Tumor Antigen-Specific Monoclonal Antibody-Based Immunotherapy, Cancer Initiating Cells and Disease Recurrence

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Abstract Immunotherapy with tumor antigen (TA)-specific monoclonal antibody (mAb) has been shown to be effective in the treatment of several types of cancer. However, its efficacy is limited by the lack of response in some of the treated patients and by disease recurrence. In this chapter, following a short description of the characteristics of cancer initiating cells (CICs) and the markers used for their identification in various types of cancer, we will provide *in vitro* evidence to suggest that disease recurrence is caused by the lack of eradication of CICs by the TA-specific mAb-based immunotherapy. In addition, we will describe potential strategies to overcome this resistance mechanism which is a major obstacle to the successful application of TA-specific mAb-based immunotherapy.

Keywords Cancer initiating cells · Monoclonal antibody · Tumor antigen · Head and neck cancer · Breast cancer · Pancreatic cancer · Osteosarcoma · Disease recurrence · Combinatorial therapy

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Abbreviations

ADCC	Antibody-dependent cell-mediated cytotoxicity
ALDH	Aldeyde dehydrogenase
BAAA	BODIPY^{TM}-aminoacetaldehyde
CDC	Complement-dependent cytotoxicity
CICs	Cancer initiating cells
CSPG4	Chondroitin sulfate proteoglycan 4
CTL	Cytotoxic T cells
DEAB	Diethylaminobenzaldehyde
Grp94	Glucose-regulated protein of 94 kDa
HSP	Heat shock protein
mAb	Monoclonal antibody
MCSP	Melanoma-associated chondroitin sulfate proteoglycan
MDSC	Myeloid-derived suppressor cells
MFI	Mean fluorescence intensity
NG2	Neuron-glial antigen 2
PDAC	Pancreas ductal adenocarcinoma
SCID	Severe combined immunodeficiency
SHH	Sonic Hedgehog Homologue
T regs	T regulatory cells
TA	Tumor antigen
TNBC	Triple negative breast cancer

Introduction

The hybridoma methodology has greatly facilitated the identification of a large number of human tumor antigens (TAs) by providing highly specific probes. Several of the identified TAs meet the criteria in terms of high expression on tumor cells, restricted distribution in normal tissues and functional properties to be used as targets of monoclonal antibody (mAb)-based immunotherapy for the treatment of malignant diseases [1]. A large body of clinical evidence in conjunction with the results of studies in animal model systems have convincingly shown that targeting TA with mAbs may represent an effective strategy for the treatment of many types of cancer [1]. The therapeutic efficacy of TA-specific mAbs is in general enhanced by combination with chemo- and/or radio-therapy [1]. Nevertheless, immunotherapy with TA-specific mAbs is not effective in all the treated patients and in most patients its efficacy is limited in time because of disease recurrence and/or metastases [1]. These clinical findings have stimulated interest in defining the molecular mechanism(s) underlying the innate and acquired resistance of tumor cells to TA-specific mAb-based immunotherapy with the expectation that this

Table 1 Mechanism of cancer resistance to antibody treatment

Mechanism of resistance	Mediated mechanism	Alteration	Cancer	References
Amplification of downstream signaling and alternative signaling pathways	Independent growth stimulatory effects	(1) KRAS mutation (codon 12 or 13) (2) BRAF mutation (V600E) (3) PIK3CA mutation (4) PTEN loss (5) Overexpression of other HER family receptors (6) Increased IGF-IR activity	Breast, colon, cancer	[40–45]
Expression of immune inhibitory molecules	Inhibition of NK cells-mediated lysis by mAbs Inhibition of ADCC	Increased HLA-G or HLA-E expression	Colon, ovarian, cancer	[46, 47]
Expression of membrane-bound complement regulatory proteins	Inhibition of CDC	Increased CD46, CD55, and CD59 expressions	Breast, lung, colon cancer and hematological malignancies	[48–51]
Impairment of proper antigen presentation	Evasion to destruction by MHC-restricted CTLs response mediated by mAbs	Down-regulation of MHC I expression	Breast cancer, colon cancer	[52–54]
Increased inhibitory signals by intratumoral Tregs or MDSCs	Suppression of the anti-cancer immune response mediated by mAbs	Apoptosis of immune effectors cells by increased production of perforin and granzyme, by expression of indolamine 2,3 dioxygenase and by production of soluble mediators such as TGF-β and IL-10	Breast cancer, hematological malignancies	[55–57]
Reduction of binding affinity of TA	Reduction of TA recognition	(1) FcγRII and FcγIII polymorphism (2) CD20 mutation (ANPS and YCYSI, at positions 170 to 173 and 182 to 185)	Breast cancer, colon cancer, hematological malignancies	[58–60]
Reduction or loss of TA expression	Reduction or loss of tumor cell recognition	(1) Genetic instability of tumor cells (2) MAPK pathway activation (3) increased histone deacetylase activity	Colon cancer and hematological malignancies	[61–65]
Impaired access to TA or production of antagonist TA	Reduction of TA recognition	(1) Expression of extracellular domain-truncated HER2 (p95 HER2) (2) Overexpression of MUC4	Breast cancer	[66, 67]

information will contribute to the rational design of targeted therapeutic strategies to counteract these resistances.

Multiple mechanisms have been shown to underlie the resistance of tumor cells to TA-specific mAb-based immunotherapy. As recently reviewed by other groups (see Table 1) and discussed in many chapters of this book, they include loss of the targeted TA, mutations in the targeted TA, upregulation of complement component inhibitors, etc. In addition, recent evidence demonstrated that the therapeutic activity of TA-specific mAbs may be mediated by induction or enhancement of TA-specific cytotoxic T cells (CTL). If this is the case, defects in antigen presentation by tumor cells to cognate CTL or suppression of their functional properties may represent mechanisms of resistance of tumor cells to mAb-based immunotherapy.

In the present paper, utilizing our recent *in vitro* data, we will discuss the possibility that disease recurrence in patients treated with TA-specific mAb-based immunotherapy in combination with radio- and/or chemo-therapy may be caused by the resistance of cancer initiating cells (CICs) to this type of therapy. Specifically, we will describe (1) the characteristics of CICs in the tumors we have investigated; (2) the effects of TA-specific mAbs with chemo- and/or radio-therapy on CICs; and (3) strategies to overcome this resistance.

Resistance of Cancer Initiating Cells from TA-Specific mAb-based Immunotherapy

1. Characteristics of CICs in Various Types of Tumors

According to the cancer stem cell theory, CICs are a subpopulation of tumor cells that, like normal stem cells, are "multipotent" since they can give rise to all cell types found in a particular cancer sample, are asymmetrically self-renewing, can divide indefinitely, and are able to generate tumor spheres and are tumorigenic in low numbers in immunodeficient mice [2]. Relevant to the data that we are going to present, CICs are chemo-and radio-resistant and are believed to play a major role in both the metastatic spread and disease recurrence, the major causes of patients morbidity and mortality [3]. Therefore, CICs cells have to be eradicated in order to "cure" a malignant disease.

Several markers have been reported to identify CICs in various types of tumors. They include $CD44^{high}CD24^{low}$ in breast cancer [4], CD133 in pancreas ductal adenocarcinoma (PDAC) [5] and in osteosarcoma [6], and the activity of the aldeyde dehydrogenases (ALDHs) in head and neck, breast, lung, pancreas, colon, prostate, and ovary cancers and osteosarcoma [2, 7–13].

According to the information in the literature and our own data, the ALDH activity appears to be a reliable marker. Since this marker has been used in studies we are going to present, we will first give some background about the ALDH activity in normal and cancer cells. ALDHs are a family of enzymes involved in the maintenance of cellular homeostasis by metabolizing both endogenous and

exogenous reactive compounds [14]. They oxidize aldehydes to the corresponding carboxylic acids using either Nicotinamide adenine dinucleotide (NAD) or nicotinamide adenine dinucleotide phosphate (NADP) as a coenzyme [15]. These enzymes are known to modulate several cell functions, including proliferation, differentiation and survival as well as the cellular response to oxidative stress. How ALDHs modulate these processes still remain to be determined. Nineteen ALDH genes have been found in the human genome [16]. They are clustered in 11 families and 4 subfamilies with distinct chromosomal locations. ALDHs, namely, ALDH1A2, ALDH1A3, ALDH1A7, ALDH2*2, ALDH3A1, ALDH4A1, ALDH5A1, ALDH6 and ALDH9A1 display high enzymatic activity in normal and cancer stem cells. Therefore, these enzymes may be considered a marker for these cells and may well play a functional role in terms of self protection, differentiation, and/or expansion of stem cell populations.

The detection of ALDH in a cell relies on the measurement of its enzymatic activity. This has been facilitated by the availability of a specific substrate which can be detected by fluorescence analysis (ALDEFLUOR). It is noteworthy that this substrate does not discriminate among different isoforms of ALDHs. Therefore, as ALDH isoforms have a wide substrate spectrum, it has been possible to determine that ALDH positivity, evidenced in various types of stem cells, including cancer stem cells, is not due to the expression of the same isoforms. It is believed that this heterogeneity indicates that the isoform responsible for Adelfluor activity in the case of normal cells depends on the type of the cell and on the tissue of origin in the case of cancer cells [17]. That is why most studies on ALDHs and stem cells do not specify the ALDH isoform, but speak rather generically of ALDH. However, ALDHA1 has been identified as a marker for the isolation and identification of normal or cancer stem cells [2], and along with ALDH3A1, which it has been shown to play a role in differentiation and cell proliferation [15]. Indeed, a reduction in cell proliferation has been observed when the enzyme is directly inhibited by the administration of synthetic specific inhibitors, antisense oligonucleotides, or siRNA.

Several lines of evidence indicate that cells which display high activity of ALDH ($ALDH^{bright}$) are normal or cancer stem cells. The enzymatic activity of ALDHs in human hematopoietic progenitor cells has been shown to be higher than in more mature hematopoietic cells [18]. In addition, it has been associated with an enhanced repopulating function and cellular resistance to cytotoxic drugs such as alkylating agents. As multipotent cells, $ALDH^{bright}$ cells isolated from bone marrow have been demonstrated to include hematopoietic, endothelial, mesenchymal, and neural progenitor cells, which are crucial in repair protocols for various pathological conditions. Like human hematopoietic progenitor cells, several other types of normal stem cells have been shown to display high levels of cytosolic ALDH expression. They include neural cells, myogenic cells, mammary cells, prostate cells, and intestinal crypt cells. These cells have been shown to be multipotent, self-renewing, and able to generate spheres and epithelial stem-like cells in culture.

Increased ALDH activity in cancer stem cells was first reported in acute myeloid leukemia. Subsequently, similar observations have been reported for several types of solid cancers, including breast, colon, prostate, lung, pancreas and

ovary. Our data as well as data from the literature demonstrate that ALDH^{bright} cells in head and neck, triple negative breast and pancreatic cancers have the characteristic of CICs, such as *in vitro* self-renewal, chemo- and radio-resistance, ability to divide indefinitely, aberrant regulation of several stem cell signaling pathways (such as Hedgehog and MAPK, Notch, PI3K/AKT, TGF-β and Wnt), expression of stem cell markers, tumor cell sphere formation, and high tumorigenicity at low cell numbers in SCID mice [19–21].

ALDH^{bright} cells are analyzed using the ALDEFLUOR^{TM} reagent kit. The activated ALDEFLUOR^{TM} Reagent, BODIPY^{TM}-aminoacetaldehyde (BAAA), is a fluorescent non-toxic substrate for ALDH, which freely diffuses into intact and viable cells. In the presence of ALDH, BAAA is converted into BODIPY^{TM}-aminoacate (BAA), which is retained inside the cells. The amount of fluorescent reaction product is proportional to the ALDH activity in the cells and is measured using a flow cytometer. The active efflux of the reaction product is inhibited by an efflux inhibitor in the ALDEFLUOR^{TM} Assay Buffer. A specific inhibitor of ALDH, diethylaminobenzaldehyde (DEAB), is used to control for background fluorescence as the gating reference standard of the ALDH^{neg} population. Thus, ALDH^{bright} cells are those ALDH^{+} cells with twice the mean fluorescence intensity (MFI) of the ALDH^{+} cell population (Fig. 1). The frequency of ALDH^{bright} cells varies among cell lines of different tumor types as well as among cell lines from the same type of tumor. Table 2 presents the results we have obtained with different human cancer cell lines analyzed for the content of ALDH^{bright} cells. It is noteworthy that this frequency is influenced by the culture conditions such as cell confluency, cell starvation and hypoxia [22]. Viable ALDH^{bright} cells can be

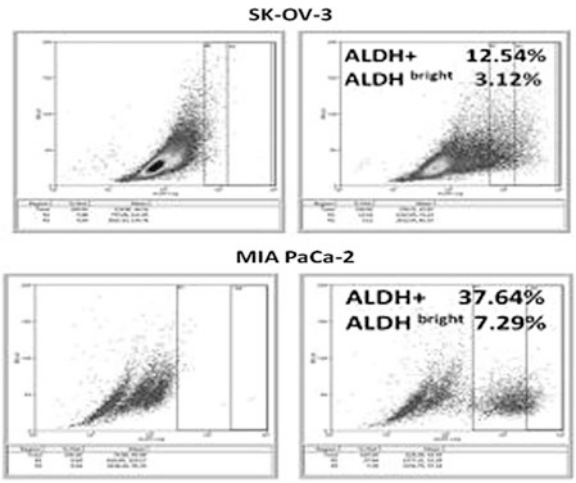

Fig. 1 ALDH activity expression as a marker of CICs in the human ovarian cancer cell line SK-OV-3 and in the human PDAC cell line MIA PaCa-2. SK-OV-3 and MIA PaCa-2 cells (2.5×10^5) were cultured in complete medium at 37 °C, 5 % CO_2. Then, the cells were harvested and stained with ALDEFLUOR with or without the DEAB inhibitor to identify ALDH^{bright} cells. ALDH^{bright} cells were identified as those ALDH^{+} cells with twice the mean fluorescence intensity (MFI) of the ALDH^{+} cell population

Table 2 Expression of $ALDH^+$ and $ALDH^{bright}$ cells in different types of cancer

Cell line	$ALDH^+$ cells (%)	$ALDH^{bright}$ cells (%)
Head and neck cancer		
PCI-4A	23.2	6.3
PCI-13	11.3	1.9
PCI-30	0.3	0.1
PCI-37A	1.2	0.1
SCC-4	5.4	1.1
SCC-90	1.6	0.2
SCC-104	0.5	0.1
UD-SCC-4	0.5	0.1
UD-SCC-6	0.3	0.1
FaDu	1.8	0.1
Breast cancer		
MDA-MB-231	18.2	0.7
SUM-149	22.1	1.5
UACC-812	4.7	0.3
Pancreatic cancer		
MIA PaCa-2	43.2	4.6
S2-LM7-AA	19.3	1.6
PDAC1	21.3	2.5
PDAC2	14.4	2.3
PDAC3	38.2	8.0
PDAC5	5.7	0.8

further isolated using a cell sorter and can be tested for their ability to form tumor spheres *in vitro* and for their tumorigenicity in low number in immunodeficient mice. As shown in Fig. 2, $ALDH^{bright}$ cells sorted from the head and neck cancer PCI-13 cell line, the PDAC MIA PaCa-2 cell line, and the TNBC MDA-MB-231-Luc cell line orthotopically injected at a low number (500 cells/mouse) in immunodeficient mice induce tumors in most if not in all of the injected mice. On the other hand, the $ALDH^{neg}$ cells failed to induce tumors.

2. Characteristics of the TA Utilized to Target Differentiated Tumor Cells and CICs in Our Laboratory

We have focused our work aiming at developing immunotherapy of solid tumors with TA-specific mAbs on three types of TA which are characterized by a high expression on the tumor cell membrane, a restricted distribution in normal tissue and the ability to modulate multiple signaling pathways. We will describe the characteristics of these TAs which are most relevant for the experiments discussed below.

A. *Chondroitin Sulfate Proteoglycan 4*

In the late 70s, utilizing mAb elicited in mice immunized with cultured human melanoma cells, we identified a TA with high expression on the membrane of melanoma cells and with a restricted distribution in normal tissues. This TA, which is highly conserved through phylogenetic evolution, consists of an N linked

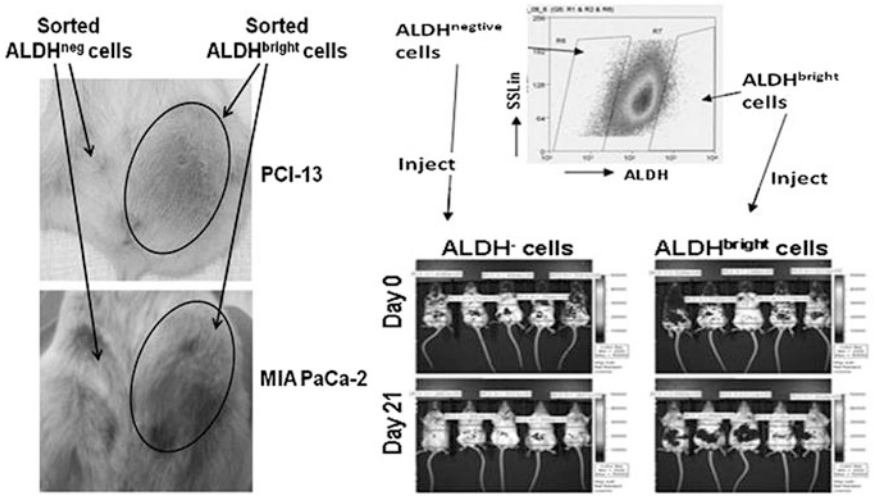

Fig. 2 Tumorigenicity of ALDH^{bright} cells sorted from human carcinoma cell lines. Immunodeficient NOD/SCID mice were challenged in the right and left flanks with 500 sorted ALDH^{bright} or ALDH^{neg} PCI-13 or MIA PaCa-2 cells isolated from human carcinoma cell lines and established xenografts and photographs of representative mice are shown. Xenografts were established in 3/3 mice challenged with ALDH^{bright} PCI-13 and 2/3 mice challenged with ALDH^{bright} MIA PaCa-2 cells (*left* panel). Sorted ALDH^{bright} and ALDH^{neg} MDA-MB-231-luc-D3H1 (Xenogen Corp) cells (500/mouse) were mixed with irradiated unsorted MDA-MB-231 cells (10,000/mouse) and Matrigel and injected (total final vol. 100 μl) intraperitoneally (i.p.) into 2 groups of NOD/SCID mice. Tumor growth was monitored in each mouse weekly by Bioluminescence imaging (*right* panel)

280 kDa glycoprotein and a 450 kDa chondroitin sulfate proteoglycan, the two components have the same polypeptide moiety [23]. Because of its large size, we named this TA high molecular weight- melanoma associated antigen. This TA, also known as cell surface chondroitin sulfate proteoglycan 4 (CSPG4), melanoma-associated chondroitin sulfate proteoglycan (MCSP) or neuron-glial antigen 2 (NG2), is expressed not only on melanoma but also on head and neck cancer, TNBC, mesothelioma, chordoma, chondrosarcoma and osteosarcoma [24]. In the tumors which have been analyzed, CSPG4 has been found to be expressed not only on differentiated cancer cells but also on CICs, defined as ALDH^{bright} cells, in head and neck cancer, triple negative breast cancer and osteosarcoma. A representative example of a double staining of ALDH^{bright} cells with a CSPG4-specific mAb in the TNBC MDA-MB-231 cell line is shown in Fig. 3. The expression of CSPG4 on CICs is also corroborated by functional data. Specifically, CSPG4 specific-mAb can inhibit the proliferation and the migration of TNBC and osteosarcoma cell lines *in vitro* (Figs. 4 and 5) and their ability to metastasize when injected in immunodeficient mice [25]. Furthermore, CSPG4-specific mAb could suppress disease recurrence and prolong survival in mice which have been grafted with the TNBC cell line MDA-MB-231 and then subjected to surgical removal of the primary tumor (Fig. 6). As already discussed, CICs play a major role in disease

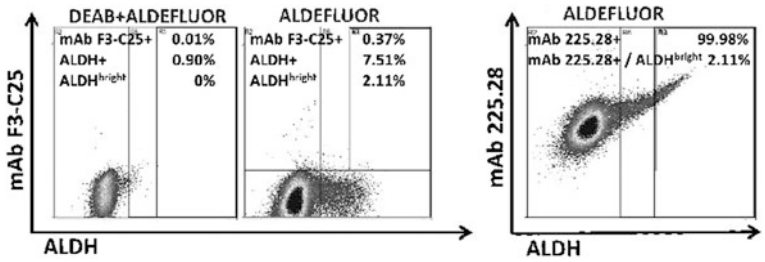

Fig. 3 CSPG4 expression on human TNBC CICs. The TNBC cells MDA-MB-231 were incubated with ALDEFLUOR (Stem Cell Technologies) to detect ALDH activity and stained with the CSPG4-specific mAb 225.28. DEAB, a specific inhibitor of ALDH, was used to establish the baseline fluorescence of these cells. The isotype matched mAb F3-C25 was used as a control for the CSPG4-specific mAb 225.28. The $ALDH^{bright}$ cells were identified as those $ALDH^{+}$ cells with twice the mean fluorescence intensity (MFI) of the $ALDH^{+}$ cell population. The % of cells stained by ALDEFLUOR and/or by mAbs is shown

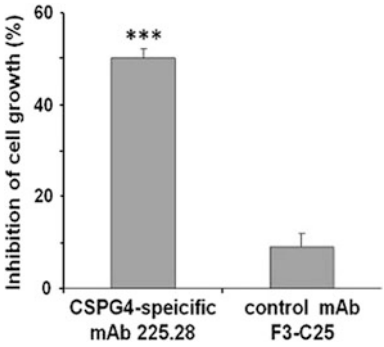

Fig. 4 Inhibition of human osteosarcoma U2OS carcinoma cell growth. Human osteosarcoma U2OS cells were serum starved 3 days and incubated either with 0.25 mg/ml CSPG4-specific mAb 225.28 or control mAb F3-C25 in a 3-D (matrigel) setting for 10 days. PBS, which was used as the solvent for both mAbs, was used as a reference for 100 % cell growth. Cells in each well were harvested from the matrigel using the Cell Recovery Solution (BD Pharmingen) and counted by two individuals using Trypan Blue. The results are expressed as % inhibition of cell growth, utilizing the values obtained for cells grown in PBS, as a reference. The values shown are the mean of three independent experiments. *** indicates $p < 0.001$

recurrence and metastatic spread. The effects of immunotherapy with the CSPG4-specific mAb are mediated by the inhibition of the activation of multiple signaling components of pathways involved in proliferation, survival and antiapoptotic signals as well as in migration of cancer cells including CICs (Fig. 7). The potential clinical relevance of these data is indicated by the beneficial effect of the clinical course of the disease of CSPG4-specific antibodies induced by CSPG4 mimics in patients with melanoma.

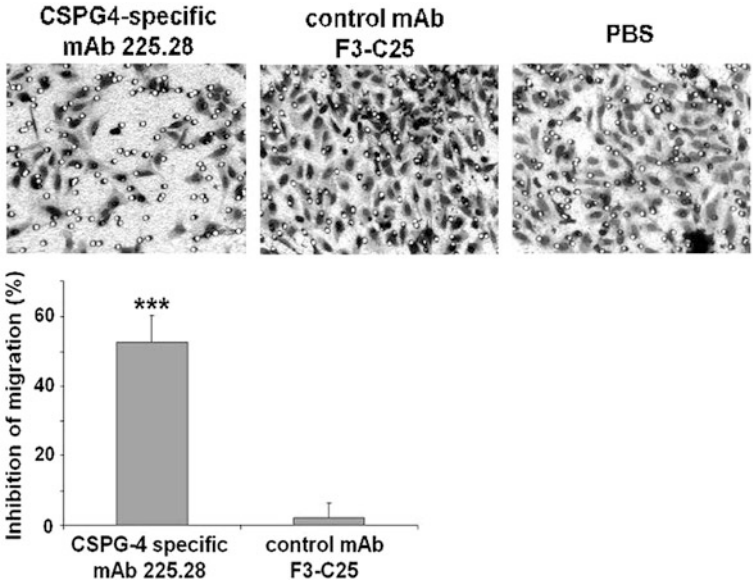

Fig. 5 Inhibition of human osteosarcoma U2OS carcinoma cell migration. U2OS cells were serum starved 3 days and seeded and incubated with either 0.5 mg/ml CSPG4-specific mAb 225.28, control mAb F3-C25 or PBS in a migration assay. Pictures of each well were taken with a Zeiss Inverted Fluorescence Microscope (AxioVision Software) (×200). The results are expressed as % inhibition of migration, utilizing the values obtained in PBS without mAb as a reference. The values shown are the mean of three independent experiments. *** indicates $p < 0.001$

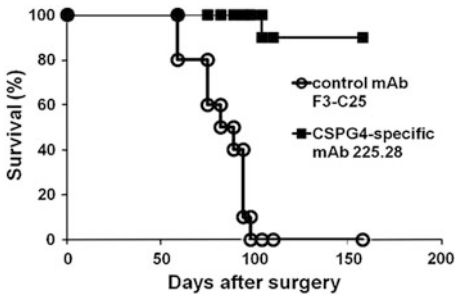

Fig. 6 Prolongation of mice survival by the CSPG4-specific mAb after surgical removal of the primary tumor derived by TNBC MDA-MB-231 cells. TNBC MDA-MB-231 cells (2×10^6 cells/mouse) were injected orthotopically into 20 SCID mice. After 63 days, mice were divided into two groups ($n = 10$/group) using a stratified randomization strategy, such that the mean tumor volume was not statistically significantly different between two groups. On day 63, all primary tumors were surgically removed. Starting from day 66, mice were treated intravenously (i.v.) with CSPG4-specific mAb 225.28 (200 μg/mouse/twice per week) or control mAb F3-C25 (200 μg/mouse/twice per week). The survival of mice was analyzed using the Kaplan–Meier survival curve

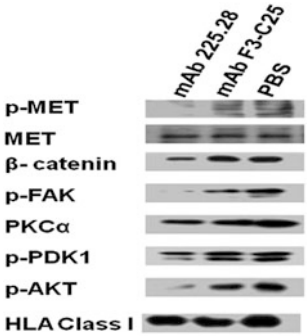

Fig. 7 Effect of the CSPG4-specific mAb 225.28 on multiple signaling pathways *in vitro* in the human osteosarcoma MG-63 cell line. The human ostersarcoma cell line MG-63 was incubated with either the CSPG4-specific mAb 225.28, the isotype control mAb F3-C25 or PBS for 72 h at 37 °C. Cell lysates were tested in western blot with anti-phosphorylated (p)-MET, MET, β-catenin, p-FAK, PKCα, p-PDK1, p-AKT antibodies. HLA class I heavy chain (HLA class I) was used as the loading control

B. *Glucose-regulated Protein of 94 kDa*

The heat shock protein (HSP) glucose-regulated protein of 94 kDa (Grp94) is a member of the HSP90 family [26, 27]. Similar to other members of this family, the molecular chaperone Grp94 is required for the stability and activity of client proteins involved in the activation of signaling pathways associated with tumor cell survival and proliferation [27–29]. These functional properties of Grp94 provide a molecular mechanism for the anti-proliferative effect and the induction of apoptosis of malignant cells by Grp94 inhibitors. The potential clinical relevance of these findings is suggested by the clinical responses observed in patients treated with inhibitors of HSP90 [30–32], which display a high degree of homology with Grp94. However, the clinical use of HSP90 inhibitors is hampered by the significant side effects they cause because of their lack of selectivity and the ubiquitous intracellular expression of HSP90. To overcome these limitations, we have isolated a Grp94-specific single chain Fv fragment from a phage display antibody library. This single chain has the unique specificity to recognize an extracellular epitope of Grp94 which is selectively expressed on the membrane of many types of malignant cells, but has a restricted distribution in normal cells. In the tumors we have analyzed, Grp94 has been found to be expressed not only on differentiated cancer cells but also on CICs, defined as $ALDH^{bright}$ cells. A representative example of a double staining of $ALDH^{bright}$ cells with a Grp94-specific mAb in the PDAC MIA-PaCa-2 cell line is shown in Fig. 8. The Grp94-specific mAb inhibits the proliferation and the migration of malignant cells and induces their apoptosis. These effects are mediated by inhibition of the activation of several components of signaling pathways involved in proliferation, survival and antiapoptosis as well as in migration of cancer cells including CICs (data not shown).

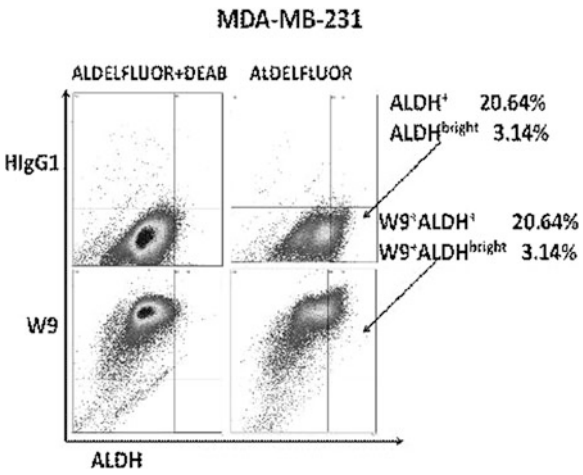

Fig. 8 Grp94 expression on human TNBC CICs. The TNBC cells MDA-MB-231 were incubated with ALDEFLUOR (Stem Cell Technologies) to detect ALDH activity and stained with the Grp94-specific mAb W9. DEAB, a specific inhibitor of ALDH, was used to establish the baseline fluorescence of these cells. The isotype matched mAb HIgG1 was used as a control for the mAb W9. $ALDH^{bright}$ cells were identified as those $ALDH^{+}$ cells with twice the mean fluorescence intensity (MFI) of the $ALDH^{+}$ cell population. The % of cells stained by ALDEFLUOR and/or by mAbs is shown

C. B7-H3

B7-H3 known also as CD276 is a member of the B7 family; it shares 20–27 % amino acid sequence homology with other members of the B7 family [33]. To detect B7-H3, we use the mAb 376.96, an IgG2a generated from a BALB/c mouse hyperimmunized with cultured human melanoma cells. The specificity of mAb 376.96 for B7-H3 has been recently shown by the following lines of evidence. First, mass spectrometry analysis of the peptides generated by trypsin digestion of the 94 kDa glycoprotein immunoprecipitated by mAb 376.96 from cultured human melanoma Colo38 cells has demonstrated a high degree of homology with that of B7-H3. Second, the mAb 376.96 specifically reacts with purified B7-H3 in binding assays. Third, the mAb 376.96 stains CHO cells transfected with human B7-H3 (2Ig) or B7-H3 (4Ig) cDNA; the epitope recognized by the mAb 376.96 is expressed on both 2 and 4Ig human B7-H3. Immunohistochemical staining with the B7-H3-specific mAb 376.96 has shown that the corresponding epitope has a broad distribution on several types of cancer including head and neck, breast, pancreatic, ovarian, prostate cancer and osteosarcoma. In contrast, this epitope has a more restricted distribution than the B7-H3 molecules in normal tissues since several tissues which express the B7-H3 molecule are not stained by the B7-H3-specific mAb 376.96. In the tumors we have analyzed, the B7-H3-specific mAb 376.96 has been found to be expressed not only on differentiated cancer cells but also on CICs, defined as $ALDH^{bright}$ cells. A representative example of a double

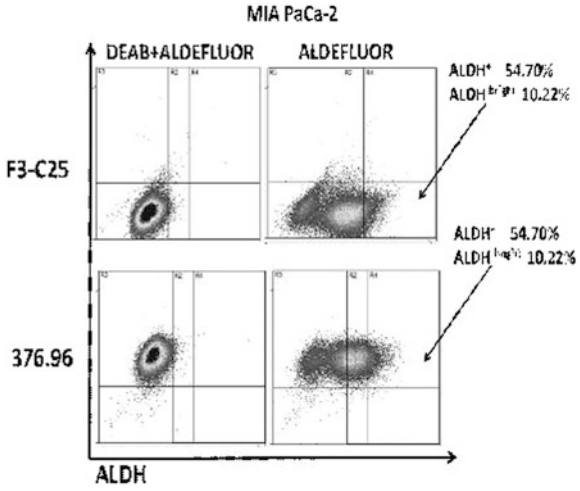

Fig. 9 B7-H3-specific mAb 376.96 expression on human PDAC MIA PaCa-2 CICs. MIA PaCa-2 cells were incubated with ALDEFLUOR (Stem Cell Technologies) to detect ALDH activity and stained with the B7-H3-specific mAb 376.96. DEAB, a specific inhibitor of ALDH, was used to establish the baseline fluorescence of these cells. The isotype matched mAb F3-C25 was used as a control for the mAb 376.96. $ALDH^{bright}$ cells were identified as those $ALDH^{+}$ cells with twice the mean fluorescence intensity (MFI) of the $ALDH^{+}$ cell population. The % of cells stained by ALDEFLUOR and/or by mAbs is shown

staining of $ALDH^{bright}$ cells with the B7-H3-specific mAb 376.96 in the PDAC Mia-PaCa-2 cell line is shown in Fig. 9. The mAb 376.96 displays an antiproliferative effect *in vitro* with various types of cell lines including TNBC, PDAC and ovarian cancers. These results are paralleled by the inhibition of PDAC cell growth grafted in immunodeficient mice (Fig. 10). Studies in progress suggest that this effect is mediated by the inhibition of the activation of several components of signaling pathways involved in proliferation, survival and antiapoptosis as well as in migration of cancer cells including CICs (Fig. 11).

Effects of TA-specific MAbs in Combination with Chemo-radio-therapy on CICs

The results shown above in the previous sections indicate that TA-specific mAbs have anti-tumor effects by inhibiting cell growth, cell migration, and inducing apoptosis *in vitro*. These effects are paralleled by an inhibition but not complete suppression of metastatic spread and disease recurrence in immunodeficient mice grafted with human cancer cell lines. These findings are likely to reflect the inability of TA-specific mAbs to eliminate CICs. This possibility is supported by our *in vitro* data which have shown that incubation of cell lines with the

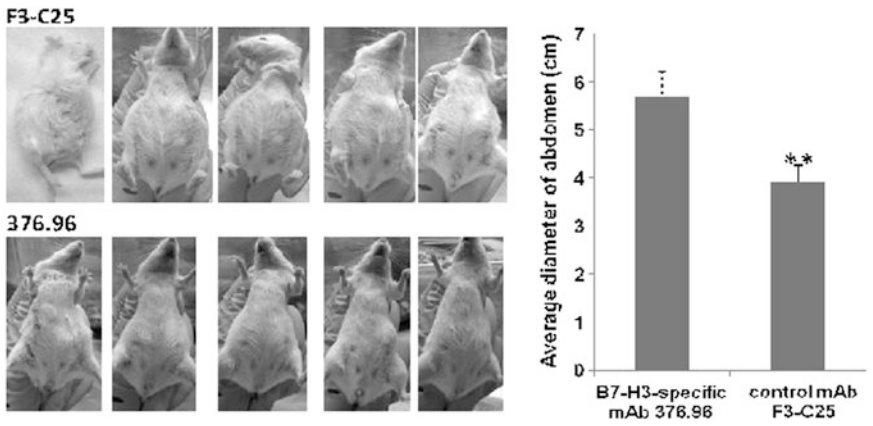

Fig. 10 Inhibition by the B7-H3-specific mAb 376.96 on the growth of tumors derived from the human PDAC MIA PaCa-2 cells grafted in SCID mice. Ten SCID mice were orthotopically challenged by the human PDAC cell line MIA PaCa-2 (2×10^6/mouse). After 28 days, the mice were randomly divided into 2 groups ($n = 5$/group). One group was treated i.v. with the B7-H3-specific mAb 376.96 (200 μg/mouse) and the other group was treated i.v. with the control mAb F3-C25 (200 μg/mouse) twice per week. After a total of 8 treatments, one mouse from the control group was dead and all other mice in the control group have ascites. In contrast, no mouse in the mAb 376.96-treated group had ascites. The pictures of mice on day 56 are shown in the left panel and the average diameters of the abdomen from both groups are shown in the right panel. **: p value < 0.01

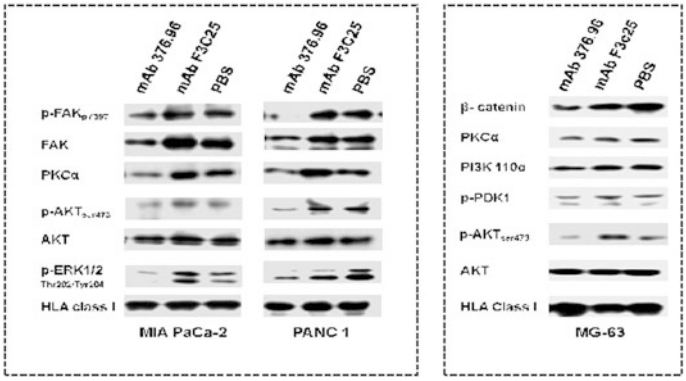

Fig. 11 Effect of the B7-H3-specific mAb 376.96 on multiple signaling pathways *in vitro* in the human osteosarcoma MG-63 cell line The human PDAC cells MIA PaCa-2, PANC 1 and the human ostersarcoma cell line MG-63 were incubated for 48 h (MIA PaCa-2 and PANC 1) or 72 h (MG-63) at 37 °C with either the B7-H3-specific mAb 376.96 or the control mAb F3-C25. Cells incubated in medium without mAbs were used as control. Cell lysates were tested by western blot with phosphorylated (p)-FAK, FAK, PKCα, p-ERK1/2, ERK1/2, p-AKT, AKT, β-catenin, PI3 K 110α and p-PDK1 mAbs. HLA class I was used as a loading control

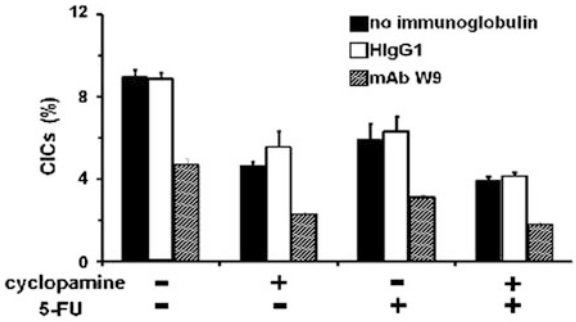

Fig. 12 Inhibition of pancreatic CICs *in vitro* proliferation by the Grp94-specific mAb W9. The PDAC MIA PaCa-2 cells were incubated with the mAb W9 (20 μg/ml), cyclopamine (20 μM), and 5-FU (10 μM) for 48 h at 37 °C. Cells were then harvested and stained with ALDEFLUOR with or without DEAB. HIgG1 was used as an isotype control. The triple combination of mAb W9, cyclopamine and 5-FU was significantly ($p < 0.05$) more effective than the triple combination of HIgG1, cyclopamine and 5-FU, double combinations and individual agents. The double combination of mAb W9 and cyclopamine was significantly ($p < 0.05$) more effective than the other combinations of two agents and individual agents. Cyclopamine was significantly ($p < 0.05$) more effective than the other individual agents

CSPG4/Grp94/B7-H3-specific mAbs can reduce the percentage of $ALDH^{bright}$ cells but not completely eliminate them. Representative examples of the effect of TA-specific mAbs on CICs are shown in Figs. 12 and 13. The detrimental effect of TA-specific mAbs on the percentage of CICs can be enhanced by combining the mAbs with chemotherapeutic agents, novel targeted agents and/or radiotherapy. Representative examples are shown in Figs. 12 and 13. The antiproliferative activity of the Grp94-specific mAb on differentiated PDAC cells as well as on pancreatic CICs is significantly enhanced when PDAC cells are incubated with the Grp94-specific mAb in combination with 5FU or with radiation (Fig. 14).

Enhancement by Signaling of the CICs Pathway Inhibitor of the Detrimental Effects of TA-specific MAb on CICs

The antitumor effects of TA-specific mAbs on both differentiated cancer cells and CICs have been shown to be enhanced by combining TA-specific mAbs with chemotherapeutic agents, targeted agents or radiotherapy. However, these types of combinatorial strategies do not completely eliminate CICs from a tumor cell population. These *in vitro* results provide an explanation for the limited duration of clinical objective responses of tumor-bearing hosts treated with TA-specific mAbs in combination with chemotherapeutic agents, targeted agents or radiotherapy. The fraction of CICs which is not eradicated by this combinatorial therapy may cause disease recurrence. These findings emphasize the need to develop novel and

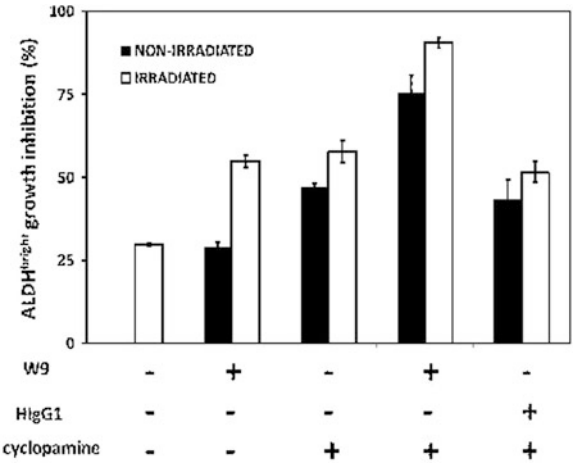

Fig. 13 Inhibition of pancreatic CICs *in vitro* proliferation by the Grp94-specific mAb W9. The PDAC MIA PaCa-2 cells were incubated with the mAb W9 (20 μg/ml), cyclopamine (20 μM), and radiotherapy (20 Gy). Following a 72 h incubation at 37 °C, the cells were harvested and stained with ALDEFLUOR with or without DEAB. HIgG1 was used as isotype control. The triple combination of mAb W9, cyclopamine and 20 Gy was significantly ($p < 0.05$) more effective than the triple combination of HIgG1, cyclopamine and 20 Gy, double combinations and individual treatment. The double combination of mAb W9 and cyclopamine was significantly ($p < 0.05$) more effective than the other combinations of two agents and individual agents. Cyclopamine was significantly ($p < 0.05$) more effective than the other individual treatments

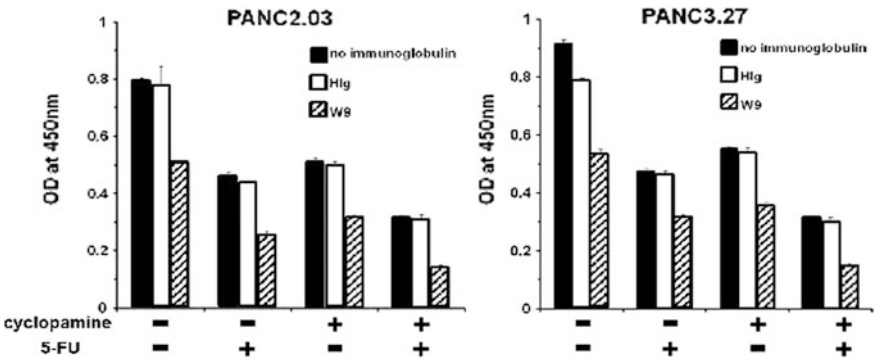

Fig. 14 Inhibition of cell growth in PDAC cells by the mAb W9, cyclopamine and 5-FU Cells (4×10^5/ml) were starved for 3 h, then incubated with the mAb W9 (20 μg/ml), cyclopamine (20 μM), and 5-FU (10 μM) in RPMI 1,640 medium containing 1.5 % FCS. Cells were then tested by the MTT assay. HIgG1 was used as a negative control. Results were shown as OD value and error bars were presented as standard deviation. The mAb W9, cyclopamine, and 5-FU combination was significantly ($p < 0.01$) more effective than the HIgG1, cyclopamine, and 5-FU combination, dual-agent combinations, and individual agents

effective therapeutic strategies which completely eradicate differentiated cancer cells and CICs. In addressing this need, we have been guided by the realization that signaling pathways involved in proliferation and survival are aberrantly activated in CICs. Inhibition of these signaling pathways has been shown to suppress CICs growth *in vitro* and in immunodeficient mice. However, also in this case, the eradication of CICs is not complete. Therefore, we have tested whether the detrimental effect on CICs by TA-specific mAbs could be enhanced by combination with chemotherapy or radiotherapy and by inhibitors of the CIC signaling pathways. Several pathways have been shown to be activated in CICs. They include the Sonic Hedgehog Homologue (SHH), MAPK, Notch, PI3 K/AKT, TGF-β and Wnt pathways [34, 35]. Since in the types of tumors that we investigated the SHH pathway has been found to be aberrantly activated in CICs, we have tested whether combining TA-specific mAbs and/or chemoradio-therapy with cyclopamine, an inhibitor of the SHH pathway, could eradicate CICs. Our *in vitro* results demonstrate that this is indeed the case at least in the tumor we have investigated. A representative example is shown in Figs. 12 and 13. Treatment of the PANC2.03, PANC3.27 and Mia-PaCa2 PDAC cell lines with the Grp94-specific mAb in combination with 5FU or radiotherapy and cyclopamine eliminate more than 90 % of the CICs in a tumor cell population. This effect is mediated by the inhibition of activation of multiple components of signaling pathways involved in proliferation, survival, antiapoptotic signals and CICs proliferation (Fig. 15).

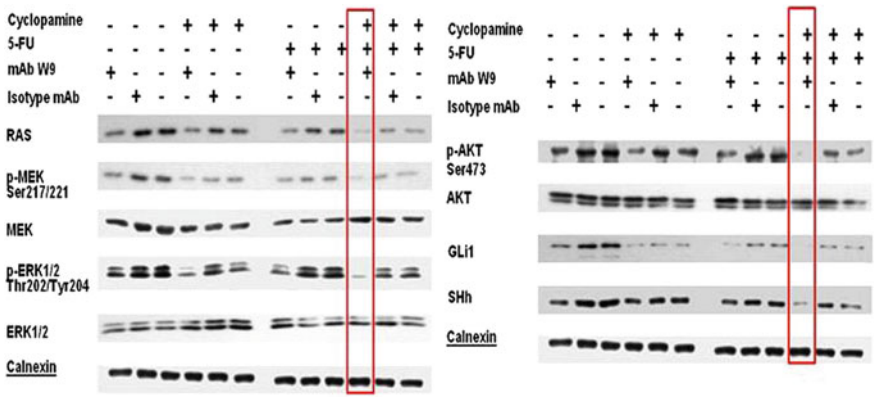

Fig. 15 Inhibition of signaling pathways in MIA PaCa-2 PDAC cells by the mAb W9, cyclopamine and 5-FU. The MIA PaCa-2 cells were incubated with the Grp94-specific mAb W9 (20 μg/ml), cyclopamine (20 μM) and 5-FU (10 μM) for 48 h at 37 °C. Cell lysates were then prepared and tested by western blot with the corresponding antibodies (Cell Signaling Technology). Calnexin was used as the loading control. Cells incubated with the mAb W9 alone, with the mAb W9 and cyclopamine, with cyclopamine alone, with the mAb W9 and 5-FU and with 5-FU alone were used as controls

Conclusions

Convincing experimental and clinical evidence indicate that TA-specific mAbs represent valuable reagents for the treatment of various types of cancer. However, a major limitation of this type of immunotherapy is its limited efficacy in time because of the occurrence of metastatic spread and/or disease recurrence, the two major causes of patients morbidity and mortality. According to the cancer stem cell theory, both complications may be mediated by CICs, a cellular subpopulation that displays stem cell properties and drives and maintains the growth of many types of cancer. In the experiments described in this chapter, we have tested this possibility by assessing the sensitivity of CICs to the detrimental effects of TA-specific mAbs. The *in vitro* data we have obtained utilizing three TA systems in several types of solid tumors indicate that CICs have a limited sensitivity to the detrimental effects of TA-specific mAbs even when they are combined with chemotherapeutic agents, targeted agents or radiotherapy. These combinations can eliminate only part of the CICs present in a tumor cell population. Whether the differential sensitivity of CICs to TA-specific mAbs in combination with chemotherapy, targeted therapy or radiotherapy reflects differences in their differentiation stage and/or epigenetic changes remains to be determined. Whatever the mechanism, if these *in vitro* findings reflect what happens in tumor-bearing hosts treated with TA based immunotherapy, then our data provide a mechanism for the disease recurrence which frequently occurs in patients treated with TA-specific mAb-based immunotherapy. Our *in vitro* data also suggest that the ability of TA-specific mAbs in combination with chemotherapeutic agents, targeted agents or radiotherapy to eradicate CICs can be markedly enhanced by inhibitors of signal transduction pathways that are aberrantly activated in CICs. These results have to be interpreted with caution since they were obtained with established cell lines and *in vitro* experiments. Nevertheless, the potential impact of this combinatorial strategy on disease recurrence in patients treated with TA-specific mAbs-based immunotherapy urges the implementation of *in vivo* experiments to assess the potential clinical relevance of the *in vitro* data we have described. Can the treatment of tumor-bearing hosts with TA-specific mAbs-based immunotherapy in combination with chemotherapeutic agents, targeted agents or radiotherapy and inhibitors of CIC signaling pathways cure a malignant disease? Can the monitoring of the percentage of CICs present in a tumor-bearing host be used to optimize the combinatorial therapy administered to a tumor bearing patient? Our results may also provide a mechanism for the failure observed in clinical trials which have tested the clinical efficacy of CIC signaling pathway inhibitors. An example is represented by the lack of clinical efficacy which has been reported for cyclopamine in patients with PDAC. Our *in vitro* results suggest that this failure can reflect the limited efficacy of cyclopamine [36] in eliminating differentiated cancer cells and CICs when it is used as a single agent. However, our data argue that this compound enhances the ability of TA-specific mAbs in combination with chemotherapeutic agents, targeted agents or radiotherapy in eliminating CICs.

This finding is not unique of cyclopamine since a recent clinical trial has demonstrated that agents which inhibit CIC signaling pathways such as cabozantinib, a c-Met inhibitor, and Trastuzumab, a HER2-specific mAb, have clinical efficacy in advanced castration-resistant prostatic cancer when they are used in combination, although the two agents failed when used as single agents [37–39].

In summary, our *in vitro* data strongly suggest that lack of complete elimination of CICs by TA-specific mAbs in combination with chemotherapeutic agents, targeted agents or radiotherapy plays a major role in disease recurrence which represents a significant obstacle to the successful clinical efficacy of TA-specific mAbs. The *in vivo* relevance of these *in vitro* data has to be tested in animal model systems since the resulting information may represent a useful background for a rational design of an effective clinical strategy.

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Conflicts of Interest

No potential conflicts of interest were disclosed.

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