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Targeting Human Cancer Stem Cells with Monoclonal Antibodies

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Abstract

Cancer stem cells (CSCs) constitute a distinct subpopulation of tumor cells that exhibit self-renewal and tumor initiation capacity and the ability to give rise to the heterogenous lineages of cancer cells that comprise the tumor. CSCs possess various intrinsic mechanisms of resistance to conventional chemotherapeutics, novel tumor-targeted drugs and radiation therapy, permitting them to survive current cancer therapies and to initiate tumor recurrence and metastasis. Different cell surface and transmembrane proteins expressed by CSCs, including CD44, CD47, EpCAM (CD326), CD123, CD133, GD2, Lgr5, insulin-like growth factor receptor I (IGF-IR), and members of the Notch and Wnt signaling pathways, have been identified and mainly used for the characterization of CSCs in experimental settings. Recently, monoclonal antibodies and antibody constructs such as Triomabs and BiTEs raised against these CSC proteins have shown efficacy against CSCs in human xenograft mice, and some of them have been demonstrated to induce tumor regression in clinical trials.

Since current cancer therapies fail to eliminate CSCs, ultimately leading to cancer recurrence and progression, selective targeting of CSCs with mAbs and antibody constructs reviewed herein may represent a novel and promising therapeutic strategy to eradicate cancer.

Keywords: Cancer stem cells (CSCs); Monoclonal antibody (mAb); Antibody construct; Novel drugs; Targeted therapy; Cancer therapy

Introduction

Tumor cell populations are organized as unidirectional cellular hierarchies in which cancer stem cells (CSCs) constitute subsets of cells, which are distinguished from the bulk of the cells that they produce by their ability to indefinitely perpetuate the growth of a malignant cell population. The experimental demonstration of CSCs in a variety of human malignant tumors has led to the conceptual hypothesis that tumors, like physiologic proliferative tissues, are hierarchically organized and propagated by limited numbers of CSCs [1-10]. According to a consensus definition, these CSCs are cells within a tumor that possess the capacity to self-renew and to give rise to the heterogeneous lineages of cancer cells that comprise the tumor [11]. CSCs can be defined experimentally by their ability to recapitulate the generation of a continuously growing tumor in serial xenotransplantation settings [11]. Moreover, recent studies provide evidence for the existence and clinical relevance of CSCs in human cancer [12-14].

CSCs harbor numerous intrinsic mechanisms of resistance to conventional chemotherapeutic drugs, radiation therapy, and novel tumor-targeting drugs that permit CSC survival of current cancer therapies and CSC-mediated initiation of tumor recurrence and metastasis [5,15,16]. Seminal studies indicate that CSCs can even be enriched by conventional chemotherapeutic drug, as demonstrated in breast cancer patients receiving systemic chemotherapy comprising conventional cytotoxic drugs [12,17-19]. Moreover, many novel tumor-targeted drugs, including tyrosine kinase inhibitors and some established monoclonal antibodies (mAbs) fail to eliminate CSCs [20-24], so that there is an urgent need for novel agents and strategies that effectively target CSCs for the use in elaborated clinical settings, preferably in combination with conventional cytostatic drugs, novel tumor-targeted agents, and radiation therapy.

During the past two decades, several mAbs have emerged as effective targeted therapies for the treatment of human malignancies, and various mAbs have been approved and established for the immunotherapy of cancer, most notably the classical mAbs rituximab (anti-CD20) and trastuzumab (anti-HER2) for the treatment of lymphoma and HER2-positive breast cancer, respectively [25,26]. Given their high target antigen specifity and generally minimal toxicity, mAbs and novel antibody constructs are well positioned as potent and specific agents for the elimination of CSCs [27]. In contrast to conventional cytostatic drugs, radiotherapy and novel tumor-targeted drugs, mAbs raised against cancer cell-specific or CSCs-specific cell surface proteins exploit the host´s immune system to eradicate the cells targeted by the mAb by using classical humoral and cellular immune mechanisms, including antibody-dependent cellular cytotoxicity (ADCC), complement-dependent cytotoxicity (CDC), inhibition of receptor-mediated signal transduction, induction of apoptosis, and priming of antigen-presenting cells and effector and memory T cells against targeted tumor antigens [28]. Therefore, targeting of cancer cells and CSCs with specific mAbs and antibody constructs appears not as a single job seen with conventional cytostatic and radiation therapy, but is substantially supported by the host´s immune system.

In the last few years, several mAbs and antibody constructs that selectively target CSCs have been developed and validated. Interestingly, some of these novel agents exhibit not only activity against CSCs, but also against regular tumor cells, as demonstrated by induction of tumor regression in xenograft mouse models and in clinical phase I-III studies (Table 1).

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Compound	Class	Targets	Clinical Status	References
H90 (anti-CD44)	Mouse IgG1 mAb	Acute myeloid leukemia (AML) SCs	Preclinical, xenograft mice	[70]
P245 (anti-CD44)	Mouse IgG1 mAb	Breast CSCs	Preclinical, xenograft mice	[77]
H4C4 (anti-CD44)	Mouse IgG1 mAb	Pancreatic CSCs	Preclinical, xenograft mice	[83]
GV5 (anti-variant 8-epitope of CD44R1)	Human recombinant IgG1 mAb	Uterine cervix and larynx CSCs	Preclinical, xenograft mice	[84]
RO5429083 (anti-CD44; targets a glycosylated, conformation-dependent epitope of CD44)	Humanized recombinant IgG1 mAb	Head and neck squamous cell carcinoma	Preclinical, xenograft mice	[86]
		Patients with metastatic and/or locally advanced, CD44-expressing malignant solid tumors	Phase I clinical study	[88]
		Patients with AML	Phase I clinical study; RO5429083 alone or in combination with cytarabine	[89]
B6H12.2 (anti-CD47, blocking)	Mouse IgG1mAb	AML SCs, bladder CSCs, glioblastoma CSCs, colon CSCs	Preclinical, xenograft mice	[100] [101] [102]
		Ovarian, breast, colon, bladder, prostate, hepatocellular, lung, kidney and gastric carcinomas, head and neck squamous cell carcinoma, sarcomas and glioblastomas	Preclinical, xenograft mice	[100]
		Non-Hodgkin lymphoma, acute lymphoblastic leukemia, multiple myeloma	Preclinical, xenograft mice	[97] [103] [104] [105]
B6H12 (anti-CD47, blocking)	Humanized IgG1 mAb	Aggressive metastatic leiomyosarcoma	Preclinical, xenograft mice	[107]
7G3 (anti-CD123)	Mouse IgG2a mAb	AML SCs	Preclinical, xenograft mice	[111]
CSL360 (anti-CD123)	Humanized recombinant chimeric IgG1 mAb	Patients with relapsed, refractory or high risk AML	Phase I clinical study	[113]
CSL362 (anti-CD123)	Humanized recombinant chimeric IgG1 mAb	Patients with AML in remission	Phase I clinical study	[114]
Fusion of anti-CD123 scFv and anti-CD3 scFv	BiTE; human recombinant bispecific mAb construct,	AML blasts and AML SCs	Preclinical, *in vitro*	[115]
Fusion of anti-CD123 scFv, anti-CD33 scFv and anti-CD16 scFv	Human recombinant trispecific mAb construct	Primary AML cells	Preclinical, *in vitro*	[116]
^{111}In-NLS-7G3 (anti-CD123 7G3 modified with 13-mer peptides harboring the nuclear translocation sequence of SV-40 large T-antigen, and labelled with ^{111}Indium)	Mouse IgG2a mAb, labelled with nuclear translocation sequence of SV-40 large T-antigen, and 111Indium	Primary AML cells	Preclinical, xenograft mice	[117]
MT110 (Solitomab) (anti-EpCAM/ anti-CD3)	BiTE; human recombinant single-chain bispecific bifunctional mAb construct	Breast CSCs, liver CSCs, colon CSCs, pancreatic CSCs	Preclinical, xenograft mice. Phase I clinical study, advanced solid tumors	[128] [129] [130] [131] [132]
Catumaxomab (Removab) (anti-EpCAM/ anti-CD3/Fcγ)	Triomab; recombinant chimeric two half antibody, each with one light and one heavy chain from mouse IgG2a and rat IgG2b isotypes. Bispecific, trifunctional mAb construct	CSCs in malignant ascites from metastatic human ovarian, gastric and pancreatic cancer. Patients with non-small cell lung cancer, and epithelial cancers	Phase I-III clinical studies, malignant pleural effusions, malignant ascites, peritoneal carcinomatosis. Non-small cell lung cancer, ovarian, gastric and epithelial cancer, intraperitoneal administration. Phase I clinical studies, lung cancer, epithelial cancers, intravenous administration	[139] [140] [141] [142] [144] [145]
AVE1642 (anti-IGF-IR)	Humanized recombinant IgG1 mAb, derived from mouse anti-IGF-IR IgG1mAb EM164	Myeloma cell lines colon CSCs. Patients with relapsed multiple myeloma and advanced solid tumors	Preclinical, in vitro, xenograft mice. Phase I clinical study, relapsed multiple myeloma, advanced solid tumors	[159] [157] [153] [154]

Figitumumab (CP-751,871) (anti-IGF-IR)	Humanized recombinant IgG2 mAb	Colon CSCs. Patients with non-small cell lung cancer and other solid tumors	Preclinical, in vitro, xenograft mice. Phase I-III clinical study, patients with non-small cell lung cancer and other solid tumors	[159] [161] [159] [158] [150] [162]
Demcizumab (OMP-12M18) (anti-Dll4)	Humanized recombinant IgG mAb	Colon CSCs, breast CSCs, colon cancer cells with KRAS mutations, pancreatic CSCs, melanoma CSCs, triple-negative breast cancer cells	Preclinical, xenograft mice. Phase I clinical study, single-agent, patients with solid tumors. Phase I clinical studies, combination with cytostatic drugs, in patients with colorectal cancer, pancreatic cancer and non-small cell lung cancer	[57] [58] [59] [60] [169] [170] [171] [172-175]
OMP-18R5 (anti-Frizzled 1, 2, 5, 7, 8)	Humanized recombinant IgG2 mAb	Pancreatic CSCs, breast CSCs, colon cancer cells, breast cancer cells, lung cancer cells, pancreatic cancer cells Patients with solid tumors	Preclinical, xenograft mice. Phase I clinical study in patients with solid tumors	[61] [62] [63] [182]

Table 1: Antibodies and antibody constructs that target human CSCs.

Resistance of CSCs to Novel Tumor-Targeted Drugs

Similar to conventional anticancer drugs, numerous novel tumor-targeted drugs were designed to target rapidly proliferating cancer cells, so that CSCs might be relatively insensitive to these drugs. For instance, imatinib has been developed as an inhibitor of the Bcr-Abl tyrosine kinase, which constitutes the fusion protein product of a chromosomal translocation (so called Philadelphia chromosome) that acts as a molecular switch for proliferation and differentiation of multipotent progenitor cells in chronic myeloid leukemia (CML) [29]. Imatinib has been shown to eliminate proliferating, committed leukemia progenitors, but not nonproliferating CML stem cells [30-33], which persist after imatinib therapy [21], and, after initial therapeutic success, many CML patients become resistant to imatinib therapy [33].

Furthermore, trastuzumab, a humanized monoclonal antibody directed against HER2 has been developed to treat patients with HER2-overexpressing breast cancers that represent one fourth of all breast cancer patients [34]. HER2 is a member of the human epidermal growth factor receptor tyrosine kinase family that is preferentially expressed in breast and ovarian cancer and that activates signaling pathways that promote tumorigenic cellular processes, such as proliferation and evasion of apoptosis [35,36]. However, trastuzumab mono-therapy in patients with HER2-overexpressing metastatic breast cancer shows a response rate of no more than 30 % [37], and primary or acquired resistance to trastuzumab occurs frequently in different clinical settings even when combination regimes are used [34,38,39]. One important mechanism of trastuzumab resistance in the therapy of HER2-overexpressing breast cancer might be the failure of trastuzumab to target breast CSCs [20,22,23,40]. Trastuzumab has been shown to exert its antitumor activities only effectively in the presence of a normal PI3K signaling pathway and in the presence of PTEN [41-44], but CSCs display aberrant PIK3K signaling and loss of PTEN [45]. A similar scenario has been reported for the small molecule dual inhibitor of EGFR tyrosine kinase and HER2 tyrosine kinase, lapatinib, which loses its therapeutic activity in breast cancers displaying loss of PTEN [46].

Finally, sorafenib, a small molecule inhibitor of multiple tyrosine kinases involved in tumor proliferation and tumor angiogenesis, including Raf, VEGFR, PDGFR and FLT3 [47] has been established in the treatment of acute myeloid leukemia (AML), because FMS-like tyrosine kinase 3 (FLT3) is overexpressed in leukemic blasts in almost all cases of AML [48]. As demonstrated recently, sorafenib is capable of reducing the number of mature AML progenitors, but fails to eradicate AML stem and primitive progenitor cells due to robust protection of these cells by the bone marrow stromal microenvironment [24], providing a further paradigm that novel tumor-targeted drugs fail to eliminate CSCs.

The Challenge of Targeting CSCs and their Progeny

According to the CSC concept of carcinogenesis [1-10], CSCs represent novel and translationally relevant targets for cancer therapy, and the identification, development and therapeutic use of compounds and drugs that selectively target CSCs is a major challenge for future cancer treatment [5,15,49]. However, the goal for any CSC-directed therapy should be the eradication of all CSCs in a patient, and the efficacy of single agents targeting CSCs may be limited by several factors. CSCs can represent a heterogenous population that may not be homogeneously sensitive to a given anti-CSC agent [9,50], and, under the selection pressure of agents targeting CSCs, therapy resistant CSC clones may emerge [51]. Therefore, the eradication of all CSCs will likely require targeting of more than one intrinsic pathway operating in CSCs to reduce the probability of escape mutants [15]. Moreover, agents causing tumor regression in advanced stages of cancer likely reflect effects on the bulk tumor population, but may have minimal effect on the CSC population. In contrast, a CSC-specific therapy would show modest effect on tumor growth of the bulk tumor population in advanced stages of cancer, but may have substantial clinical benefit in early stages of cancer as well as in neoadjuvant and adjuvant clinical settings [52]. Ultimately, cure of cancer will require the eradication of all malignant cells within a patient´s cancer: CSCs and their progeny. Therefore it will be important and promising to combine in sophisticated clinical settings CSC targeting mAbs with novel tumor-targeted drugs and conventional cytotoxic drugs. Such combinations

may act in concert to eradicate CSCs, differentiated progenitors and bulk tumor cells in cancer patients [53].

Compounds and Drugs that Target CSCs

Various compounds and drugs that selectively target CSCs have been discovered recently [53]. These agents include microbial-derived and plant-derived biomolecules [16,54,55], small molecule inhibitors targeting key components of intrinsic signaling pathways of CSCs, some classical drugs, such as metformin, tranilast and thioridazine [53], and mAbs and antibody constructs directed against CSC-specific cell surface molecules [27,56].

Although these compounds, including mAbs and antibody constructs, have been shown to effectively target signaling pathways and/or molecules selectively operating in CSCs, some of them are also capable of killing other types and subpopulations of cancer cells, which do not display CSC properties. Finally, recent data obtained in xenograft mice with human cancers indicate that mAbs and antibody constructs targeting CSCs are most effective in eradicating CSCs and their progeny when these agents are combined with conventional cytostatic drugs [57-63], envisioning the use of complex combination therapies for the future treatment of cancer.

Antibodies against CSC Surface Molecules, Tested for Anti-CSC Activity

These mAbs and antibody constructs have been demonstrated to exhibit significant anti-CSCs activity *in vitro* and in human xenograft mice (Table 1).

Anti-CD44

CD44 is a transmembrane glycoprotein and the receptor for hyaloronic acid, osteoponitin, collagens, fibronection, selectin and laminin that mediates adhesive cell to cell and cell to extracellular matrix interactions through binding to hyaloronic acid and its other ligands [64]. Overexpression of CD44 is observed in many tumor cells and is associated with aggressive tumor growth, invasion and metastasis [65]. CD44 was first described as a CSC marker in breast cancer [66] and has subsequently been shown to be expressed on CSCs in bladder, gastric, prostate, pancreatic, ovarian, colorectal and hepatocellular carcinomas and head and neck squamous cell carcinomas [67]. CD44 plays an important role in the regulation of normal and malignant myelopoiesis and is abundantly expressed on leukemic blasts in all human acute myeloid leukemia (AML) subtypes and on AML CSCs [27,68-70]. Moreover, a number of recent studies suggest that CD44 fulfil some of the special properties that are displayed by CSCs, including self-renewal, niche preparation, EMT and resistance to apoptosis [71]. Therefore, targeting CD44 by monoclonal antibodies appears as a reasonable strategy to eliminate CSCs [27,72].

H90 is a mouse IgG1 monoclonal antibody (mAb) directed against human CD44 [73]. Ligation of CD44 by H90 activates CD44 signaling, reverses myeloid differentiation blockage and induces myeloid differentiation in AML blasts of subtypes M1 to M5 obtained from different patients [74]. H90 also inhibits proliferation, induces terminal differentiation and mediates apoptosis in human myeloid leukemia cell lines [75,76]. Notably, H90 is the first mAb that has been shown to target CSCs. As demonstrated in a seminal study in 2006, H90 induces terminal differentiation and inhibits engraftment, homing, proliferation and the repopulation capacity of human AML SCs in NOD-SCID mice engrafted with CD34+ CD38- AML SCs isolated from AML patients [70]. By contrast, H90 treatment of NOD-SCID mice engrafted with human bone marrow CD34+ hematopoietic progenitor cells or cord blood had only a minor effect on inhibition of normal hematopoietic engraftment, indicating the specificity of H90 for AML SCs [70]. Colletively, this study reveals for the first time that CD44 is a key regulator of AML SC function that is essential for proper homing of AML SCs to microenvironmental niches and for maintaining AML SCs in a primitive state [70].

Another mouse IgG1 mAb raised against human CD44 is P245, which has been shown to reduce tumor growth and to eliminate breast CSCs in xenograft mice with human triple negative basal-like breast cancer [77]. Triple-negative (negative expression of estrogen receptor, progesterone receptor, and HER2) basal-like breast cancer cells resemble many features of breast CSCs, including expression of CD44high, CD24low and ALDH-1. The triple negative basal-like subtype of breast cancer is characterized by a high content of breast CSCs, aggressive proliferation, high metastatic capability and poor overall survival of patients [78-81]. P245 has been shown to significantly reduce tumor growth in xenograft mice with human triple negative basal-like breast cancer [77]. Treatment of the mice with doxorubicin and cyclophosphamide, a cytostatic drug combination commonly used for the therapy of triple negative basal-like breast cancer, resulted in complete histological tumor regression, but residual breast CSCs survived the doxorubicin/cyclophosphamide therapy and could be detected by virtue of their CD44 expression [77]. Tumor relapse mediated by the residual CD44 breast CSCs occurred 4-6 weeks after complete histological regression, but the relapse could be effectively prevented when P245 was systemically injected during the tumor regression period [77]. These data provide substantial evidence that anti-CD44 mAbs such as P245 can eliminate human breast CSCs and can prevent relapse of aggressive breast cancer.

H4C4 is a mouse IgG1 mAb that recognizes a constant region of the human CD44 receptor. The antibody was originally developed against human peripheral blood adherent cells and displayed the property of inducing *in vitro* homotypic aggregation of several types of human hematopietic cells and cell lines [82]. Recently, it was demonstrated that H4C4 markedly decreases tumosphere formation of human pancreatic cancer stem-like cells *in vitro* and inhibits human pancreatic tumor growth, metastasis and tumor recurrence in xenograft mice [83]. In particular, pretreatment of human MiaPaCa-2 pancreatic cancer cells with H4C4 dramatically decreased tumorsphere formation of $CD44^{+}$ MiaPaCa-2 cells and totally blocked tumorigenesis after implantation of the cells into nude mice. In xenograft mice bearing orthotopic human pancreatic cancer established by implatation of MiaPaCa-2 cells, treatment with H4C4 for 5 weeks resulted in significant inhibition of tumor growth and metastatsis as well as in inhibition of tumor recurrence after tumor elimination by radiation therapy with a total dose of 24 Gy. Finally, mechanistic experiments *in vitro* revealed that H4C4 inhibits CD44-STAT3 signaling pathways that is accompanied by the elimination of the pancreatic cancer stem-like cells [83].

A recombinant fully human IgG1 mAb recognizing the extracellular domain of an alternative splicing variant of CD44, CD44R1(v8-v10), was produced recently by genetical reshaping and class-switching from a human CD44R1-detecting IgM to a recombinant human CD44R1-detecting IgG1, termed GV5 [84]. GV5 showed preferential binding to the CD44R(v8-v10) isoform, which is overexpressed in colorectal, bladder, lung and larynx cancers as well as in basal-like breast cancers resembling breast CSCs [84,85]. In a xenograft mouse models, GV5 completely inhibited tumorigenesis and tumor formation of human ME180 uterine cervix carcinoma cells subcutaneously implanted with

orthotopic GV5 administration. Moreover, intraperitonal injections of GV5 markedly inhibited the growth of established tumors from human HSC-3 larynx carcinoma cells implanted into xenograft mice one week before the first GV5 treatment [84]. Results from *in vitro* experiments presented in the study strongly suggest that GV5 exerts its anti-tumor activity by induction of antibody-dependent cellular cytotoxicity (ADCC) and internalization of CD44R1 [84].

RO5429083 is a novel functional recombinant humanized IgG1 mAb that targtes a glycosylated, conformation-dependent extracellular epitope of human CD44 [85]. The antibody has recently been shown to markedly inhibit tumor growth in xenograft mice with human head and neck squamous cell carcinomas (HNSCC) induced by human CAL 27 HNSCC cells, whereas cisplatin, a cytostatic drug clinically used for HNSCC treatment, showed only a little effect on tumor growth inhibition [85]. Moreover, treatment of xenograft mice with RO5429083 inhibited constitutive epithelial growth factor receptor (EGFR) phosphorylation in CAL 27 HNSCC xenografts [85]. This is of considerable clinical importance because constitutive EGFR phosphorylation has been shown to be associated with early relapse and poor prognosis in patients with HNSCC [86,87]. Finally, RO5429083 treatment of peripheral mononuclear cells isolated from healthy human donors resulted in an expansion of cytolytic natural killer (NK) cells, demonstrating that RO5429083 is able to activate effector cells of the innate immune system [86]. These promising preclinical results have very recently led to the initiation of two phase I clinical studies: a study of RO5429083 in patients with metastatic and/or locally advanced, CD44-expressing, malignant solid tumors [88], and a study of RO5429083 alone or in combination with cytarabine in patients with acute myelogenous leukemia [89].

Anti-CD47

CD47 is a widely expressed transmembrane protein and a receptor for thrombospondin family members that also serves as the ligand for signal regulatory protein alpha (SIRPα) [90-92]. SIRPα is expressed on phagocytic cells, including macrophages and dendritic cells that when bound and activated by CD47 initiates a signal transduction cascade resulting in inhibition of phagocytosis [91,93,94]. Therefore, the CD47/SIRPα interaction has been attributed as a mechanism that provides a “don´t eat me”-signal [95]. Abundant expression of CD47 is exploited by tumor cells to avoid phagocytosis by tumor-associated macrophages and is required for the survival, growth and metastasis of hematopoietic and solid tumors [96-100].

A seminal study revealed that CD47 is abundantly expressed on human AML SCs and that CD47 is much more highly expressed on AML SCs than on their normal counterparts, such as hematopoietic stem cells (HSCs) and multipotent progenitor cells [101]. As investigated in a large cohort of AML patients, increased CD47 expression in human AML is associated with poor clinical outcome and worse overall survival, providing evidence that increased CD47 expression on AML SCs substantially contributes to the pathogenesis and fate of human AML [101]. It was shown in the study that the CD47-binding and CD47-blocking mouse IgG1 mAb B6H12.2 preferently enables phagocytosis of human AML SCs by human and mouse macrophages in vitro, whereas non-blocking anti-CD47 mAbs failed to enable phagocytosis of the AML SCs by human and mouse macrophages [101]. Similar results were obtained in a study using a blocking anti-CD47 mAb and human bladder CSCs highly expressing CD47 [102]. Further, in a xenograft mouse model, B6H12.2 prevented the engraftment of human AML SCs, and treatment of human AML xenograft mice with B6H12.2 completely eradicated AML cells by the mechanism of phagocytosis *in vivo*, whereas normal HSCs were not depleted [101]. Thus, human AML SCs can be targeted and eradicated with blocking anti-CD47 antibodies such as B6H12.2 capable of enabling phagocytosis of AML SCs.

A recent comprehensive study impressively shows that CD47 is highly expressed on virtually all human solid tumor cells, as demonstrated in ovarian, breast, colon, bladder, prostate, hepatocellular, lung, kidney and gastric carcinomas, sarcomas and glioblastomas obtained from patients [100]. The study further demonstrates that anti-CD47 blocking mAb B6H12.2 enables phagocytosis of patient-derived ovarian, breast and colon carcinoma cells as well as of glioblastoma CSCs and colon CSCs by mouse and human macrophages *in vitro*. Administration of B6H12.2 prevented and inhibited tumor growth in xenograft mice with patient-derived glioblastoma and ovarian, breast, colon and bladder cancer, and increased the survival of the xenograft mice over time. In xenograft mice transplantated with aggressive and spontaneously metastasizing patient-derived bladder cancer and head and neck squamous cell carcinoma, administration of B6H12.2 effectively prevented lung and lymph node metastases and also induced a significant inhibition of primary site tumor growth [100]. This study was extended to examine the effect of anti-CD47 B6H12.2 on human hematopoietic malignancies. In xenograft mice with human non-Hodgkin lymphoma [103], acute lymphoblastic leukemia [104] and multiple myeloma [105], B6H12.2 was able to inhibit tumor growth and dissemination by the mechanism of blocking the CD47-SIRPα interaction and enabling phagocytosis of CD47-expressing tumor cells. Interestingly, B6H12.2 did not induce ADCC or complement-dependent cytotoxicity (CDC) and exerted its anti-tumor activity exclusively by promoting phagocytosis of CD47-expressing tumor cells [97,105].

Finally, a fully humanized blocking anti-CD47 mAb (B6H12) [106] has recently been shown to effectively inhibit tumor growth and metastasis in xenograft mice with human aggressive metastatic leiomysarcoma [107]. These results collectively indicate that all human solid and hematopoietic tumor cells require CD47 expression to suppress phagocytic innate immune surveillance and elimination, and that blockade of CD47 function by anti-CD47 mAbs such as B6H12.2 and B6H12 represents a novel and effective strategy for cancer therapy.

Anti-CD123

The mouse IgG2a mAb 7G3 recognizes the N-terminal domain of the human interleukin-3 (IL-3) receptor α chain (CD123) and functions as a specific IL-3 receptor antagonist that antagonizes IL-3 biologic activities, such as histamine release from basophil granulocytes or IL-6 and IL-8 secretion from endothelial cells [108]. In comparison to normal hematopoietic stem cells (HSCs), CD123 is overexpressed on AML blasts, $CD34^+$ leukemic progenitors and AML SCs, and confers growth advantage of AML cells over HSCs [109-111]. Clinically, high CD123 expression in AML is associated with higher blast counts at diagnosis and a lower complete remission rate that results in poor prognosis and reduced survival [110,112], ultimately defining CD123 as a promising cell-surface target for the elimination of AML SCs and the eradication of AML.

In fact, the CD123-targeting antibody 7G3 has been shown to eliminate human AML SCs [111]. *Ex vivo* treatment of primary human AML cells with 7G3 selectively inhibited engraftment, repopulation ability, and bone marrow and spleen homing of the cells in NOD/SCID mice, whereas 7G3 treatment of normal HSCs derived from human cord blood or bone marrow resulted in significant engraftment

and hematopoietic differentiation of the human HSCs in NOD/SCID mice. Moreover, 7G3 selectively eradicated human AML SCs in NOD/SCID mice engrafted with primary human AML cells. It was further demonstrated that 7G3-mediated inhibition of engraftment and homing of AML SCs in NOD/SCID mice is dependent on ADCC induced by the Fc fragment of 7G3, and that 7G3 inhibits spontaneous and IL-3-induced proliferation of AML SCs *in vitro*. These pleiotropic activities of 7G3 against human AML cells and AML SCs finally led to the reduction of AML burden and to an improved long-term survival of NOD/SCID mice engrafted with human AML [111].

CSL360 is a recombinant chimeric IgG1 mAb derived from 7G3 that binds to the same epitope of the (IL-3) receptor α chain (CD123) as 7G3. In a phase I clinical study, 40 patients with relapsed, refractory or high-risk AML received 12 weekly intravenous infusions of CSL360 at dose levels from 0.1 to 10 mg/kg [113]. CSL360 treatment with a dose of ≥ 3 mg/kg resulted in sustained saturation and down-regulation of CD123 on AML blasts as well as in inhibition of *ex vivo* proliferative responsiveness of AML blast to IL-3. However, changes of the percentage of AML SCs in the patients´ bone marrow were not observed during the study. Only one patient became leukemia-free and achieved a complete remission after 12 doses, indicating that CSL360 has no significant anti-leukemic activity in high-risk AML patients, and suggesting that blockade of IL-3 signaling is an ineffective therapeutic strategy for these patients [113]. Nevertheless, a phase I study of CSL362, a second generation mAb targeting the IL-3 receptor α chain, in patients with $CD123^+$ acute myeloid leukemia in remission, has been started in September 2012 [114].

Some novel and interesting antibody constructs targeting CD123 and CD3 (bispecific antibody) or CD123, CD33 and CD16 (Fcγ-receptor III) (trispecific antibody) have been generated recently. The bispecific antibody constructs belong to the bispecific T cell engager (BiTE) class of antibodies and consist of a single chain variable region fragment (scFv) targeting CD123 and a scFv targeting the T cell receptor protein complex CD3. When expressed from CHO-S cells, CD123 scFv and CD3 scFv form a homodimer that closely resembles the structure of a natural antibody. This bispecific antibody construct is capable of redirecting and activating resting T cells ($CD3^+$) via its CD3 scFv and simultaneously binding to CD123, thereby inducing T-cell mediated lysis and apoptosis of $CD123^+$ AML blasts and AML SCs [115]. The trispecific constructs consist of scFvs binding to CD123, CD33 and CD16, and are able to target CD123/CD33 AML blasts and to mediate ADCC via binding of the Fcγ-receptor III (CD16) expressed on macrophages and NK cells. These antibody constructs have recently been shown to induce potent ADCC of primary leukemia cells isolated from peripheral blood or bone marrow of AML patients [116].

Finally, the anti-CD123 mAb 7G3 has recently been used for the generation of an Auger electron radioimmunotherapeutic agent for targeting $CD123^+$ AML SCs. The antibody was modified with 13-mer peptides harboring the nuclear translocation sequence (NLS) of SV-40 large T-antigen and with the chelating agent DTPA for labelling with the Auger electron-emitting isotope ^{111}Indium [117]. This radioimmunotherapeutic agent, termed 111In-NLS-7G3, binds effectively to primary $CD123^+$ AML cells from patients and is rapidly imported into the nucleus where it induces DNA double-strand breaks leading to apoptosis. As demonstrated in xenograft mice with human $CD123^+$ AML, ^{111}In-NLS-7G3 can also be used for highly sensitive imaging of $CD123^+$ AML cells or AML SCs in SPECT/CT [117].

Anti-EpCAM

EpCAM (epithelial cell adhesion molecule, CD326, ESA) is an integral transmembrane glycoprotein composed of a large extracellular domain, one transmembrane region, and a small 26 amino acid intracellular domain. Beyond its adhesion function, EpCAM can transduce multiple oncogenic signaling and gene expression pathways to the nucleus after regulated intramembrane proteolysis of its ectodomain and subsequent nuclear translocation of its intracellular domain [118]. Overexperession of EpCAM in combination with CD44 was first demonstrated for CSCs in human breast carcinomas [66], colorectal carcinomas [119], and pancreatic carcinomas [120], and can be regarded as a universal marker exclusively expressed by epithelial cancers and their CSCs [121-123].

MT110 (Solitomab) is bispecific bifunctional single-chain antibody construct of the BiTE class that binds to EpCAM (CD326) and to the T cell receptor protein complex CD3 [124]. MT110 is a recombinant protein construct that consists of 2 single-chain antibodies linked such that a single polypeptide chain of approximately 55 kDa is created. In contrast to bispecific trifunctional antibody constructs (Triomabs), MT110 and other BiTE class antibodies lack an Fcγ portion [125]. MT110 activates and redirects resting human peripheral $CD4^+$ and $CD8^+$ T cells to induce specific lysis and apoptosis of target cells expressing EpCAM [124-127]. Initially, it was demonstrated that MT110 is able to eradicate human colon cancer cells and patient-derived metastatic ovarian cancer in NOD/SCID xenograft mice [124]. Moreover, *ex vivo* treatment with MT110 of malignant pleural effusions obtained from patients with advanced breast cancer resulted in a specific lysis of pleural $EpCAM^+$ breast cancer cells by activated and redirected autologous $CD4^+$ and $CD8^+$ T cells, indicating that breast cancer patients with malignant pleural effusions might benefit from targeted therapy with MT110 [126]. Next, EpCAM-expressing CSCs derived from human liver and breast cancer tissues could be eradicated in soft agar colonies by MT110 in the presence of human T cells [128]. CSCs isolated from patient-derived primary colon or pancreatic cancers injected together with allogeneic or autologous (donor-derived) peripheral mononuclear cells into NOD/SCID mice were effectively eradicated by MT110, and the CSCs did not establish significant tumor growth in the NOD/SCID xenograft mice treated with MT110 [129,130]. These promising results suggest that EpCAM-expressing CSCs and cancer cells can effectively be eradicated by MT110. Consequently, a phase I clinical study of MT110 in patients with locally advanced, recurrent or metastatic solid tumors expressing EpCAM has been initiated in 2008 and is ongoing and recruiting participants [131]. Interim results from this study have been reported recently. Sixteen patients who received a dose of ≥ 24 μg/day MT110 were evaluable for anti-tumor response: best response of stable disease was observed in 38% of the patients, median duration was 155 days [132], pointing out an encouraging anti-tumor activity of MT110 in patients with advanced solid tumors.

Catumaxomab (Removab) is a chimeric antibody construct consisting of two half antibodies, each with one light and one heavy chain that originate from parental mouse IgG2a and rat IgG2b isotypes [133]. This antibody construct belongs to a novel family of bispecific, trifunctional antibodies termed Triomabs, and has two binding specifities directed against EpCAM and one against the T cell receptor protein complex CD3. With its Fc fragment, catumaxomab additionally binds Fcγ-receptor type I and III positive dendritic cells, macrophages and natural killer cells. Therefore, the anti-tumor activity of catumaxomab and other Triomabs such as Ektomun (see below) results from T-cell-mediated lysis and apoptosis induction,

ADCC, and phagocytosis via activation of Fcγ-receptor-positive accessory cells. Importantly, no additional activation of immune cells is necessary for effective tumor elimination by catumaxomab, which therefore represents a self-supporting system [134,135]. In contrast to mAbs of the BiTE class, Triomabs exert a vaccination effect and induce long-lasting antitumor immunity, most likely due to their Fc-mediated interaction with Fcγ-receptors expressed on dendritic cells and macrophages [136-138].

Catumaxomab has recently been shown to induce regression of malignant pleural effusions, malignant ascites and peritoneal carcinomatosis in patients with advanced epithelial cancers resistant to conventional chemotherapy [139-141]. In addition, catumaxomab is able to effectively eliminate CD133$^+$/EpCAM$^+$ CSCs from malignant ascites of patients with advanced ovarian, gastric and pancreatic cancer [142], indicating that catumaxomab can be therapeutically used to eradicate CSCs of epithelial cancers. Therefore, catumaxomab is currently in phase I-III clinical studies for local therapy in patients with advanced ovarian, gastric and non-small cell lung cancer, and is in the European Union on the market for the therapy of malignant ascites caused by epithelial cancers [143]. In a phase I clinical study of a single intravenous dose of catumaxomab at 3 dose levels (2 µg, 5 µg, and 7.5 µg) in 15 patients with advanced non-small cell lung cancer, catumaxomab exhibited considerable antitumor activity and favourable overall survival as well as low general toxicity and only transient elevation of liver enzyme levels (AST, ALT, and γGT) [144]. A phase I clinical study of ascending intravenous doses of catumaxomab in patients with EpCAM-overexpressing epithelial cancers has been initiated in 2011 and is ongoing and recruiting participants [145].

Other anti-EpCAM antibodies, including edrecolomab (Panorex®, mouse IgG2a mAb) and adecatumumab (MT201, a recombinant fully human IgG1 mAb) [146-148] have been tested in clinical studies, but have not yet been investigated for their activity against CSCs.

Anti-insulin-like growth factor receptor I (anti-IGF-IR)

Insulin-like growth factors (IGFs) constitute polypeptide hormones with anabolic and mitogenic activities that regulate cell growth and differentiation. Deregulation of IGF signaling has been widely demonstrated in the development and progression of multiple types of human cancer, consequently leading to the development and validation of therapeutics that target IGF signaling [149,150]. Various humanized recombinant mAb targeting the insulin-like growth factor receptor I (anti-IGF-IR), a transmembrane tyrosin kinase receptor for IGF-I and IGF-II that is overexpressed in many cancers [151,152], have been developed and tested in phase I-III clinical studies, alone or in combination with conventional cytostatic drugs and novel tumor-targeted drugs [150]. Although some IGF-IR-targeting mAbs, including figitumumab (CP-751.871), cixutumumab (IMC-A12), dalotuzumab (MK-0646), ganitumab (AMG-479), BII022 and AVE1642 showed rather disappointing results in clinical studies [150,153-156], AVE1642 and figitumumab have recently been demonstrated to target human colon CSCs *in vitro* and in xenograft mice [157,158].

AVE1642 is a humanized recombinant IgG1 mAb derived from mouse anti-IGF-IR IgG1 mAb EM164. AVE1642 inhibits growth and increases bortezomib-induced apoptosis of aggressive human myeloma cells *in vitro* [159]. Based on these results, a phase I clinical study of AVE1642 in combination with the proteasome inhibitor bortezomib was conducted in patients with relapsed multiple myeloma. Despite the favorable toxicity profile of the antibody, the response rates for patients treated with AVE1642 alone or in combination with bortezomib were considered insufficient to merit further development of AVE1642 in multiple myeloma [153]. However, AVE1642 has been shown to inhibit IGF-IR signaling and proliferation of colon CSCs enriched from the human colon cancer cell line HT29. Moreover, the antibody was able to induce significant regression of tumors established by the colon CSCs in xenograft mice [157], indicating a potential of AV1642 to eradicate colon CSCs.

Figitumumab (CP-751,871) is a fully human IgG2 mAb against the IGF-IR that have been tested in phase I-III clinical trials in patients with non-small cell lung cancer and other solid tumors with a rather disappointing outcome, leading to the discontinuation of two phase III trials due to insufficient clinical activity of the antibody [150,160-162]. Despite its rather weak anticancer activity in human solid tumors, figitumumab inhibited IGF-IR signaling, IGF-IR-induced Akt activation and growth of colon CSCs with enhanced IGF signaling that were enriched from human colon cancer cell lines [158]. Figitumumab also markedly inhibited growth of tumors established by the human colon CSCs in xenograft mice [158]. These results emphasize that IGF-IR targeting with mAbs may be used for the eradication of CSCs displaying deregulated IGF signaling, as demonstrated hitherto for human colon CSCs.

Anti-delta-like 4 ligand (anti-Dll4)

The Notch pathway is an evolutionary conserved signaling system that regulates cell fate specification, tissue patterning and morphogenesis by modulating cell differentiation, survival, proliferation and apoptosis. In mammals, the system consists of five canonical ligands, Delta-like (Dll) 1, 3, 4, Jagged 1, 2, and four single-pass transmembrane receptors, Notch 1-4 [163]. Aberrant Notch signaling has been implicated in promoting tumorigenesis and has been shown to critically regulate self-renewal, survival and multiresistance of CSCs [164,165]. Overexpression of Dll4 is found in tumor vasculature and in tumor cells to activate Notch signaling [166], and blockade of Dll4 signaling in tumors results in excessive, non-productive tumor vascularity, paradoxically associated with poor tumor perfusion, hypoxia and decreased tumor growth [167,168].

A humanized recombinant IgG mAb (OMP-21M18, demcizumab) that blocks Dll4 was shown to have anti-tumor activity independent of an angiogenic mechanism in xenograft mice with patient-derived colon and breast cancers [57]. Moreover, OMP-21M18 was able to reduce the frequency of CSCs in colon and breast tumors *in vitro* and *in vivo*, the latter was demonstrated in explanted tumors of xenograft mice treated with OMP-21M18. The combination of OMP-21M18 with the cytostatic drugs irinotecan and paclitaxel significantly reduced the frequency of colon CSCs and breast CSCs, respectively. This was observed *in vitro* and in explanted colon and breast tumors of xenograft mice treated with the antibody and the respective cytostatic drug [57]. OMP-21M18 also exhibited strong anti-tumor activity in patient-derived colon cancers with oncogenic *KRAS* mutations that are intrinsically resistant to epidermal growth factor receptor (EGFR) blockade with the mAb cetuximab [58]. In xenograft mice with patient-derived mutant *KRAS* colon cancers, OMP-21M18 was able to significantly reduce tumor growth that was further enhanced when OMP-21M18 was combined with irinotecan. Further analysis of mutant *KRAS* tumors explanted from xenograft mice indicated that OMP-21M18 in combination with irinotecan produced a significant decrease in colon CSC frequency while promoting apoptosis in tumor cells [58]. Similar results were obtained recently with OMP-21M18 alone and OMP-21M18 combined with the cytostatic drug gemcitabine in xenograft mice with patient-derived pancreatic cancer [59]. Furthermore, recent studies indicate that OMP-21M18 is able to inhibit tumor growth, recurrence and metastases, and to reduce the frequency of CSCs in xenograft mice

with patient-derived melanomas [169]. Even in xenograft mice with B-RAF V600E melanomas resistant to B-Raf inhibitors, OMP-21M18 was able to significantly reduce tumor growth and the frequency of melanoma CSCs [170]. In xenograft mice with patient-derived triple-negative breast cancer, which constitutes a progesterone-receptor-negative, estrogen-receptor-negative and HER2-negative aggressive subtype of human breast cancer containing high contents of CSCs [81], OMP-21M18 and OMP-21M18 combined with paclitaxel targets the tumor vasculature, inhibits tumor growth, delays tumor recurrence, and decreases the frequency of CSCs [60]. Finally, a recent study demonstrates that OMP-21M18 delays tumor recurrence, overcomes drug resistance, inhibits EMT, and decreases the frequency of CSCs in xenograft mice with patient-derived breast cancer and pancreatic cancer [171]. All these promising results from preclinical studies in xenograft mice with various patient-derived cancers have recently led to the initiation of phase I/Ib clinical studies of OMP-21M18 in patients with solid tumors [172], metastatic colorectal cancer [173], pancreatic cancer [174] and non-small cell lung cancer (NSCLC) [175].

Another humanized IgG mAb against Dll4 is REGN421, which has been developed on the basis of results of a study with polyclonal anti-mouse Dll4 antibodies exhibiting strong anti-tumor activity and induction of non-productive angiogenesis in mouse tumor models [167]. REGN421 is currently tested in a phase I clinical study in patients with advanced solid tumors [176].

Anti-Frizzled

The Frizzled (FZD) family of transmembrane G protein-coupled receptors consists of 10 members (FZD 1-10), which are activated by secreted lipoglycoproteins of the Wnt family [177]. The highly conserved Wnt/FZD signaling pathway has multiple functions in animal development and cell fate determination and polarization [178]. Deregulation of the Wnt pathway and up-regulation of FZD receptors play important roles in tumorigenesis and CSC maintenance that is observed in a variety of human cancers, including colorectal cancer, hepatocellular carcinoma and triple-negative breast cancer [164,178-181].

OMP-18R5, a humanized recombinant IgG2 mAb binding to a conserved epitope within the extracellular domain of FZD1, FZD2, FZD5, FZD7 and FZD8 has recently been developed. Through binding to FZD receptors, OMP-18R5 blocks Wnt3A-induced phosphorylation of the Wnt co-receptor LPR6 and accumulation of Wnt signaling protein β-catenin, thereby inhibiting canonical Wnt signaling [62]. Importantly, in contrast to the cytostatic drugs gemcitabine and paclitaxel, OMP-18R5 is able to reduce the frequency of CSCs in patient-derived pancreatic and breast carcinomas established in xenograft mice [61-63]. OMP-18R5 alone, and more effectively in combination with cytostatic drugs, inhibits tumor growth of patient-derived colon, breast, lung and pancreatic cancer in xenograft mice [61-63], pointing out that OMP-18R5 should be regarded as promising agent for the elimination of CSCs and the future treatment of solid tumors. Consequently, a phase I dose escalation study of OMP-18R5 in patients with solid tumors has been initiated in 2011 and is still recruiting participants [182].

Antibodies against CSC Surface Molecules, Not Yet Tested for Anti-CSC Activity

These mAbs and antibody constructs bind to cell surface molecules expressed by CSCs, but have not yet been demonstrated to exhibit anti-CSCs activity *in vitro* and in human xenograft mice (Table 2).

Anti-CD133

Human CD133 (prominin-1) is a transmembrane single-chain glycoprotein with two large extracellular loops containing four N-linked glycosylation sites on each extracellular loop, and two small intracellular loops [183,184]. Originally identified as a cell surface antigen present on CD34+ hematopoietic stem cells [183], CD133 has recently been established as marker for the isolation and analysis of CSCs in solid tumors, including brain tumors, and colon, prostate, lung, ovarian, pancreatic and hepatocellular carcinomas [184,185]. CD133 exhibits several splice variants and different poorly characterized glycosylated isoforms, such as CD133-1 and CD133-2, which are bound by the mouse IgG1 mAbs AC133 and AC141, respectively [183,185,186]. Although AC133 has been shown to be unsuitable for the detection of CSCs in glioblastoma, because glioblastoma CSCs can solely express non-glycosylated isoforms of CD133 not detectable by AC133 or AC141 [185,187,188], colon CSCs selectively express a distinct CD133 epitope which is bound by AC133 and which is lost upon colon CSC differentiation [189]. Therefore, AC133 can be used for the selective detection and isolation of colon CSCs, whereas its specificity for the detection of CD133+ CSCs in other solid tumors is uncertain [184,185].

Two mAbs, 32AT1672 (mouse IgG1) and C24B9 (rabbit IgG) that recognize unmodified non-glycosylated epitopes of CD133 are commercially available for research purpose [185]. C24B9 has recently been shown to detect a truncated variant of the CD133 protein expressed by glioblastoma cells that could not be detected by AC133 [187], ultimately indicating that CD133 exhibits numerous variants and epitope modifications detected by different mAb species. Therefore, it is still questionable whether CD133 represents a specific marker for CSCs and a therapeutic target for antibody-mediated elimination of CSCs.

Anti-GD2

Gangliosides represent a family of sialic acid-containing glycosphingolipids that are anchored to the plasma membrane by a lipophilic ceramide segment, with the antigenic hydrophilic carbohydrate section extending extracellularly. Through interactions with carbohydrate-binding proteins on apposing cells, gangliosides function as receptors in cell to cell recognition and also interact laterally in their own membranes to regulate the responsiveness of various signaling proteins [190]. GD2 is a b-series disialoganglioside selectively, but weakly expressed in the fetal and adult central nervous system, umbilical mesenchymal stem cells, peripheral nerves, and skin melanocytes [191,192]. However, GD2 is highly expressed in human tumors of neuroectodermal origin, including neuroblastomas, melanomas, gliomas, small cell lung carcinomas (SCLC), Ewing sarcomas as well as in osteosarcomas and some soft-tissue sarcomas [191]. Recently, it has been shown that GD2 is expressed on highly tumorigenic CSCs isolated and enriched from human breast cancer cell lines or breast cancer patient samples [193], thus identifying GD2 as a putative cell surface target for mAb-mediated eradication of breast CSCs. Because of the relatively selective expression of GD2 on the cell surface of neuroectodermal tumors and breast CSCs, GD2 is an attractive and promising target for tumor-specific antibody therapy. In fact, several mAbs against GD2 have been developed for clinical use and treatment of neuroectodermal tumors over the past two decades [194].

3F8 is a mouse IgG3 mAb raised against human GD2 that has undergone extensive preclinical and clinical testing [191,195]. Initially, it was demonstrated that 3F8 binds with high affinity to GD2 expressed on human melanoma cell lines as well as on human neuroblastoma and osteosarcoma cells from patient samples [195]. The mechanisms of tumor cell killing by 3F8 were shown to be mediated by ADCC and CDC [191,195]. In two clinical studies, one phase I study in patients with neuroblastoma and melanoma [196] and one phase II study in patients with stage IV neuroblastoma [197], intravenous administration of 3F8 displayed manageable toxicity and adverse side effects as well as marked anti-tumor activity. To circumvent human

Compound	Class	Targets	Status/Use	References
AC133, (anti-CD133-1); AC141 (anti-CD133-2)	Mouse IgG1 mAb	Poorly characterized glycosylated CD133 extracellular epitopes of uncertain specificity; colon CSCs	Experimental research, *in vitro* detection of CD133+ cells	[183] [185] [189]
32AT1672 (anti-CD133); C24B9 (anti-CD133)	Mouse IgG1 mAb; rabbit IgG mAb	Unmodified (non-glycosylated) extracellular epitopes of CD133	Experimental research, *in vitro* detection of CD133+ glioblastoma cells	[185] [187]
3F8 (anti-GD2)	Mouse IgG3 mAb	Human neuroblastoma and osteosarcoma samples and melanoma cell lines; patients with advanced neuroblastoma and melanoma	Phase I and II clinical studies in patients with neuroblastoma and melanoma	[195] [196] [197]
Hu3F8 (anti-GD2)	Humanized IgG1 and IgG4 mAbs	Human neuroblastoma cells, neuroblastoma xenograft mice; patients with neuroblastoma	Phase I clinical study in patients with high risk neuroblastoma and GD2-positive tumors	[198] [199]
3F8BiAb (anti-GD2/anti-CD3)	Heteroconjugate of 3F8 and mouse IgG2a mAb OKT3	Human neuroblastoma cell lines	Experimental research, in vitro killing of neuroblastoma cells by redirected activated T cells	[200]
Ch14.18 (anti-GD2)	Chimeric mAb: Fab of mouse anti-GD2 IgG2a mAb 14G2, fused with human Fc constant IgG1 regions	Patients with melanoma and high-risk neuroblastoma	Phase I clinical studies in patients with neuroblastoma and melanoma; phase III clinical studies, combination with cytokines and cytostatic drugs in neuroblastoma patients	[202] [206] [203] [204] [208] [205]
TRBs07, Ektomun, Ektomab (anti-GD2 and anti-GD3/ anti-CD3/Fcγ)	Triomab; recombinant chimeric two half antibody, each with one light and one heavy chain from mouse IgG2a (Me361) and rat IgG2b (26II6) isotypes. Bispecific, trifunctional mAb construct	Human melanoma cell lines; mouse melanoma model treated with Triomab Surek (anti-mouse-CD3/anti-GD2, anti-GD3/Fcγ)	Experimental research, *in vitro* killing of melanoma cells by redirected activated T cells; induction of tumor eradication and tumor immunity in a mouse melanoma model by Surek	[209] [137] [138]
KM4056 (anti-Lgr5)	Rat IgG2b mAb	CHO cells expressing the human CSC-marker Lgr5	Preclinical, xenograft mice	[217]
1D9, 4D11, 9G5 (anti-Lgr5)	Rat IgG mAbs	Colon CSCs isolated and enriched from patient-derived colorectal cancers	Preclinical, *In vitro*, xenograft mice, identification of colon CSCs	[218] [219]

Table 2: Antibodies and antibody constructs against CSC surface moelcules, not yet tested for anti-CSC activity.

anti-mouse antibody response against 3F8 and to enhance the ADCC properties of 3F8, humanization of 3F8 was performed, and humanized recombinant IgG1 and IgG4 mAbs (Hu3F8-IgG1 and Hu3F8-IgG4) were produced [198]. Hu3F8-IgG1 was intensively tested *in vitro,* and displayed more potent ADCC mediated by both peripheral blood mononuclear cells and polymorphonuclear leucocytes than 3F8. Moreover, Hu3F8-IgG1 was able to induce a stronger anti-tumor effect in human neuroblastoma xenograft mice than 3F8 [198]. This superior therapeutic activity of Hu3F8-IgG1 compared to 3F8 has recently led to the initiation of a phase I clinical study of Hu3F8 in patients with high-risk neuroblastoma and GD2-positive tumors [199]. Further, a bispecific mAb targeting both GD2 and CD3 was generated by chemical heteroconjugation of 3F8 and OKT3 (mouse anti-human CD3ε IgG2a mAb). This bispecific antibody construct, termed 3F8BiAb, redirects and activates non-MHC restricted human peripheral CD3+ T cells to induce specific lysis of target cells expressing GD2, as demonstrated in vitro for various human neuroblastoma cell lines [200].

The human-mouse chimeric anti-GD2 mAB ch14.18 consists of the Fab portion of the mouse anti-GD2 IgG2a mAb 14G2a fused with the human Fc constant regions of an IgG1 immunoglobulin [201]. Ch14.18 retains the anti-GD2 specificity and the ability to target GD2-expressing tumors observed with 14G2a, but mediates ADCC of GD2-positive melanoma cells *in vitro* about 100-fold more efficiently than 14G2a [202]. Early phase I clinical studies and retrospective analysis of single-agent ch14.18 treatment in patients with melanoma and neuroblastoma revealed that ch14.18 has considerable antitumor activity in neuroblastoma [203-205], but not in melanoma [206]. However, combination of ch14.18 with interleukin-2 [207] and GM-CSF plus IL-2 plus isotretinoin [208] results in enhancement of antitumor activity of ch14.18 in melanoma and neuroblastoma patients, respectively.

TRBs07 (Ektomun, Ektomab) is a bispecific, trifunctional antibody construct (Triomab) comparable to catumaxomab that originate from parental mouse anti-GD2 and anti-GD3 IgG2a mAb Me361 and rat anti-CD3 IgG2b mAb 26II6 [209]. This Triomab binds human CD3 as well as the gangliosides GD2 and GD3 with a preference for GD2, thereby redirecting and activating CD3-positive T-lymphocytes, which induce specific lysis of target cells expressing GD2, such as human melanoma cells and other neuroectodermal tumor cells [209]. Similar to catumaxomab, TRBs07 additionally binds via its Fc fragment to Fcγ-receptors on dendritic cells, macrophages and natural killer cells, and induces the secretion of cytokines such as IFN-γ, TNF-α, IL-2, IL-6 and IL-10 by peripheral blood mononuclear cells *in vitro* [209]. The Triomab Surek, which possesses the identical anti-GD2 binding arm as TRBs07, but binds to mouse CD3 instead of human CD3, has recently been shown to induce tumor eradication and protection against a second tumor challenge in a mouse melanoma model. Eradication of mouse melanoma was accompanied by the induction of tumor-specific T cells with defined melanoma-associated antigen specificity, induction of a humoral anti-tumor immune response, and generation of a long-term memory response against a second tumor challenge, indicating that Triomabs can generate potent tumor immunity against melanoma cells [137,138].

In view of the recent discovery that GD2 is expressed on human breast CSCs and promotes tumorigenesis in human breast cancer xonograft mice [193], mAbs and antibody constructs targeting GD2 may represent powerful therapeutic tools for the eradication of breast CSCs and the treatment of human breast cancer and other GD2-expressing tumors.

Anti-Lgr5

Lgr5 (leucine-rich-repeat containing G-protein-coupled receptor 5) is a seven-span transmembrane protein and the receptor and functional target for R-spondin proteins, which constitute secreted agonists of the canocial Wnt/β-catenin signaling pathway. Lgr5 therefore is a facultative Wnt receptor that mediates Wnt signal enhancement by soluble R-spondin proteins [210,211]. Lgr5 is expressed on Wnt/β-catenin-dependent adult stem cell populations of the intestine, colon and hair-follicle [212]. Expression of Lgr5 has been demonstrated on intestinal crypt stem cells, which are the cells-of-origin of intestinal cancer [213], indicating that Lgr5 is a functional marker of intestinal CSCc involved in intestinal and colorectal carcinogenesis [214,215]. In patients with colorectal cancer, Lgr5 expression positively correlates with histological grade, invasion and metastasis, and inversely correlates with survival of patients, revealing an important role of Lgr5 in the progression and prognosis of colorectal cancer [217].

KM4056, a rat IgG2b mAb that specifically recognizes the N-terminal extracellular domain of human Lgr5, has been generated recently [217]. KM4056 binds with high affinity to human Lgr5 genetically expressed on the surface of Chinese hamster ovary (CHO) cells. In xenograft mice, KM4056 significantly inhibits tumor growth of human Lgr5-expressing CHO cells, demonstrating a strong anti-tumor activity of KM4056 *in vivo* [217].

Three rat IgG mAbs, 1D9, 4D11 and 9G5 that selectively bind endogenously expressed Lgr5 on colon CSCs isolated and enriched from human colorectal cancer samples have been developed and investigated very recently [210,218,219]. Using these mAbs, it was demonstrated that Lgr5 is a selective marker for human colon CSCs that is suitable for the identification, isolation, enrichment and functional characterization of human colon CSCs *in vitro* and in xenograft mice [218]. Although 1D9, 4D11 and 9G5 have been used hitherto for the identification and characterization of colon CSCs, it is conceivable that these or similar mAbs raised against Lgr5 will be exploited in the future as therapeutic tools to eradicate colon and intestinal CSCs [219].

Conclusions and Future Directions

The last years have highlighted the challenges and opportunities of selectively targeting CSCs, which are regarded as one of the main driving force in carcinogenesis and cancer. Although the rather novel CSC concept of carcinogenesis is mostly accepted to date, other relevant mechanisms and driving forces of carcinogenesis, including epigenetic modifications, genome instability, replicative immortality, immune evasion, reprogramming of energy metabolism, and most likely, a sophisticated conjunction of all of these mechanisms appears to be essential for the genesis of cancer [220-223].

Nevertheless, in line with the CSC concept of carcinogenesis [1-10], CSCs constitute adequately characterized cells and represent novel and translationally relevant targets for cancer therapy [5,15,49]. Unfortunately, CSCs exhibit a variety of intrinsic mechanisms of resistance to conventional chemotherapeutic drugs and radiation therapy, allowing them to survive standard cancer therapies and to initiate tumor recurrence and metastasis [5,15,16]. Fortunately, CSCs express virtually private cell surface proteins, including CD44, CD47, EpCAM, CD123, GD2, Lgr5, IGF-IR, Dll4 and FZD receptors, which can be targeted specifically and readily with novel mAbs and antibody constructs, without inducing a considerable extent of collateral tissue damage and even an enrichment of CSCs observed with conventional cytostatic drugs and radiation therapy.

Significant advances have been made recently in the discovery, development and validation of novel mAbs and antibody constructs that selectively target CSCs, and the future clinical use of these novel immunotherapeutics may represent a powerful strategy for eradicating CSCs in cancer patients by exploiting the host´s immune system, thereby preventing tumor recurrence and metastasis, and, hopefully, contributing to the cure of cancer. There is recent evidence that the novel class of Triomab antibody constructs not only can effectively eradicate their target cells in a spational and temporally fashion, but also are capable of inducing a long-lasting immunity against tumor and CSCs antigens, thereby fulfilling the criteria of a cancer vaccination effect. Other classes of antibodies targeting CSCs have recently been demonstrated to induce robust tumor regression in xenograft mice and in clinical studies, pointing out their potent activity against CSCs and cancer cells. Finally, the combination of mAbs and antibody constructs targeting CSCs with conventional cytostatic drugs, novel tumor-targeted drugs and radiation therapy as well as the establishment of appropriate biomarkers and the definition of novel clinical endpoints for monitoring the efficacy of combined and multimodal therapeutic strategies will be a challenge to improve future cancer treatment [224,225].

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