptor⁺. This contrasted to an SCA-1⁻, CD24^{hi}, ER⁻ progenitor/transit amplifying (TA) cell type distinct from the basal cell compartment [38].

Self-renewal of human and mouse normal and malignant mammary stem cells involves a diverse network of regulatory mechanisms, including the signaling pathways of Notch [39], Hedgehog [40], Wnt/ β -catenin [41], epidermal growth factor (EGF)-like/EGF receptor (EGFR)/Neu [42], leukemia inhibitory factor (LIF) [43], TGF- β [44], integrins [45], telomerase [46], SDF-1/CXCR4 [47], prolactin/growth hormone (GH) [42], the insulin-like growth factor-1 (IGF-1) system [48] and ER/PR [49] (Figure 2). The Notch, Wnt, Hedgehog and HER2/Neu signaling pathways and the PCG transcription factor BMI-1 govern the differentiation and self-renewal of normal breast stem cells, and their deregulation is known to promote breast cancer. The Notch signaling pathway is implicated in cell differentiation and self-renewal of mammary stem cells. Overexpression of the active form of Notch 4 inhibits differentiation of breast epithelial cells and might contribute to breast carcinogenesis [39]. Musashi-1 (Msi-1) is a positive regulator of Notch signaling, and both Msi-1 and

Notch 1 are key regulators of asymmetrical cell division in human breast epithelial stem cells [49]. The Hedgehog pathway is required for normal and neoplastic development of the mammary gland. Overexpression of the ligands or activated β -catenin leads to an increased number of stem cells and poor prognosis in breast cancer [40,50]. The PCG transcription factor BMI-1 is overexpressed in human breast cancer cell lines and is known to induce telomerase activity [51]. BMI-1 effects on stem-cell renewal appear to involve epigenetic silencing of the p16^{INK4a} gene [52,53] and, by activating Hedgehog signaling pathways, BMI-1 impacts on self-renewal of normal and malignant human mammary stem cells [54]. In normal and neoplastic mammary epithelial cells, the overexpression of HER2/Neu results in an increase in the stem/progenitor cell and CSC pool.

Mammary CSCs are a small subpopulation of breast cancer cells (0.1–1%) but have high self-replicating potential and can promote carcinogenesis when transplanted into NOD/SCID mice [50]. These qualities make them prime targets for anticancer treatment [55]. Less than 100 mammary CSC cells with a phenotype of CD44⁺, CD24^{-low}, Lin⁻ were able to generate palpable tumors in NOD/SCID mice, whereas CD44⁻, CD24⁺ breast cancer cells, even when injected at 100-fold higher cell densities,

Figure 2. Factors and signaling pathways involved in the regulation of self-renewal of stem cells. See main text for details. Self-renewal of human and mouse normal and malignant mammary stem cells involves a diverse network of regulatory mechanisms, including the signaling pathways of Notch [39], Hedgehog [40], Wnt/ β -catenin [41], EGF-like/EGFR/Neu [42], LIF [43], TGF- β [44], integrins [45], telomerase [46], SDF-1/CXCR4 [47], prolactin/growth hormone (GH) [42], the IGF-1 system [48], and ER/PR [49]. Alterations in one or more of these pathways can contribute to deregulated cancer stem cell renewal.

were unable to induce tumor formation [50,56]. Similar results were obtained with breast CSCs expressing CD133⁺. There was no overlap in CD markers between the CD133⁺ and the CD44⁺, CD24⁻ CSCs, but both populations displayed markedly enhanced self-renewal capacity and shared the expression of stemness genes (*OCT4*, *NOTCH1*, *ALDH1*, *FGFR1*, *SOX1*) [57,58]. The LA7 cell line, which is derived from a rat breast adenocarcinoma (CD133⁺, CD49f⁺, CD29⁺, CD24^{-low}), provides further evidence for different CSC subpopulations in breast cancer. LA7 is tumorigenic in NOD/SCID mice, shares CD29 and CD49f with normal rodent breast stem cells and a single LA7 cell is capable of generating all three lineages of the mammary gland [59]. The CD44⁺, CD24^{-low}, Lin⁻ subpopulation of human breast CSCs can be further subdivided based on ESA expression. The ESA⁺, CD44⁺, CD24^{-low}, Lin⁻ breast CSCs, but not ESA⁻, CD44⁺, CD24^{-low}, Lin⁻ cells, were capable of generating breast tumors in immunodeficient mice, and a small population of former cells was able to reconstitute mammary tissue heterogeneity of the initial tumor in NOD/SCID mice [50]. Despite successive injections into SCID mice, CD44⁺, CD24^{-low}, Lin⁻ breast CSCs remained tumorigenic, could be re-isolated from the tumors and displayed plasticity in generating breast cancer cells with different phenotypes. Of the 13 breast cancer cell lines examined, CD44⁺, CD24⁻ breast cancer cells also expressed the highest levels of pro-invasive genes (*CXCR4*, *IL-11*, *CTGF*, *MMP-1*, *ADAMTS1*, *UPA*), invaded matrigel *in vitro* and were more likely to be ER⁻ [60]. Furthermore, as part of a dynamic hierarchy, CD44⁺, CD24⁻ breast cancer cells were shown in NOD/SCID mice to progress to CD44⁺/CD24⁺, CD44⁻/CD24⁺ and CD44⁻/CD24⁻ phenotypes [50]. Selected based on their ability to efflux Hoechst 33342

stain, a side population (SP; see ‘CSCs and implications for therapeutic applications’ below for further explanation) of ER⁺, MCF-7 breast cancer cells expressed the luminal epithelial marker MUC-1, and this SP produced MUC⁺ tumors *in vivo* [61], thus identifying MUC-1 as a new potential stem/progenitor cell marker. Recently, the cell surface receptor PROCR (protein C receptor, CD201) was found to be present on 100% of CD44⁺, CD24⁻ breast cancer cells, and CD44⁺, CD24⁻, PROCR⁺ cells were enriched for genes involved in cell motility, chemotaxis, hemostasis and angiogenesis. By contrast, CD44⁻, CD24⁺ cells were enriched for genes implicated in carbohydrate metabolism and RNA splicing [62]. Importantly, tumors composed of mainly CD44⁺, CD24⁻, PROCR⁺ cells had worse clinical outcome than tumors with a CD44⁻, CD24⁺ signature.

Prostate stem/progenitor cells and CSCs

The prostate epithelium is composed of three epithelial cell types, luminal, basal and rare neuroendocrine cells, and all three cell types might contribute to carcinogenesis [63]. Terminally differentiated luminal cells constitute the inner epithelial cell layer and express CK8, CK18, androgen receptor (AR), lipoxygenase 2, secret prostate-specific antigen (PSA) and prostate-specific alkaline phosphatase (PAP) into the glandular lumen in an androgen-dependent manner [64]. The basal cell compartment of the adult prostate gland accounts for >75% of mitotic activity in the prostate and is AR⁻ [65] but expresses CK5, CD44, BCL-2, $\alpha_2\beta_1$, p63, telomerase and glutathione S-transferase [66–68] (Tables 1 and 2). Androgen-deprivation experiments provided evidence for the presence of adult stem cell populations within the basal layer of the prostate gland (Table 1) [69]. Marked prostate atrophy with extensive apoptosis of luminal cells and preferential survival of basal cells is a hallmark in castrated rodents. Despite repeated rounds of androgen deprivation and subsequent supplementation or androgen replacement years after castration, a fully functional prostate epithelium is reconstituted in the presence of androgens [69]. Recent findings revealed a high proteasomal turnover rate of functional AR in a CD133⁻ subset of $\alpha_2\beta_1^{\text{hi}}$ -enriched basal cell population, resulting in androgen-responsiveness at very low to

Table 2. Markers for the *in vitro* and *in vivo* identification of putative prostate cancer stem cells

Marker	Refs
Integrins α_2/β_1	[88]
BCL-2	[92]
β -Catenin	[56]
BMI-1	[89]
BrdU, Ki67	[80,92]
CD44	[89]
CD133	[72,88]
CD49f (integrin α_6)	[76,77]
CK5/14; CK8/18	[117]
GST- π	[118]
ABCG2/Hoechst 33342	[56,101]
OCT3/4	[49,89]
P63	[68,92]
P27	[92]
SCA-1	[75]
SMO (Smoothered)	[89]

undetectable cellular levels of AR protein [70]. Additional evidence implicates the basal layer as an important source of prostate stem cells. Mice with a targeted deletion of the basal cell marker p63 show agenesis of the prostate and mammary gland [68], and complementation of p63^{-/-} blastocysts with p63^{+/+} β -galactosidase-positive ESCs generates chimeric animals with prostates composed of β -galactosidase-positive basal and luminal epithelial cells [71]. Candidate prostate stem/progenitor cell populations have been identified by their preferential expression of the surface markers CD44 [30], CD133 [72], $\alpha_2\beta_1^{\text{hi}}$ [73], CK5/14, CK8/18 [74], SCA-1 [75], CD49f [76,77], and ABCG2 [78]. CD44 is present in most basal cells, whereas CD133, $\alpha_2\beta_1^{\text{hi}}$, CK5/14, CK8/18 and ABCG2 are expressed in ~1% of the basal cell populations, and there is overlap between the $\alpha_2\beta_1^{\text{hi}}$, CD44⁺, and ABCG2⁺ cell populations [79]. The basal cell layer might not be the exclusive site for prostate stem/progenitor cells. Pulse-chase bromodeoxyuridine (BrdU) incorporation studies conducted after androgen withdrawal and replacement identified BrdU labels in both the basal and luminal epithelial compartment [80]. In a possible hierarchical stem cell model for the basal-to-luminal organization of the prostate epithelium, an androgen-independent immature basal stem cell pool (CD44⁺, $\alpha_2\beta_1^{\text{hi}}$, CD133⁺, AR⁻ [70]) might eventually generate an AR⁺ intermediate TA stem cell subpopulation ($\alpha_2\beta_1^{\text{hi}}$, CD133⁻ [70]), and progenies thereof might populate both the basal and luminal compartment. Support for the presence of cell phenotypes intermediate between basal and luminal cells comes from analyses of CK and neuroendocrine marker profiles in normal, hyper- and neoplastic human prostate [74,81], primary cell [79,82] and organ cultures [82], as well as *in vitro* differentiation studies [83]. However, modifications to this linear differentiation scheme might be warranted. The marked increase in prostate growth during puberty is largely attributable to a strong proliferation of luminal prostate cells, with the majority of basal cells remaining inactive [80]. [³H] thymidine uptake studies showed that basal cells with high levels of AR are not actively involved in proliferation [84]. In addition, the recent discovery of a luminal-restricted progenitor cell population in the mammary gland supports the concept of separate basal- and luminal-derived progenitor cell populations responding independently to various growth and differentiation cues [85].

Prostate cancer is the most common malignancy in men, and the two-layered epithelium of the postpubertal prostate gland is the site of development of human prostate cancer, which initiates from prostatic intraepithelial neoplasia (PIN) and proceeds through a series of defined stages into invasive androgen-dependent to androgen-independent cancer [86]. There is more than one stem/progenitor cell population present in prostate cancer. The majority of human prostate adenocarcinoma cells express the luminal epithelial cell markers CK8/18, PSA and PAP [87] but lack basal cell markers. A small cancer cell fraction in primary prostate carcinoma tissues and the majority of prostate cancer metastases express the basal cell markers CK5/14, CD44 and p63 (Table 2) [25]. A subpopulation of 0.1% of cancer cells isolated from primary prostate cancer with the basal cell marker profile CD44⁺ [30], CD133⁺ [72],

collagen receptor $\alpha_2\beta_1^{\text{hi}}$ [73], AR⁻ displayed high *in vitro* proliferative potential [88], increased clonogenic, tumor-initiating and metastatic activity in xenograft tumor models [89] and the ability to generate glandular tissue structures *in vivo* [72,88]. This putative CD44⁺, AR⁻ tumor progenitor cell population was ~100-fold more tumorigenic than the CD44⁻ prostate cancer cell population, expressed stem-cell-related genes such as OCT3/4, BMI-1, β -catenin or Smoothed (SMO) and ~1% of the CD44⁺ prostate cancer cell pool engaged in asymmetric cell division [89]. Similar to breast CSCs, CD44⁺, CD24⁻ prostate cells were recently reported and might represent tumor-initiating cells [90]. Moreover, higher Gleason grades (6–9) of primary prostate cancer correlated with enhanced numbers of CD44⁺ cells in these tumor tissues [50]. A hierarchical system of progenitor populations has been proposed, where ~80% of CD44⁺ cells express $\alpha_2\beta_1^{\text{hi}}$ and all ABCG2⁺ cells are CD44⁺ [79].

Various mouse models have been established for the study of prostate cancer. PTEN is a suppressor gene that is often mutated in human prostate cancer. Loss of function of PTEN and activation of AKT signaling are strongly correlated with human prostate cancer, and PTEN prostate-specific knockout mice develop PIN, which progresses into invasive and metastatic adenocarcinoma [91]. Although both basal and luminal cells have a PTEN gene deletion, prostate cancers in this model were largely composed of basal cells (P63⁺, SCA-1⁺, BCL-2⁺), suggesting expansion of a tumorigenic stem cell population unable to differentiate into a luminal phenotype [92]. Lentiviral targeted knockdown of PTEN by a siRNA construct also caused PIN lesions in prostate cells [49], as did lentiviral overexpression of both AR and Akt, causing prostate cancer [93]. In the Rb^{-/-}, p53^{-/-} prostate-specific deletion mouse model, SCA-1 was detected in invasive prostate cancer cells [94] at proximal regions of the prostate where murine prostate stem and progenitor cell populations had previously been described [80]. In contrast to PTEN mice, these prostate cancer lesions were positive for luminal and neuroendocrine markers but failed to express basal cell markers. Finally, SCA-1⁺ prostate epithelial cells with constitutively active AKT can initiate prostate carcinogenesis [95].

CSCs and implications for therapeutic applications

The successful elimination of a cancer requires anticancer therapy that affects the differentiated cancer cells and the potential CSC population [96]. At present, conventional anticancer therapies include chemotherapy, radiation and immunotherapy [97–99] and kill rapidly growing differentiated tumor cells, thus reducing tumor mass but potentially leaving behind cancer-initiating cells (Box 2). Therapies that exclusively address the pool of differentiated cancer cells but fail to eradicate the CSC compartment might ultimately result in relapse and the proliferation of therapy-resistant and more aggressive tumor cells, causing the death of the patient. An ideal drug regime would kill differentiated cancer cells and, at the same time, specifically, selectively and quickly target and kill CSCs to avoid toxic side effects for other cell types and to counteract the mutagenic evasive potential of CSCs

Review

Box 2. Future CSC-targeting therapies

Anticancer drugs kill cancer cells and cause a reduction in tumor mass. Frequently, however, the cancer re-emerges after a few months or even years because the therapy failed to kill the CSCs. Future anticancer drugs need to be tested for their efficacy in killing CSCs rather than the bulk population of cancer cells. Leukemia and lymphoma chemotherapies are more successful than other anticancer treatments, probably because they include the destruction of the resident hematopoietic stem cell population in the bone marrow of the patient, thus killing all CSCs. New generations of anticancer drugs will be developed to selectively and specifically seek and destroy various different populations of CSCs but leave the normal stem cell population unharmed to allow for complete regeneration of the specific tissues.

(Figure 3). The identification of CSC markers and their exploitation in targeted chemotherapy is an ultimate goal and responsibility of present day cancer research. Specific therapeutic targeting of CSCs requires an intricate knowledge of the biology of these cells.

Current anticancer therapeutic strategies include surgery, chemotherapy, radiation and hormonal ablation. Classical androgen ablation therapy has been used in prostate cancer and eliminates androgen-dependent prostate cancer cells. However, this treatment is controversial and does not take into account the renewal and growth potential of androgen-independent prostate cancer stem/progenitor cells. It is also possible that hormonal depletion therapy might support the development of androgen-independent, highly aggressive and incurable prostate cancer [100].

The presence of active transmembrane ABC transporter family members, such as multidrug resistance transporter 1 (MDR1) and ABCG2, can facilitate the efflux of DNA-binding dyes such as Hoechst 33342 in cells with (cancer) stem cell activity known as the 'side population' (SP). The SP fraction has been identified in numerous cancers, including neuroblastoma, breast cancer, prostate cancer and gastrointestinal carcinoma, and their chemoresistance is due to the ability of multidrug-resistant stem/progenitor cells to efflux anticancer drugs such as mitoxanthrone, gemcitabine, doxorubicin or 5-fluorouracil [44,101–103]. Moreover, in the presence of mitoxanthrone, the SP cell frequency in neuroblastoma was increased [102]. Enhanced resistance of CD133⁺ brain tumor stem cells to the chemotherapeutic agents paclitaxel (TaxolTM), carboplatin, etoposide (VP16), and temozolomide has been attributed to higher expression of ABCG2, increased activity of DNA mismatch repair genes and an altered balance of intracellular pro-/antiapoptotic factors [104]. Recently, a molecular link between the hyaluronan receptor and the stem cell markers CD44 and MDR-1 was reported in breast and ovarian cells [45]. Treatment of SK-OV-3.ipl (a human ovarian tumor cell line) and MCF-7 (human breast cancer) cells with hyaluronic acid (HA) resulted in (i) the nuclear accumulation of signal transducer and activator of transcription protein 3 (STAT-3)–NANOG complexes and stimulation of STAT-3-specific genes, including *MDR-1* gene expression, and (ii) the formation of a functional complex between CD44, MDR-1 and the cytoskeletal factor ankyrin. Thus, blockage of HA binding to CD44 might target stem/progenitor cells, inhibit

Figure 3. Dynamic effects of therapeutic strategies directed against cancer stem cells (CSCs). A tumor tissue is a complex mix of cancer cells at various stages of differentiation, from uncommitted CSCs through various stages of cancer progenitor cells to matured cancer cells, with a concomitant decrease in the levels of proliferative and/or metastatic potential. Both the CSC niche with supporting cell types and the matured cancer cell compartment (supportive cells are not shown) create an intricate network of interdependency. Cancer therapy should ideally address both the CSCs and the matured cancer cells by slowing down proliferation and production of differentiated cancer cells and increasing apoptosis in both CSCs and matured cancer cells. In a fast-growing cancer, tumor therapy might come too late and/or be ineffective (1) or reduce tumor mass by killing matured cancer cells without targeting the CSC niche (2). The latter effect might stimulate CSC proliferation and increase the CSC pool (3), which would consequently result in a resurgence of even larger numbers of matured cancer cells (4). In another scenario, therapeutic intervention itself might provoke an enlargement of the CSC pool by selecting for more radio- and chemoresistant CSC clones (5). These CSCs will have a superior ability to repair DNA damage upon radiotherapy and/or overexpress members of the ABC transmembrane pumps, resulting in the swift efflux of certain chemotherapeutics. Over time, this new generation of CSCs could also include new mutant CSCs with even more aggressive signatures (5). CSC therapy targets the CSC niche itself by attenuating the self-replicating potential of CSCs and disturbing cellular crosstalk within the CSC niche (6). Increased apoptosis of CSCs will result in a significantly smaller number of matured cancer cells, which can then be addressed successfully with common anticancer therapies (7). Thus, anticancer therapy that only results in apoptosis of the matured cancer cells and/or only inhibits the proliferation of CSCs provides a potential window of opportunity for new and more aggressive CSC mutants to occur and might be unsuccessful if not dangerous. It is expected that the elimination of cancer should target the CSC pool, and successful treatment regimes would need to be the result of an orchestrated 'target and destroy mission' aimed at CSCs and matured cancer cell populations.

MDR-1-mediated efflux and increase drug retention [45]. CD44⁺, CD24^{-/low} mammosphere cell cultures established from MCF-7 human breast cancer cells and isolated mammary gland progenitor cells are also less sensitive to radiation [105].

The overexpression of the Wnt/ β -catenin pathway in isolated progenitor cells and in the Sca-1⁺ immortalized COMMA-D β -geo murine mammary gland cell line further enhanced the radio-resistance of these progenitor cells [105,106]. Inhibition of the hedgehog pathway by the SMO signaling inhibitor cyclopamine or an anti-SHH antibody has been shown *in vitro* and *in vivo* to result in the arrest of growth and invasion of metastatic prostate cancer cells without affecting normal prostate epithelial cells [107,108].

These new therapeutic strategies target signaling pathways that are involved in the self-renewal processes of cancer stem/progenitor populations (Figure 3) and block the growth of differentiated tumor cells (Table 3). Thus, novel small molecules and specific antibodies have the potential not only to reduce tumor mass but also to eradicate the self-renewable source of CSCs [109,110]. Examples include the reduction of the SP fraction of metastatic UMSSC10B and HN12 head and neck cancer cell lines by (i) the targeting or inhibiting of membrane-anchored tyrosine kinase receptor signaling by EGFR and Her2/Neu [111,112] and (ii) the supra-additive combinatorial treatment of prostate cancer cell PC-3 xenographs in mice, which combines docetaxel or the anti-EGFR antibody cetuximab with sunitinib malate (SU11248), an oral multi-tyrosine kinase receptor block targeting vascular endothelial growth factor (VEGF)-1, -2 and -3/platelet-derived growth factor (PDGF)- α and - β /KIT/FLT-3 [113]. A new class of peptide molecules, apoptin, brevinin-2R, E4orf4 and hamlet, are emerging that have the capacity to (semi)specifically kill cancer cells [53,114,115]. Some of them even appear to 'hijack' signaling pathways that normally promote cell survival and proliferation and redirect them to promote cell death [53,114]. Elucidating the mechanisms by which these promising anticancer peptides might kill CSCs could provide the basis for new gene- and peptide-based therapies.

Table 3. A selection of inhibitors of signaling pathways involved in self-renewal, growth and survival of stem/progenitor cells

Target	Inhibitor
Tyrosine kinase receptors	
ErbB1	Gefitinib, erlotinib, lapatinib, PKI-166, mAB-C225, EKB-569, cetuximab
ErbB2/Neu	Trastuzumab, PKI-166, lapatinib, TAK165
ErbB1-4	CI1033
PDGFR β /KIT/ABL	Imatinib mesylate, sunitinib malate (SU11248)
PDGFR β /FLT3	Sorafenib, SU11248
VEGFR1-3/PDGFR β /KIT	CEP-7055, AZD2171, AMG-706, Vatalanib, SU11248
Hedgehog	Cyclopamine, anti-SHH antibody
Wnt/β-catenin	Anti-Wnt antibody, WIF-1
Notch	DAPT, GSI-18
ABC multidrug efflux transporters	
MDR1/ABCB1	Gefitinib, CI1033, tamoxifen, MS-209, Cyclopamine
ABCG2	Gefitinib, CI1033, tamoxifen, MS-209, Cyclopamine
MRP1/ABCC1	MS-209

Box 3. Outstanding questions

Little is known about how many of the stem cell markers function:

- What are the (potential) short-term and long-term side effects of (cancer) stem-cell-directed therapies?
- Will such therapies, if not selective for CSCs, lead to premature, selective senescence of the targeted tissue or organ due to the depletion of the 'reserve' stem cells?
- Would such treatment lead to an acute and irreversible organ failure, as is sometimes observed with classical chemotherapies (e.g. depletion of bone marrow upon methotrexate therapy)?
- Is trans-differentiation a common phenomenon or rather a sign of contamination of committed stem cells with pluripotent stem cells?
- Can current clinically available chemotherapies be adapted for targeting of stem cells?

Conclusions

The rapid development of the CSC field, combined with genome-wide screening techniques, has allowed the identification of important new (cancer) stem cell markers, and these discoveries have contributed to one of the most important quests of modern medicine, a cure for cancer. Several important issues still remain to be resolved (Box 3), and little is known about how many of the stem cell markers function and affect stem cells. Few experiments illustrate the specific role of a single marker among the combination of markers that define a given committed stem cell stage and how selected targeted disruption of a single marker impacts CSCs. Attempts have been made to target CSCs with specific antibodies and/or small molecules (e.g. targeting the IL4-CXCR4 axis in CD133⁺ colon CSCs [29]), and initial results are promising, but little is known about the potential short- and long-term side effects of these (cancer) stem-cell-directed therapies. Will such therapies, if not specific for CSCs, lead to tissue and/or organ damage due to the depletion of the 'reserve/regenerative' stem cells? Would such treatment lead to an acute and irreversible organ failure, as is sometimes observed with classical chemotherapies (e.g. during depletion of bone marrow upon methotrexate therapy)?

Major challenges in the CSC field still lie ahead (Box 4). We need to discover more specific markers and understand their physiological roles to better define the transition from pluripotency to various stages of tissue commitment and apply this knowledge to novel therapeutic targeting strategies. Research on the selective targeting of CSCs is still in its infancy. The development of tools for the modulation of expression and precise targeting of specific subpopulations of CSCs will emerge as the major focus of drug development not only for cancer treatment but also for regenerative medicine. As for *Rb*, *p53* and other major (proto)oncogenes, there is an acute need for the mapping of gene mutations and the identification of the importance of post-translational protein modifications [116] in stem cell markers. This will not only facilitate the development of more sophisticated therapies but will also refine detection methods. When a sufficient number of CSC markers becomes available, CSC-specific therapies might be developed that spare healthy stem cells and thus reduce side effects and retain regenerative tissue capacities.

Undoubtedly, discoveries made in the CSC field will feed back into other areas of stem cell research because

Box 4. Goals ahead

- The development of therapeutics that can selectively target CSCs at various stages of differentiation.
- The discovery of additional CSC markers to allow more specific targeting.
- Better characterization of the biological function of known CSC markers.
- The discovery and characterization of mutational 'hot spots' within known CSC markers.
- The development of new therapy monitoring methods based on screening of CSC markers.
- The development of therapeutic systems that would target only CSCs with well-defined markers, thus sparing healthy stem cells and reducing side effects.

many marker gene products (non-modified and non-deregulated forms) found in CSCs are shared with the normal stem cell population. It is also expected that a better understanding of the processes that control autonomous growth, differentiation and cell migration will contribute to novel regenerative-medicine-based treatments that will revolutionize therapeutic strategies and bring renewed hope to cancer patients.

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