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Photochemical internalisation, a minimal-invasive strategy for light-controlled endosomal escape of cancer stem cell-targeting therapeutics

Pål Kristian Selbo[1,2,*], Monica Bostad[1,2], Cathrine Elisabeth Olsen[1,2], Victoria Tudor Edwards[1,2,3], Anders Høgset[2,3], Anette Weyergang[1] and Kristian Berg[1]

[1]Department of Radiation Biology, Institute for Cancer Research, The Norwegian Radium Hospital, Oslo University Hospital, Montebello, Norway
[2]Cancer Stem Cell Innovation Center (SFI-CAST), Institute for Cancer Research, Norwegian Radium Hospital, Oslo University Hospital, Oslo, Norway
[3]PCI Biotech, Lysaker, Norway

[*]Corresponding author address: Department of Radiation Biology, Institute for Cancer Research, Norwegian Radium Hospital, Oslo University Hospital, P. O. Box 4950 Nydalen, N-0424 Oslo, Norway. Tel.:+47 22 78 14 69
E-mail address: selbo@rr-research.no

Abstract

Despite progress in radio-, chemo- and photodynamic therapy (PDT) of cancer, treatment resistance still remains a major problem for patients with aggressive tumours. Cancer stem cells (CSCs) or tumour-initiating cells are intrinsically and notoriously resistant to conventional cancer therapies and are proposed to be responsible for the recurrence of tumours after therapy. According to the CSC hypothesis it is imperative to develop novel anticancer agents or therapeutic strategies that take into account the biology and role of CSC. The present review outlines our recent work pointing out photochemical internalisation (PCI) using the clinically relevant photosensitiser $TPCS_{2a}$/Amphinex® as a rational, non-invasive strategy for light-controlled endosomal escape of CSC-targeting drugs. PCI is an intracellular drug delivery method based on light-induced ROS-generation and a subsequent membrane-disruption of endocytic vesicles leading to cytosolic release of the entrapped drugs of interest. In different proof-of-concept studies we have demonstrated that PCI of CSC-directed immunotoxins targeting CD133, CD44, CSPG4 and EpCAM is a highly specific and effective strategy for killing cancer cells and CSCs. CSC overexpressing CD133 are PDT-resistant, however, this is circumvented by PCI of CD133-targeting immunotoxins. In view of the fact that $TPCS_{2a}$ is not a substrate of the efflux pumps ABCG2 and P-glycoprotein (ABCB1), the PCI-method is a promising anti-CSC therapeutic strategy. Due to a laser-controlled exposure, PCI of CSC-targeting drugs will be confined exclusively to the tumour tissue, suggesting that this drug delivery method has the potential to spare distant normal stem cells.

1. Introduction

In contrast to differentiated cancer cells with restricted proliferative potential, cancer stem cells (CSCs) are immortal and highly aggressive malignant cells.[1-3] By mimicking normal stem cell biology, CSCs have the capacity to self-renew and differentiate into phenotypically diverse cancer cells and initiate and thereby recapitulate the original human tumour histology in immunodeficient mice models. Although the clinical impact of CSCs remains to be directly proven clinically, a number of pre-clinical experimental evidence in different transplantation assays have provided support to the CSC hypothesis.[4-12] Several recent independent reports on genetic lineage tracing to track CSCs *in vivo* provide direct, functional validation to the CSC hypothesis in natural unperturbed mice tumour models.[13-15] CSCs represent the most resistant population(s) in the tumour. CSCs are intrinsically and notoriously resistant to conventional cancer therapies and hence, it has been suggested that this surviving population of cells are the major drivers of tumour recurrence after therapy. Given that eradication of CSCs is hypothesized to be a prerequisite to achieve cure of cancer, there is an urgent need for novel strategies that directly target and kill the CSCs.[3,16-18] However, is targeting of exclusively one CSC population the right way to go? A change in the tumour microenvironment may result in de-differentiation of differentiated cancer cells resulting in gain of a progenitor or CSC phenotype, confirming the high plasticity of tumors, however, challenge the proposed strategy of targeting only CSC to obtain therapeutical success.[19] In addition, in the same tumour there may exist independent CSC populations.[20] If such parallel CSC populations have a different expression pattern, specific targeting of one of the CSC populations may lead to survival of the non-targeted CSC population. Consequently, this may have implication for the success of novel CSC targeting strategies. Hence, it is

plausible to evaluate simultaneous targeting of both CSCs and the bulk population of the tumour. This may be obtained by using a combination of drugs that both eliminate the distinct CSC populations and differentiated cells as opposed to current conventional therapies that are mostly based on monotherapies or combination therapies lacking specificity for the CSC population.

We have previously demonstrated that the light-triggered drug delivery technology photochemical internalisation (PCI) is able to induce complete responses of highly aggressive tumors in vivo.[21,22] In addition, PCI-based photosensitizers, including the clinical relevant $TPCS_{2a}$/Amphinex® are neither substrates of P-glycoprotein/MDR-1/ABCB1 nor ABCG2/BCRP (breast cancer resistance protein).[23-25] Furthermore, we have demonstrated that PCI-based targeting of EpCAM, a marker used to enrich CSCs, resulted in significant kill of cancer cells of various cancer types.[26-28] Based on these studies, we hypothesized that PCI of immunotoxins targeting CSC-markers may represent a rational approach to eradicate CSCs.

In this review, we present our recent work on the PCI-based targeting of CSCs. We first discuss important factors that make CSCs highly resistant to chemo-, radio- and photodynamic therapy. Then we present the intracellular drug delivery technology PCI and we discuss our recent data revealing that PCI of immunotoxins targeting different CSC-markers, including CD133, CD44, EpCAM and CSPG4, is highly specific and efficient. In addition, others works on the putative implication of CSCs in PDT will be briefly discussed.

2. Hallmarks of CSCs that render them resistant to ROS-generating therapies

Mimicking the nature of normal stem cells that protect themselves against xenobiotics and mutagenic insult, CSCs are thought to be highly resistant to conventional cancer therapies and are proposed to cause relapse of tumour after end of therapy.[1,29-32] There are several factors that make CSCs hard to kill by reactive oxygen species (ROS)-generating therapies, including chemo-, radio,- and photodynamic therapy:

2.1 CSCs thrive in hypoxic conditions.

As for hematopoietic stem cells, CSCs thrive in hypoxic conditions,[33-37] which may *per se* represent a barrier for cancer therapies including radiotherapy, PDT and some chemotherapeutics (e.g. doxorubicin and bleomycin) that are dependent on oxygen for the generation of cytotoxic levels of ROS. Yeung et al. reported that hypoxia enriched and preserved the CSC phenotype of colorectal cancer-derived cell lines and induced undifferentiated dense colonies, whereas normoxia promoted differentiation into complex spheroids with lumen-containing structures.[36] Li et al. also demonstrated that hypoxia-inducible factors are important driver of glioma CSCs.[35] In contrast, distinct glioma CSCs seem to be dependent on factors produced by the vasculature, and hence remain in the perivascular niche indicating that hypoxia is not a general driver of stemness.[38,39]

2.2. CSCs have a strong anti-ROS defence system.

Normal stem cells have upregulated non-enzymatic and enzymatic ROS-scavenging systems as crucial mechanisms to deal with intracellular stress and ROS accumulation. These antioxidant defence mechanisms play important roles as redox regulators of self-renewal and differentiation.[40-44] In addition, CSCs have also been shown to possess an elevated ROS defence capacity that renders them resistant to

chemo-, radiotherapy and potentially photodynamic therapy.[45-48] Pharmacological depletion of ROS scavengers such as the major intracellular antioxidant reduced glutathione (GSH) upregulated in CSCs was first shown by Diehn et al. to significantly attenuate the clonogenicity of CSCs, which resulted in enhanced radiosensitization in vivo.[49] Of high interest, Ishimoto demonstrated that overexpression of the CSC marker CD44 together with xCT, a subunit of the cystine-glutamate antiporter system xc^-, promoted cystine uptake and thereby increased the intracellular synthesis of GSH.[50] GSH is known to be the most important non-enzymatic ROS-scavenger and indeed, several independent reports have demonstrated its important role in reducing the efficacy of PDT.[51-53]

2.3. CSCs efficiently efflux small drugs and some photosensitizers

Multidrug resistance (MDR) is a major barrier for small drug-based anti-cancer agents and is proposed to play a central role for protection and survival of CSCs.[54] The ATP-binding cassette (ABC) efflux transporter ABCG2 (CD338/BCRP/MXR) over-expressed in several types of normal stem cells is suggested to be an important driver of MDR in CSCs.[55] A key physiological role of the efflux transporter ABCG2 is to protect normal stem cells from xenobiotics and intracellular stress.[56] Of potential relevance for several photosensitizers used in clinical PDT, it has been indicated that one important function of ABCG2 in normal stem cells in hypoxic environments is to prevent intracellular accumulation of heme and porphyrins, which can be stress- and harmful for the cells.[57] ABCG2 is up-regulated by hypoxia and provides for normal but also CSCs a strong survival advantage in such a stressful environment.[58] ABCG2 has also been shown to be a molecular determinant of the Hoechst 33342 side-population (SP) phenotype that is detected as a distinct cell population exerting efflux

of Hoechst 33342 and high ABCG2 activity.[59,60] CSCs have been found enriched in the SP of several tumour cell lines of human,[61-63] mouse[64,65] and rat[66] origin, however, this is not established as a general phenomenon as CSCs are also detected in the non-SP.[60] A number of anti-cancer agents are substrates of ABCG2 including mitoxantrone, topotecan, doxorubicin, daunorubicin, methotrexate, irinotecan and its active metabolite SN-38, and the tyrosine kinase inhibitors erlotinib, imatinib and gefitinib.[55,67] Originally, MDR-1 (P-glycoprotein or ABCB1) was thought to promote PDT-resistance,[68] however, this is not the case for most photosensitizers.[23,69-71] In a study by Jonker et al. it was discovered, for the first time, that ABCG2 knockout mice fed with chlorophyll-enriched food develop a new type of protoporphyria.[72] Light-exposure of the skin resulted in some cases to lethal phototoxicity. The serum and the bile of the mice had elevated levels of pheophorbide a, a potent photosensitizer and degradation-product of chlorophyll. In addition, a 10-fold increase of protoporphyrin IX (PpIX) was detected in erythrocytes, compared to wild type mice. Pheophorbide a and PpIX as substrates of ABCG2 has been verified in subsequent studies.[24,73-75] In fact, several other pre-clinical and world-wide clinically approved photosensitizers used in PDT, including pyropheophorbide-a methyl ester (PPME)[73], 2-(1-Hexyloxyethyl)-2-devinyl pyropheophorbide-a (Photochlor)[75,76], Porfimer sodium (Photofrin®)[77], benzoporphyrin derivative monoacid ring A (BPD-MA/ Verteporfin/Visudyne®)[75], chlorin e_6 (Ce_6)[73], Hypericin[78] are to a variable extent substrates of ABCG2. Liu et al. demonstrated, however, that inhibition of ABCG2 with the tyrosine kinase inhibitor imatinib improved the efficacy and selectivity of PDT using some of these clinically approved photosensitizers,[75] although the clinical impact of ABCG2 in cancers subjected to PDT remains to be documented. In contrast, PCI-photosensitizers are not substrates of ABCG2, and hence we proposed that PCI

of CSC-targeting drugs can be used as a strategy to circumvent ABCG2-based resistance and to kill MDR-cells and CSCs.[24]

On the contrary, the following photosensitizers are not substrates of ABCG2 are meso-tetra(3-hydroxyphenyl)porphyrin (m-THPP), meso-tetra(3-hydroxyphenyl)chlorin (m-THPC, Foscan®)[73,74], hematoporphyrin IX (HpIX)[73] and HPPH-galactose[76].

2.4 CSCs have enhanced DNA repair capacity

CSCs are chemo- and radiotherapy resistant partly because of rapid activation of the DNA damage sensor and repair machinery resulting in an extreme capacity in protection of genome integrity.[79,80] In a seminal study by Bao et al., it was shown that CSCs display a basal amplified checkpoint (Chk1/2 kinases) activation that prime CSCs to defend against genomic insults such as ionizing radiation.[46] Notably, it was demonstrated that pharmacological inhibition of Chk1/2 kinases diminished the radioresistance of $CD133^{high}$ glioma CSCs both in vitro and in vivo, indicating that checkpoint activation is critical to the radioresistance of the CSC population.

Enhanced DNA damage repair in CSCs may also have implications for PDT-based targeting of DNA, although clinical photosensitizers have been designed and developed to avoid nuclear localization and potential mutagenic insults. With a few exceptions, chemo- and radioresistance do not affect sensitivity to PDT, [81] and given that clinically based PDT and PCI photosensitizers are not localized in the nucleus, these technologies are suggested to confer rational strategies to kill therapy resistant cancer cells. Indeed, we have demonstrated that chemo- and radioresistant sarcoma cells are highly sensitive to PCI of the ribosome-inactivating protein gelonin.[23,25]

2.5 CSCs have hyper-activation of ROS-sensitive survival pathways

Another important aspect of CSCs that render them ROS therapy resistant is their upregulated pathways for self-renewal and survival also known to be central for the integrity of normal stem cells and progenitor cells such as BMI-1[82], Sonic hedgehog{Ruiz i Altaba, 2011 9793 /id}, Wnt[8,84], Notch[85] , Stat3[86], SOX2[87] HER2[88] and EGFR[89] and for that reason it has been suggested that novel CSC-targeting drugs should take one or several of these regulators into account to obtain significantly improved cancer therapies. Since the expression of these pathways will vary between patients and cancer types, precision medicine will be needed to guide future therapeutic interventions including CSC-targeted drugs.

We have shown in several studies that PCI-based targeting of EGFR, using either EGF-saporin or cetuximab-saporin, is a promising approach providing potent cytotoxic responses in different types of cancer cell lines.[24,90-92] In these studies we did not examine if enrichment of CSCs was due to overexpression of EGFR. However, one of the MDR models, the ABCG2^{high} and EGFR^{high} MA11 breast cancer cell line was extremely sensitive to PCI of the EGFR-targeting toxin EGF-saporin.[24] Furthermore, Berstad et al. demonstrated the feasibility of PCI of a recombinant immunotoxin targeting EGFR in vivo,[93] suggesting that this targeting method is a potential therapeutic approach for the eradication of aggressive head and neck squamous cell carcinoma (HNSCC).

It has also been demonstrated that CSCs display higher VEGF-expression than differentiated cancer cells both in normoxic and hypoxic conditions.[94,95] PDT using different clinically relevant photosensitizers, e.g. Photofrin, BPD-MA and ALA-PpIX, induce overexpression of VEGF.[96,97] However, this may be controlled by co-treatment with celecoxib[98], bevacizumab[99], p38-inhibitors[97] or vascular targeting

TKIs.[100] Conversely, blocking VEGF by bevacizumab in combination with BPD-PDT in two different tumour models using the chorioallantoic membrane of the chicken embryo did not improve the effect of PDT,[100] also experienced in our preclinical studies (unpublished data). Furthermore, in several studies it has been revealed that PDT, using different clinical photosensitizers, induced expression of NF-κB, Erk1/2, COX-2, iNOS and survivin, where all were correlated with PDT resistance.[101-105] Interestingly, these mediators have also been shown to be important for the survival of CSC.[79,106-108] Of relevance to this, we have shown strong cytotoxic synergy after combining a MEK1/2 inhibitor (PD98059) and an EGFR inhibitor (Tyrphostin AG1478) with PDT in A431 cells.[109] In addition, recently PCI of the recombinant fusion toxin VEGF/rGel was found to be a potent approach to target VEGFR-expressing cells in tumors.[110]

2.6 Quiescent CSCs are chemo- and radiation therapy resistant.

Quiescence or dormancy is an important state that preserves the self-renewal of normal stem cells,[111] but is also suggested to be an essential reason for drug- and radiotherapy resistance of CSCs, [112-114] although this cannot be generalized since undifferentiated testicular germ cell tumour cells are more sensitive to cisplatin or radiation therapy than differentiated daughter cells. [115] Expression of forkhead box O (FOXO), hypoxia-inducible factor 1α (HIF1α) and liver kinase B1 (LKB1) is central for metabolic functions and protect quiescent normal stem cells challenged with ROS.[114,116] Hence, targeting of the molecular mechanisms that cause dormancy or keeping the CSCs in an undifferentiated state may induce the sensitization of CSCs to therapeutic agents. Of notice, it was shown in a study by Ortel et al. that differentiation therapy, e.g. using vitamin D analogues, rendered LNCaP prostate

cancer cells highly sensitive to ALA-PDT.[117] Even though this study did not explore potential CSC-phenotypes, it is plausible, in the light of the inhibitory effect of D-vitamin (calcitriol) on Wnt-signalling, associated with the CSC phenotype,[118-120] that this approach may represent a therapeutic strategy by forcing CSCs to differentiate and subsequently kill them by photosensitisation. Since all quiescent or dividing eukaryotic cells undergo endocytosis,[121] we suggest that PCI-based targeting utilizing CSC-directed immunotoxins that is efficiently taken up in cells by endocytosis has the capacity to kill dormant CSCs.

In summary, these different resistant mechanisms, separate or in concert, call for novel therapeutical strategies that take into account the biology of notoriously therapy-resistant CSCs. This could be obtained either by directly or indirectly targeting of the CSCs or by modifying the tumour microenvironment in a way that force the CSCs to differentiate thereby render them more sensitive to ROS-generating therapies. We propose that PCI-based targeting of CSCs may represent a novel approach to eradicate CSCs.

3. Eradicating only a distinct CSC population may not be the right way to go

In two independent studies by Morel et al.[122] and Mani et al.[123] it was shown that activation of the Ras/MAPK signalling pathway in non-tumourigenic mammary epithelial cells gave rise to a cell population that displayed $CD44^{+}CD24^{-}$ stem-like signatures and exhibited an epithelial-mesenchymal transition (EMT) phenotype that was characterized by the loss of E-cadherin and gain of vimentin expression, strongly suggesting a link between EMT and stemness. Indeed, molecular connections between EMT and cancer stemness were later demonstrated in two important reports.[124,125] Yang et al., showed that overexpression of Twist induced EMT and enhanced CSC-

properties in HNSCC.[124] Vermeulen et al. demonstrated that stromal cells surrounding the CSC preserved a high Wnt activity in the CSCs, and of high interest, stromal cell secreted hepatocyte growth factor stimulated differentiated tumour cells to express high Wnt resulting in gain of cancer stemness of colon carcinoma cells.[125] Recently, it was demonstrated by Rizvi et al. that flow induces upregulation of EGFR activity, vimentin and downregulation of E-cadherin which resulted in EMT in 3D ovarian cancer nodules.[126] Altogether, these reports indicate that differentiated cells gain CSC properties via the EMT process upon shift in the microenvironment of the tumour, and thereby challenge the cancer stem cell hypothesis. The implication of these seminal studies suggests the importance of not only targeting the CSC population of the tumour, but also emphasizes the necessity to establish therapeutic interventions that target both CSCs and differentiated cells, with the microenvironment of the tumour in mind, to gain successful therapy. Due to a Darwinian evolution of cancer, high-risk heterogeneous tumors may have co-existence of independent clones with distinct genotype and phenotype. Hence, the CSC story gets even more complex: Several reports indicate the existence of independent sub-clones of CSCs in a tumour which carry distinct phenotypic profiles.[3,127-129] These are dynamic CSC populations that alter their phenotypes with the change of the microenvironment of the tumour over time. Such variations, both intratumourally but also most truly between patients, point to the importance of moving cancer treatments from stratified to personalised cancer medicine. If these observations are reflected in clinical situations, developing successful anticancer therapeutic interventions that take into account the tremendous heterogeneity of high risk tumors will be extremely challenging.

4. PCI is an efficient and specific light-controlled intracellular drug delivery method providing endosomal escape of entrapped therapeutics

PCI is a minimal invasive, highly specific and efficient technology for light-enhanced cytosolic release of membrane-impermeable molecular therapeutics entrapped in endocytic vesicles (Fig. 1).[130-132] The PCI photosensitizer $TPCS_{2a}$/Amphinex® is currently in a Phase II clinical trial in combination with the chemotherapeutic drug bleomycin (PCI of Bleomycin) for the treatment of inoperable HNSCC at 10 cancer centres in Europe. In addition, a Phase I/II study for the treatment of bile duct cancer, with gemcitabine ($TPCS_{2a}$-PCI of gemcitabin) followed by systemic cisplatin/gemcitabine, in patients with inoperable bile duct cancer is running at several hospitals in Europe.

Briefly, the drug or molecule/complex to be delivered co-localizes with the PCI-photosensitizer in endocytic vesicles. [22,130,133] Upon exposure to light matching the absorbance peak(s) of the PCI-photosensitizer (in vitro: blue lamp with main peak around 420nm, in vivo: 652nm red laser), the light is absorbed by the PCI-photosensitizer from the triplet excited state, the energy is transferred to molecular oxygen (O_2) generating the ROS singlett oxygen ($^1O_2$), also named the Type II reaction in photochemistry.[134] Alternatively, at low oxygen levels, the triplet excited state undergo a Type I reaction by electron exchange between the PS and the substrate, e.g. a biomolecule.[81] Light-triggered ROS-generation induces lipid peroxidation of the membranes of endosomes and lysosomes resulting in membrane rupture or destabilisation with a subsequent endosomal escape of the drug into cytosol. PCI has been demonstrated to be a feasible drug delivery technology in >80 different cancer cell lines and in >10 different animal models.[132] PCI has been shown

to be a highly efficient technology for induced endolysosomal escape for several cancer targeted therapeutics,[21,132,135] and to increase the efficacy of some conventional anti-cancer agents.[132,136,137] Parallel to the clinical evaluations of PCI, we are currently elucidating the potential of PCI to enhance the efficacy of anti-cancer targeting therapeutics[23,135] and vaccines[138,139].

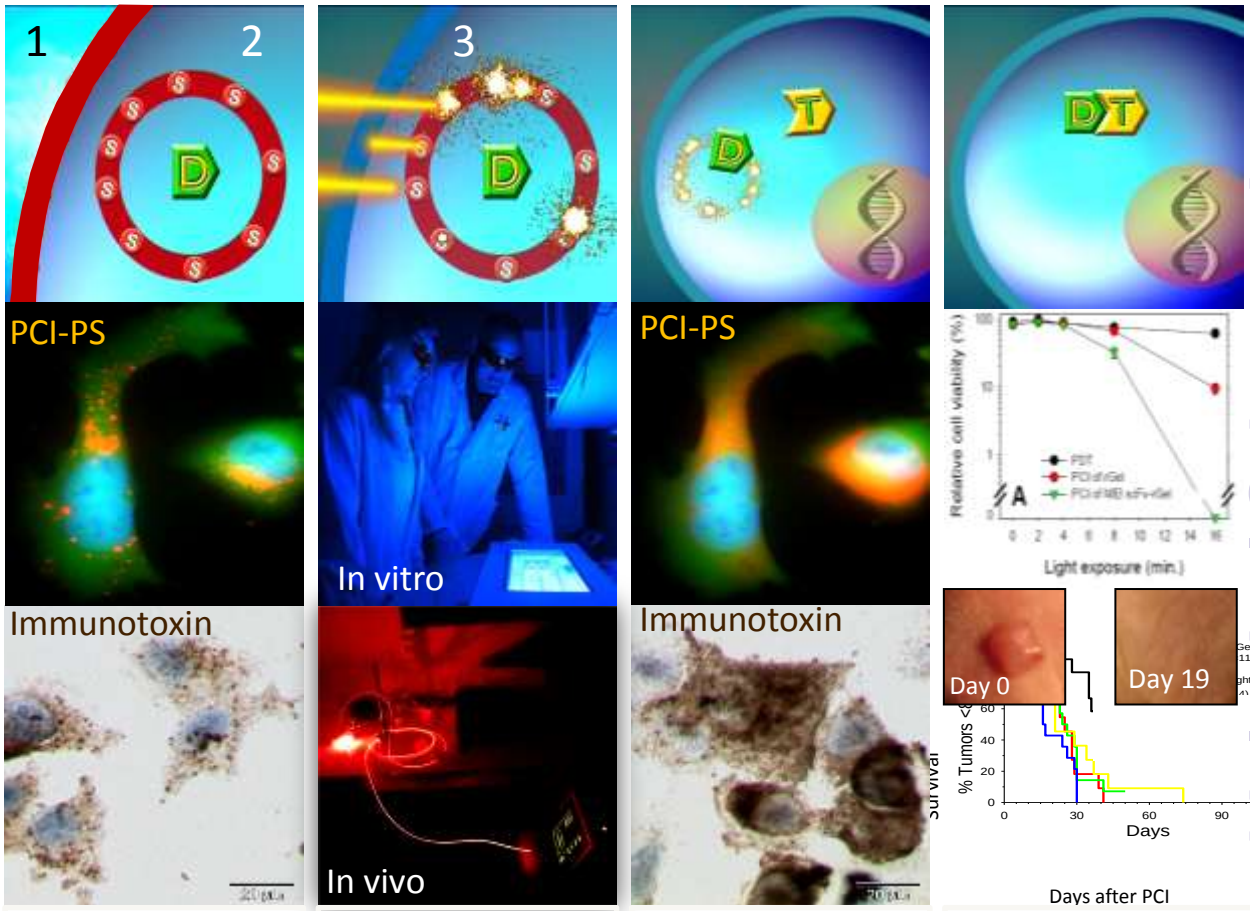

Figure 1. Principle of the PCI drug delivery technology. 1, Following local or systemic administration of PCI-photosensitizer (S) and drug of interest (D, e.g. CSC-targeting immunotoxin), both agents accumulate in tumour and are subsequently taken up in tumour cells by endocytosis and accumulates in endosomes and lysosomes (panels under). 2, Light activation, e.g. LumiSource blue light exposure in vitro or red laser exposure of tumour in vivo, and excitation of PCI-photosensitizer entrapped in endocytic vesicle membranes leads to generation of reactive oxygen species (mainly singlet oxygen), which 3, burst or destabilise the membranes resulting in endosomal escape and

cytosolic delivery of both photosensitizer and drug. 4. PCI of drug which attacks its biological target (T) resulting in potent cytotoxic responses in vitro or ablation of tumour xenograft (panels under). The figure is partly based on experimental data published in PLOS ONE.[21]

4.1 Strongly amphiphilic photosensitizers used for PCI-based drug delivery are not substrates of the CSC marker ABCG2

Over-expression of active ABCG2 in CSCs may explain that some tumour types are resistant to a broad range of anti-cancer drugs[140] and potentially PDT. Of high relevance, a number of clinical and pre-clinical relevant PDT-photosensitizers have been reported to be substrates of ABCG2 as described above. However, ABCG2-mediated transport of photosensitizers can be blocked by utilizing ABCG2 inhibitors, e.g. fumitremorgin C (FTC),[73-75] but potential neurotoxic effects of FTC does not make it applicable in humans.[141] Moreover, since ABCG2 has an important physiological function in protecting many types of normal tissues (e.g. brain, mammary gland, testis, gastrointestinal tract, kidney, liver, the biliary tract and hematopoietic stem cells) from xenobiotics,[55] systemic inhibition of ABCG2 may lead to severe adverse effects. Conversely, the tyrosine kinase inhibitors (TKI) imatinib mesylate (STI571, Gleevec/Glivec), gefitinib (ZD1839, Iressa) and nilotinib (AMN107, Tasigna) have been shown pre-clinically to reverse resistance to chemotherapeutics by antagonizing ABCG2-mediated MDR.[142-145] Liu demonstrated that also HPPH efflux can be abrogated by using imatinib, which lead to increased PDT efficiency due to enhanced selectivity and uptake of HPPH in 4T1 mouse breast carcinoma tumors.[75]

We demonstrated that strongly amphiphilic photosensitizers used for PCI-based drug delivery, including a porphine ($TPPS_{2a}$), a chlorin ($TPCS_{2a}$) and a phtalocyanine ($AlPcS_{2a}$), are neither substrates of ABCB1/p-gp/MDR1[23,25] nor ABCG2/BCRP[24], both important efflux pumps for normal and CSCs. In these studies, and in the work of Lou et al, [137] it was clearly demonstrated using a variety of MDR models that PCI is a promising strategy to circumvent drug resistance. To evaluate the possible interaction of PCI-photosensitizers, including $TPCS_{2a}$, with ABCG2, we utilized flow cytometry to measure and compare both Hoechst 33342 and photosensitizer accumulation, with or without presence of the specific ABCG2-inhibitor fumitremorgin C (FTC) in the $ABCG2^{high}$ breast cancer cell line MA11. The MA11 cell line was found to have a SP of >50%, however no shift in fluorescence of PCI-photosensitizers was found when FTC was co-incubated with the PCI-photosensitizers. However, a strong FTC-enhanced fluorescence was obtained both for Hoechst 33342 and the control photosensitizer pheophorbide a,[24] in line with previous reports.[72-74] The flow cytometry data were verified by fluorescence microscopy and confirmed in the glioma cell line U87. Finally, no significant effect upon addition of FTC was observed on cell viability after treatment with $TPCS_{2a}$ or $TPPS_{2a}$ based photochemical treatment. In contrast, FTC significantly enhanced the cytotoxic effect of pheophorbide a-PDT, [24] thereby verifying previous data by Robey and co-workers.[74] In this review we also present experimental data demonstrating that blocking ABCG2 efflux activity by using imatinib (Glivec/Gleevec) and for the first time nilotinib (Tasigna) resulted in strongly enhanced pheophorbide a-PDT (PhA-PDT) (Fig.2), while no change in survival was observed using these inhibitors in combination with $TPCS_{2a}$ and light, validating that $TPCS_{2a}$ is not a substrate of ABCG2 (data not shown) as earlier shown.[24]

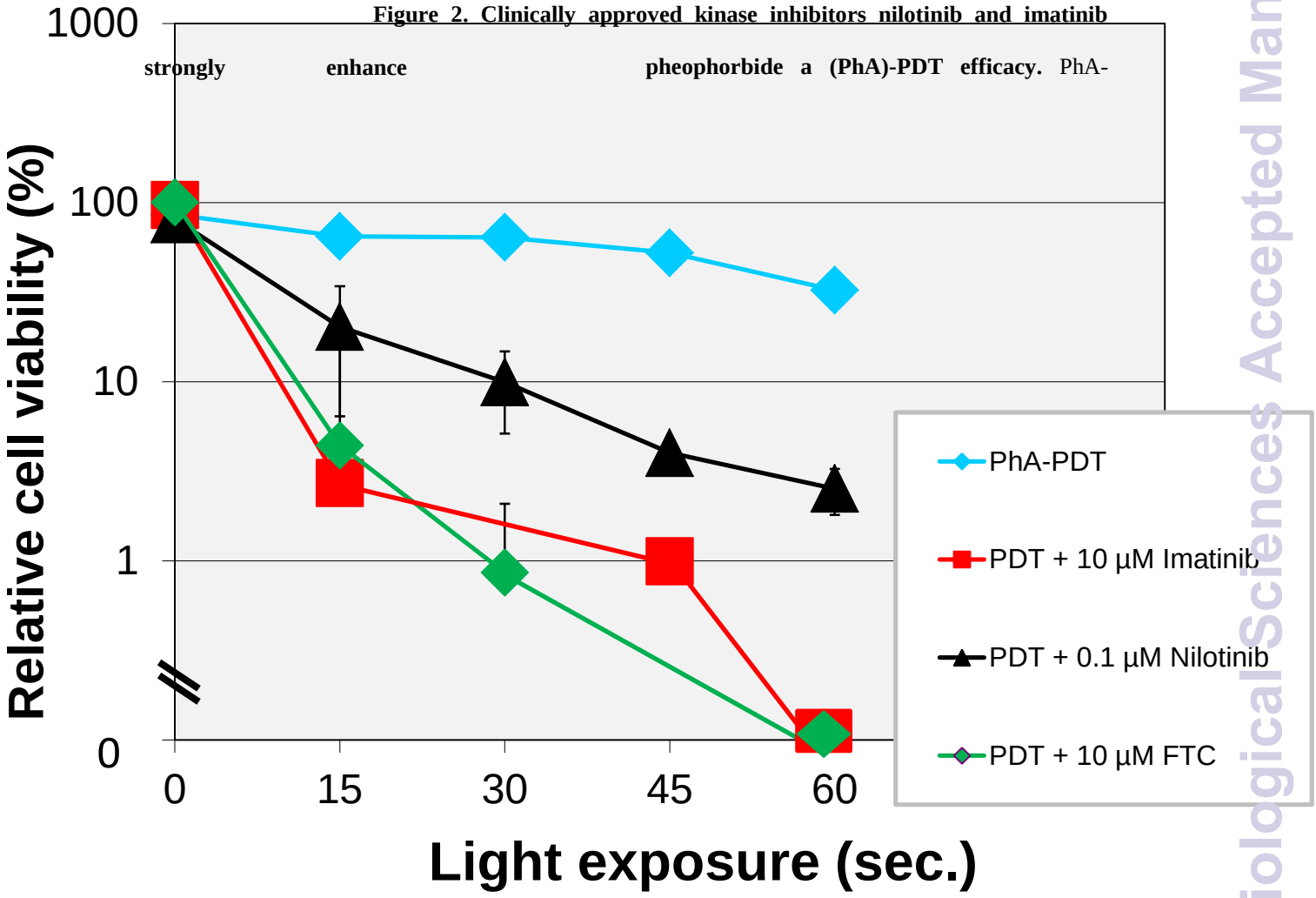

Figure 2. Clinically approved kinase inhibitors nilotinib and imatinib strongly enhance pheophorbide a (PhA)-PDT efficacy. PhA-

Figure 2

PDT-resistant breast carcinoma MA11 cells overexpressing ABCG2 were first pre-treated for 1 hour with nilotinib or imatinib or the ABCG2 specific inhibitor fumitremorgin C (FTC) and then co-incubated with 1 µM PhA for 1 hour prior to LumiSource light exposure (Irradiance 12.5 mW/cm^2). Cytoxicity was evaluated by the MTT-assay 24 hours post PDT. Co-treatment with imatinib resulted in similar cytotoxic response as for FTC treatment. Data points are average of triplicates from MTT-assay and were reproduced in three independent experiments. Error bars, SD.

Epidermal growth factor receptor (EGFR) is highly efficiently endocytosed[146] and its co-expression with ABCG2 is associated with drug resistance in lung cancer patients.[147] EGFR and Akt pathways are important regulators of ABCG2 expression and function, however, various tyrosine kinase inhibitors (TKIs) may be used to downregulate ABCG2-activity.[148-150] PCI-based cytotoxic targeting of EGFR in the ABCG2^{high}-expressing MA11 cells by PCI of 1 pM of the targeting-toxin EGF-saporin resulted in a very strong cytotoxic response compared to toxin or photochemical treatment alone in the MA11 cells, thereby validating our previous observations on the PCI-based targeting of EGFR in other cancer cell lines, including carcinomas of colon, prostate, epidermis and ovary.[90,91] The strong cytotoxicity obtained with picomolar levels of EGF-saporin indicates efficient endocytosis and suggests a favourable therapeutic window for this treatment regimen. The importance of EGFR-blockage in combination with PDT has previously been shown by del Carmen at al. and us,[92,109,151] although these studies did not investigate the role of ABCG2.

By evaluating both single layer cells (2D) and spheroids (3D) we found that all MA11 cells were positive for ABCG2. In addition to localization of ABCG2 on the plasma membrane, we observed that all cells had an elevated intracellular content of ABCG2. Interestingly, in a small fraction of the MA11 cells, ABCG2 was also for the first time detected in the cell nucleus suggesting a new role of ABCG2, subsequently revealed by Bhatia et al.[152]

Hence, based on our important data demonstrating that amphiphilic PCI-photosensitizers are neither substrates of ABCG2 nor ABCB1, we went further on to explore $TPCS_{2a}$ as an attractive candidate for PCI-based targeting of CSC-markers.

4.2 PCI of CSC-targeting therapeutics

Pre-clinically, we are currently exploring PCI as a rational strategy for light-controlled targeting of CSC. In this setting we enhance the intracellular delivery of a CSC-targeting drug, e.g. a monoclonal antibody (mAb) or a single chain Fv fragment (scFv) of the mAb targeting a CSC-marker expressed on the plasma membrane, linked to the protein-toxins saporin or gelonin (making an immunotoxin) by using $TPCS_{2a}$ -PCI (Fig.3). We have established in vitro that the PCI method can be utilized for specific and efficient delivery of model drugs targeting the CSC markers CD133, CD44, CSPG4 and EpCAM[21,26-28,153-155] and in addition we have experimental data indicating that CD24, CD271 and CD90 may also represent potential CSC targets for PCI (unpublished data). We have also obtained data demonstrating the potential of PCI-based CD133-targeting in vivo (Manuscript in preparation). In this section we will highlight our recent studies demonstrating that PCI of CSC-targeting therapeutics may represent a rational strategy to kill CSC.

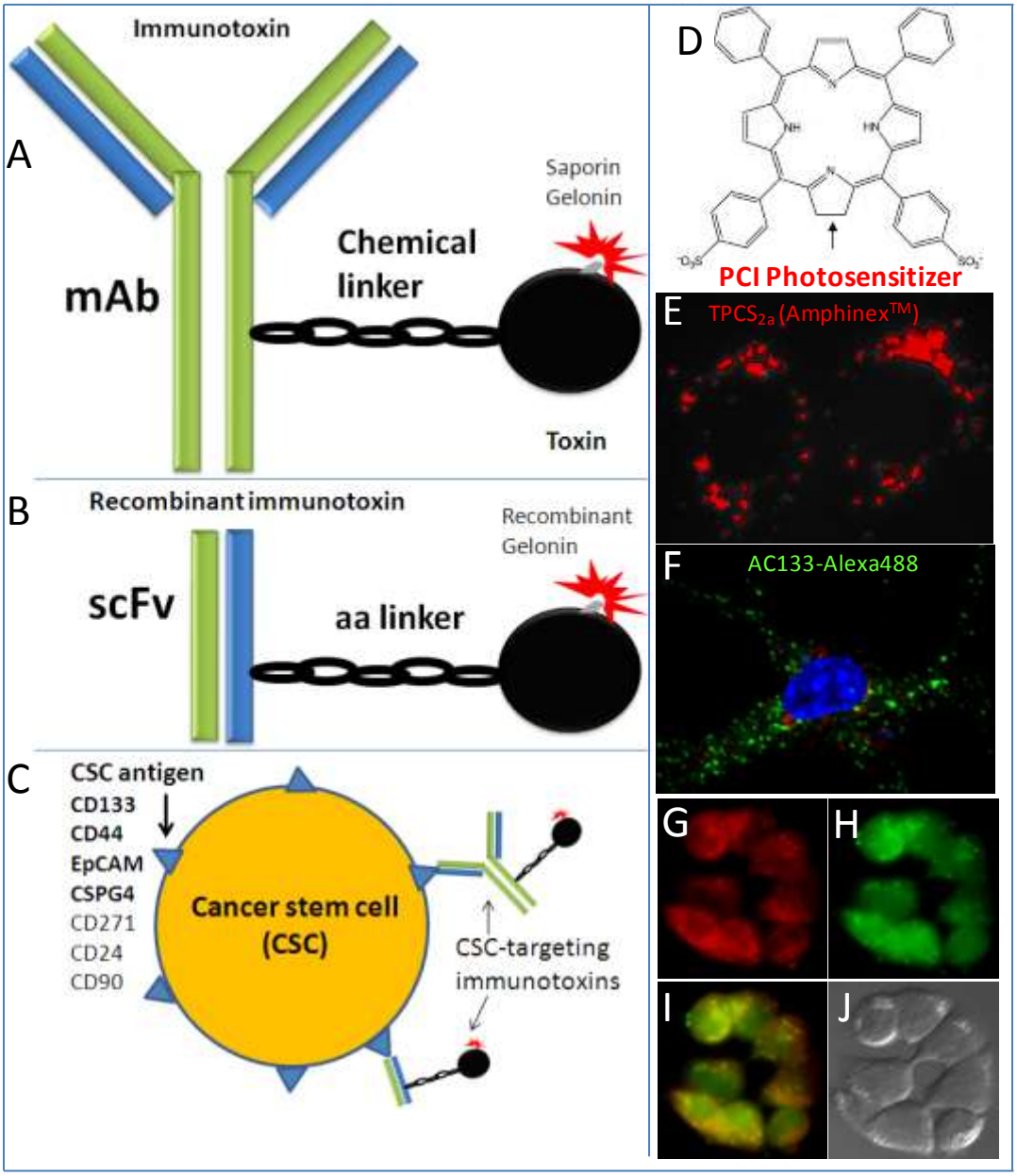

Figure 3. Drugs used in PCI-based targeting of CSCs. A, Immunotoxin consisting of a mAb chemically linked with a disulfide bond to a ribosomal inactivating toxin, e.g. saporin or gelonin. The linker can also be a streptavidin-biotin bridge. B, Recombinant immunotoxin or fusion toxin based on a scFv of the mAb fused to gelonin via an amino acid (aa) linker thereby achieving a 3-5-fold reduction of the molecular weight of the immunotoxin compared to an antibody-toxin conjugate. C, Specific binding of CSC-targeting immunotoxins to CSC-antigens on the cell surface prior to endocytosis. D, Chemical structure of $TPCS_{2a}$/Amphinex®. E, typical intracellular fluorescence pattern of $TPCS_{2a}$ and E, of CD133-targeting mAb AC133 labelled with Alexa-488 prior to PCI. G, PCI-induced endosomal

escape of $TPCS_{2a}$ and H, CD133-targeting immunotoxin. I, merge of G and H. J, DIC micrograph of WiDr colorectal adenocarcinoma cells.

The low cure rates by conventional cancer therapies used today are not due to lack of efficiency per se, but mainly due to reduced specificity, often resulting in dose-limiting toxicity and severe adverse effects. Immunotoxins have in some cases provided high selectivity, but still, a major limitation of immunotoxins is limited tumour tissue penetration of monoclonal antibodies or antibody-drug-conjugates. In addition, the treatment involves multiple injections causing induction/generation of neutralizing antibodies.[156] Furthermore, both specific and unspecific off target effects cause adverse events such as vascular leak syndrome and damage to tissues in the body that expresses the target antigen [157]. Most of these obstacles may be prevented by using PCI to enhance intracellular delivery of immunotoxins. The PCI-method provides drug delivery with a primarily localized effect due to the light-controlled activation of the PCI-photosensitizer and endosomal escape of the drug to the cytosol only in the illuminated tumour tissue.[132] In addition, as previously demonstrated, only one treatment is required, i.e. one injection of anti-cancer drug and one following light exposure,[21] thus the potential impact of this is that patients may avoid multiple drug injections and treatment-induced resistance. Additionally, since all eukaryotic cells undergo endocytosis, [121] the PCI-based targeting will also affect quiescent cancer cells, including CSCs. Since the putative CSC markers CD133, CD44 and EpCAM are also expressed in normal stem and progenitor cells,[158] it is of high importance that drugs targeting these receptors are specifically and efficiently delivered and activated only in malignant tissues to prevent severe adverse effects, as observed with systemic anti-CD44v6 treatment.[159] By employing site-specific strategies, such as PCI, which activates CSC-targeting drugs only in malignant tissues, in a spatio-temporal laser-

controlled manner, this can be achieved. Hence, we propose the potential to shield normal stem cells by use of the PCI method to deliver CSC-targeting drugs.

4.2.1 PCI-based targeting of CD133

CD133 (Mouse version: Prominin-1) was originally shown to be a marker of human hematopoietic progenitors[160,161] and mouse embryonic and adult neuroepithelium.[162] CD133 has also been proposed as a novel therapeutic target for the eradication of CSC in tumour tissues from colon[4-6], brain[46,163,164], pancreas[165], prostate[166], ovary[167] non-melanoma skin[168] and sarcoma[169,170]. CD133 is an independent prognostic factor for poor clinical outcome in patients with colon carcinomas and brain gliomas.[171-173] However, this is probably not due to its role as a CSC marker, but rather that CD133 is related to hyperactivation of the Ras–Raf–MEK–ERK pathway.[173] Elevated expression of CD133 has been linked to development of tumour resistance in colon cancer and gliomas, where gliomas it was shown that $CD133^{high}$ cells had increased expression of ABCG2, DNA repair enzymes and inhibitors of apoptosis and restricted proliferative potential.[174] CD133 overexpression has also been linked to radioresistance in medulloblastoma cells.[34] CD133 as a marker of CSC has been under debate since plasmamembrane $CD133^{neg}$ tumour cells seem to have tumour initiating capacity.[175,176] This can be attributed to a down-regulation of the glycosylated epitope AC133 of the CD133 protein recognized by the mAb CD133/1, which takes place when the CSCs differentiate. In parallel, neither mRNA nor intracellular and surface CD133 protein expression is changed.[177] Hence, by using the CD133/1 (AC133), such cells are considered to be CD133 negative although they have plasma membrane expression of CD133 or a cytosolic expression of the antigen [178,179]. The AC133 glycosylated epitope may re-occur on the plasmamembrane of cells

that were originally deemed CD133^{neg} indicating CSC plasticity[180] (also observed in our lab, data not published).

In two different studies, we recently demonstrated that CD133^{high} CSCs are very sensitive to PCI of CD133-targeting toxins.[153,154] In the first study, we showed that PCI-based targeting and eradication of CD133^{high} colorectal cancer stem-like cells (WiDr cell line) was highly efficient at femtomolar levels of the targeting toxin AC133-saporin, indicating a potential wide therapeutic window for tumour ablation. Interestingly, we also obtained similar strong cytotoxic responses using the alternative CD133-targeting immunotoxin CD133/2-saporin, based on the mAb clone 293C (CD133/2) as the carrier for saporin. The WiDr cell line that we have used as a CD133^{high} cell line model originates from the same tumour as the HT29 cell line[181] and is both p53[182] and BRAF mutant[183] and it was previously shown to be resistant against both chemo-[184] and radiotherapy.[185] Over 95% of the WiDr cells are CD133+,[186] the cells have an undifferentiated phenotype[187] and are highly tumorigenic.[188] By using FACS, we isolated CD133^{low} and CD133^{high} WiDr cells and demonstrated that the CD133^{high} WiDr cells had enhanced stemness due to enhanced ability to form 2D and 3D colonies, and most importantly, this sub-population had a very high tumour-initiating capacity since as few as 10 CD133^{high} WiDr cells injected s.c. were tumorigenic in athymic nude mice as compared to CD133^{low} that did not initiate aggressive tumors. In this study, we also revealed for the first time that CD133^{high} cancer stem-like cells are resistant to PDT. Conversely, PCI of the CD133-targeting immunotoxin AC133-saporin circumvented the PDT-resistance of the WiDr cells.[153]

High cytotoxic responses after PCI of AC133-saporin were also shown in the high grade, undifferentiated colon carcinoma cell line HCT116, previously reported to

have a large population of cancer stem-like cells[189] and a CD133^{high} population that is radiotherapy resistant.[190] To prove that PCI-based CD133-targeting is also feasible in other cancers, we also demonstrated efficient targeting by PCI of AC133-saporin in the pancreas adenocarcinoma cell line BxPc-3. Despite a very low expression (<1%) of CD133 in the BxPc-3 cells,[165] we obtained a significant and synergistic effect when combining PCI and AC133-saporin, suggesting the importance of the CD133- population in this cell line.[153]

In another work we used a sarcoma cell model derived from a SW872 xenograft where the CD133-population is in average ~2% and when co-expressed with aldehyde dehydrogenase (ALDH), a marker of cancer stem-like cells (CSCs) in various tumour types, express CSC-properties.[191] In this sarcoma model, we demonstrated that in vitro purging of the CD133 cell population by PCI of CD133/2-saporin efficiently depleted SW872 cells with stem-like properties and significantly reduces tumour-initiating capacity of the surviving CD133^{neg} cells.[154]

In summary, PCI of CD133-targeting immunotoxin may represent a promising strategy to eradicate CD133-expressing CSCs. Based on these studies we have now initiated projects on the PCI-based in vivo targeting of CD133.

4.2.2 PCI-based targeting of CD44

CD44 has been suggested as a therapeutic target for eradication of CSCs in several cancers.[192-195] Over-expression of CD44 is associated with aggressive tumour growth, proliferation and metastasis and linked to both chemo- and radiation therapy resistance in breast[45,196], colorectal[197,198], pancreatic[199] and prostate cancer[200-202]. However, due to the expression of CD44 on normal cells, and its important role in many physiological processes in normal cells, it is of high importance to develop

therapeutic strategies that only target and kill CD44-expressing cancer cells.[203] Expression of CD44, in particular CD44v variant isoforms, contributes to ROS resistance due to upregulation of the primary intracellular antioxidant GSH.[204] CD44 has also been associated to MDR,[205] which is suggested to be regulated and assisted by a complex partnership with hyaluronic acid and downstream mediators.[206]

We have demonstrated that the androgen-independent DU145 CD44^{high} prostate cancer cells with strong anti-ROS activity are resistant to PDT compared to the PDT-sensitive CD44^{neg} LNCaP prostate cancer cells. However, the DU145 CD44^{high} cells are hypersensitive to PCI of the CD44-targeting immunotoxin IM7-saporin.[155] The IM7 mAb clone recognizes an epitope common to alloantigens and all isoforms of mouse and human CD44. Moreover, we also showed that PCI of IM7-saporin was highly specific and efficient for the eradication of cancer cells having CD44^{high} populations of pancreatic (BxPc-3 and Mia-Pa-Ca), colon (WiDr), breast (MDA-MB-231) and sarcoma (SW872) origin. Very low cytotoxicity of IM7-saporin alone and no cytotoxicity of IM7 mAb alone was observed in all cell lines, highlighting the promising potential of light-controlled CD44-targeting drug delivery of the PCI technology.[155]

As for the light-controlled and specific elimination of CD133-expressing cancer cells, we propose that this PCI-based CD44-targeting approach may spare distant normal cells expressing CD44, suggesting a novel strategy for selectively eliminating CD44-positive CSCs in vivo. The PCI method has the potential to be used in the treatment of various cancers as a minimally invasive method for targeting aggressive and ROS-resistant CD44 expressing tumors.

4.2.3 PCI-based targeting of EpCAM

The epithelial cell adhesion molecule EpCAM (CD326, ESA, EGP-2, TROP-1, 17-1A) is expressed in near all carcinomas and is of this reason one of the best-studied target antigens of human solid tumors.[207,208] EpCAM^{high} expression has been used in combination with CD44^{high}/CD24^{-} for the identification of CSCs from breast[209], colorectal[192] or pancreatic carcinomas[210]. EpCAM has an important role in signalling function and mitotic activity where it induces the proto-oncogene c-myc and the cell cycle regulating genes cyclin A and E, thereby initiating cell cycle and proliferation.[211] Of high relevance, Maetzel et al. demonstrated that EpCAM has also a direct effect on Wnt signalling and regulation of gene transcription in the nucleus resulting in cell proliferation and tumour formation in mice, probably explaining the wide expression of EpCAM in CSCs in different cancers.[212] EpCAM is expressed on both CSCs and differentiated tumour cells, thus by EpCAM-targeting there is a potential to target the whole tumour population.

EpCAM was selected for the first documentation for PCI-based targeting.[26] Here we used the monoclonal chimeric antibody MOC31 which was chemically conjugated to the powerful ribosome-inactivating protein toxin gelonin. PCI of MOC31-gelonin was shown to be specific and relative efficient in colon adenocarcinoma (WiDr and KM20L2), small cell lung carcinoma (NCI-H146) and ductal carcinoma of the breast (T47D), whereas the EpCAM negative THX melanoma cell line was found non-responsive. However, by confocal microscopy we revealed that the MOC31 mAb was not efficiently taken up in EpCAM-positive cells, which may explain that the PCI-effects was not as efficient as anticipated. This was in accordance with the relative high immunotoxins doses needed to achieve significant cytotoxic therapeutic effects.

One obstacle associated with clinical EpCAM-targeting is the expression of the target on a wide range of normal epithelia. Systemic intolerability and damage of normal EpCAM-expressing tissue is seen with high-affinity antibodies. Hence, there is need for the development of new anti-EpCAM antibodies with improved therapeutic potential. Recently, we used a novel fully human EpCAM-targeting mAb, 3-17I, for the PCI-induced delivery of saporin. The human monoclonal antibody 3-17I was discovered using Affitech's human naïve phage display library, maturation techniques and proprietary CBAS technology.[213] The reduced binding to normal tissues compared to that of MOC31, in addition to the improved antibody dependent cell-mediated cytotoxicity (ADCC)-and complement dependent cytotoxicity (CDC)-activities that 3-17I displays over the EpCAM-targeting mAb MT201, makes 3-17I an attractive candidate for therapeutic and diagnostic applications and for engineering and development of bispecific antibodies, immunotoxins, or antibody-drug conjugates.[28] By confocal microscopy we found that the 3-17I mAb was sequestered together with $TPCS_{2a}$ in endosomes and lysosomes, after receptor-mediated endocytosis. $EpCAM^+$ human cancer cell lines MCF-7 (breast), BxPC-3 (pancreas), WiDr (colon), and the $EpCAM^-$ COLO320DM (colon), were treated with $TPCS_{2a}$-based PCI of 3-17I-saporin.[28] No cytotoxicity was observed after treatment with 3-17I-saporin and $TPCS_{2a}$ without light exposure. However, cell viability, proliferation and colony-forming capacity was strongly reduced in a light-dependent manner after PCI of 3-17I. The promising binding profile of 3-17I in patient samples suggest a potential clinical application for detection and diagnosis of $EpCAM^+$ cancers.[28] PCI-controlled cytosolic delivery of 3-17I-based drug conjugates has a very interesting therapeutic potential, and it would be of high interest to further explore this strategy in vivo.

4.2.4 PCI-based targeting of CSPG4

Chondroitin sulfate proteoglycan 4 (CSPG4, NG2, HMW-MAA, MCSP), is a trans-membrane chondroitin sulfate proteoglycan,[214] expressed in a majority of melanoma lesions and initially associated with melanoma therapy resistance and aggressiveness.[214,215] CSPG4 induces signalling cascades through FAK and MAPK potentially promoting survival and chemoresistance, invasion, migration, proliferation and epithelial-mesenchymal transition.[215] In addition, CSPG4 has been shown to be a novel marker of normal epidermal stem cells of the skin.[216] Recently, CSPG4 was shown to be associated with triple-negative breast cancer (TNBC), where the CSPG4 was over-expressed in 75% of the lesions (n=42) tested, and in several cell lines and tumour cells from pleural effusions.[217] CSPG4 was strongly associated with the proposed cancer stem cell phenotype $CD44^{high}/CD24^{low}$ in TNBCs,[217] suggesting CSPG4 as a potentially valuable target for eradication of TNBC and malignant melanomas. Previously, we showed that PCI of a CSPG4-targeting recombinant fusion toxin, MEL/scFv-rGel resulted in efficient and specific elimination of $CSPG4^{+}$ A-375 amelanotic melanoma cells. In vivo PCI of MEL/scFv-rGel caused 50% survival (at day 120 post PCI) of athymic nude mice with A-375 tumors.[21] Complete tumour responses were obtained in the amelanotic melanoma xenograft by PCI of the recombinant CSPG4-targeting fusion toxin. Based on this we have now initiated a study were we aim to target TNBC cells overexpressing CSPG4.

4.2.5 Other putative CSC markers for PCI-based targeting

We are also evaluating PCI-based targeting of other putative CSC targets, including CD90, CD271 (data not published) and two signalling pathways that are drivers of

stemness: the Wnt-β-catenin (data not published) and the PIK3-mTOR pathway, the latter shown to be targeted by photosensitization using the PCI-photosensitizer $AlPCS_{2a}$.[218] CD90 has been proposed as a CSC marker candidate in sarcoma, glioma and liver cancer and has been shown to be over-expressed in drug resistant cancer cells after chemotherapy.[219-222] Recently, CD271 was suggested to be a marker of malignant melanoma CSCs.[223,224] We have now experimental data demonstrating that targeting of CD271 is feasible with the PCI-technology (data not published).

5. Summary

Efficient endocytosis of plasma-membrane receptors over-expressed on stem-like cancer cells may represent an 'Achilles heel' which may be exploited therapeutically by PCI of CSC-targeting toxins. The combined cytotoxic effects of the PCI technology, including direct kill of CSCs and differentiated cell, vascular shutdown and the potential vaccination effect makes it a plausible approach that warrant further pre-clinical and future clinical investigations.

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Reference List

(1) Reya T, Morrison SJ, Clarke MF, Weissman IL. Stem cells, cancer, and cancer stem cells. Nature 2001;414:105-11.

(2) Clarke MF, Dick JE, Dirks PB, Eaves CJ, Jamieson CH, Jones DL, et al. Cancer stem cells--perspectives on current status and future directions: AACR Workshop on cancer stem cells. Cancer Res 2006;66:9339-44.

(3) Visvader JE, Lindeman GJ. Cancer stem cells: current status and evolving complexities. Cell Stem Cell 2012;10:717-28.

(4) O'Brien CA, Pollett A, Gallinger S, Dick JE. A human colon cancer cell capable of initiating tumour growth in immunodeficient mice. Nature 2007;445:106-10.

(5) Ricci-Vitiani L, Lombardi DG, Pilozzi E, Biffoni M, Todaro M, Peschle C, et al. Identification and expansion of human colon-cancer-initiating cells. Nature 2007;445:111-5.

(6) Todaro M, Alea MP, Di Stefano AB, Cammareri P, Vermeulen L, Iovino F, et al. Colon cancer stem cells dictate tumor growth and resist cell death by production of interleukin-4. Cell Stem Cell 2007;1:389-402.

(7) Vermeulen L, Todaro M, de Sousa MF, Sprick MR, Kemper K, Perez AM, et al. Single-cell cloning of colon cancer stem cells reveals a multi-lineage differentiation capacity. Proc Natl Acad Sci U S A 2008;105:13427-32.

(8) Malanchi I, Peinado H, Kassen D, Hussenet T, Metzger D, Chambon P, et al. Cutaneous cancer stem cell maintenance is dependent on beta-catenin signalling. Nature 2008;452:650-3.

(9) Murat A, Migliavacca E, Gorlia T, Lambiv WL, Shay T, Hamou MF, et al. Stem cell-related "self-renewal" signature and high epidermal growth factor receptor expression associated with resistance to concomitant chemoradiotherapy in glioblastoma. J Clin Oncol 2008;26:3015-24.

(10) Visvader JE, Lindeman GJ. Cancer stem cells in solid tumours: accumulating evidence and unresolved questions. Nat Rev Cancer 2008;8:755-68.

(11) Zhou BB, Zhang H, Damelin M, Geles KG, Grindley JC, Dirks PB. Tumour-initiating cells: challenges and opportunities for anticancer drug discovery. Nat Rev Drug Discov 2009;8:806-23.

(12) Frank NY, Schatton T, Frank MH. The therapeutic promise of the cancer stem cell concept. J Clin Invest 2010;120:41-50.

(13) Driessens G, Beck B, Caauwe A, Simons BD, Blanpain C. Defining the mode of tumour growth by clonal analysis. Nature 2012;488:527-30.

(14) Schepers AG, Snippert HJ, Stange DE, van den BM, van Es JH, van de WM, et al. Lineage tracing reveals Lgr5+ stem cell activity in mouse intestinal adenomas. Science 2012;337:730-5.

(15) Chen J, Li Y, Yu TS, McKay RM, Burns DK, Kernie SG, et al. A restricted cell population propagates glioblastoma growth after chemotherapy. Nature 2012;488:522-6.

(16) Rosen JM, Jordan CT. The increasing complexity of the cancer stem cell paradigm. Science 2009;324:1670-3.

(17) Hanahan D, Weinberg RA. Hallmarks of cancer: the next generation. Cell 2011;144:646-74.

(18) Diehn M, Cho RW, Clarke MF. Therapeutic implications of the cancer stem cell hypothesis. Semin Radiat Oncol 2009;19:78-86.

(19) Chaffer CL, Brueckmann I, Scheel C, Kaestli AJ, Wiggins PA, Rodrigues LO, et al. Normal and neoplastic nonstem cells can spontaneously convert to a stem-like state. Proc Natl Acad Sci U S A 2011;108:7950-5.

(20) Piccirillo SG, Combi R, Cajola L, Patrizi A, Redaelli S, Bentivegna A, et al. Distinct pools of cancer stem-like cells coexist within human glioblastomas and display different tumorigenicity and independent genomic evolution. Oncogene 2009;28:1807-11.

(21) Selbo PK, Rosenblum MG, Cheung LH, Zhang W, Berg K. Multi-modality therapeutics with potent anti-tumor effects: photochemical internalization enhances delivery of the fusion toxin scFvMEL/rGel. PLoS ONE 2009;4:e6691. doi:10.1371/journal.pone.0006691.

(22) Selbo PK, Sivam G, Fodstad O, Sandvig K, Berg K. *In Vivo* Documentation of Photochemical Internalization, a Novel Approach to Site Specific Cancer Therapy. Int J Cancer 2001;92:761-6.

(23) Selbo PK, Weyergang A, Bonsted A, Bown SG, Berg K. Photochemical internalization of therapeutic macromolecular agents: a novel strategy to kill multidrug resistant cancer cells. J Pharmacol Exp Ther 2006;319:604-12.

(24) Selbo PK, Weyergang A, Eng MS, Bostad M, Mælandsmo GM, Høgset A, et al. Strongly amphiphilic photosensitizers are not substrates of the cancer stem cell marker ABCG2 and provides specific and efficient light-triggered drug delivery of an EGFR-targeted cytotoxic drug. J Control Release 2012;159:197-203.

(25) Olsen CE, Berg K, Selbo PK, Weyergang A. Circumvention of resistance to photodynamic therapy in doxorubicin-resistant sarcoma by photochemical internalization of gelonin. Free Radic Biol Med 2013;65:1300-9.

(26) Selbo PK, Sivam G, Fodstad O, Sandvig K, Berg K. Photochemical internalization increases the cytotoxic effect of the immunotoxin MOC31-gelonin. Int J Cancer 2000;87:853-9.

(27) Selbo PK, Kaalhus O, Sivam G, Berg K. 5-Aminolevulinic Acid–based Photochemical Internalization of the Immunotoxin MOC31-gelonin Generates Synergistic Cytotoxic Effects In Vitro. Photochem Photobiol 2001;74:303-10.

(28) Lund K, Bostad M, Skarpen E, Braunagel M, Kiprijanov S, Krauss S, et al. The novel EpCAM-targeting monoclonal antibody 3-17I linked to saporin is highly cytotoxic after photochemical internalization in breast, pancreas and colon cancer cell lines. MAbs 2014;6:1038-50.

(29) Dean M, Fojo T, Bates S. Tumour stem cells and drug resistance. Nat Rev Cancer 2005;5:275-84.

(30) Baumann M, Krause M, Hill R. Exploring the role of cancer stem cells in radioresistance. Nat Rev Cancer 2008;8:545-54.

(31) Maugeri-Sacca M, Vigneri P, De MR. Cancer stem cells and chemosensitivity. Clin Cancer Res 2011;17:4942-7.

(32) Colak S, Medema JP. Cancer stem cells--important players in tumor therapy resistance. FEBS J 2014;281:4779-91.

(33) Suda T, Takubo K, Semenza GL. Metabolic regulation of hematopoietic stem cells in the hypoxic niche. Cell Stem Cell 2011;9:298-310.

(34) Blazek ER, Foutch JL, Maki G. Daoy medulloblastoma cells that express CD133 are radioresistant relative to CD133- cells, and the CD133+ sector is enlarged by hypoxia. Int J Radiat Oncol Biol Phys 2007;67:1-5.

(35) Li Z, Bao S, Wu Q, Wang H, Eyler C, Sathornsumetee S, et al. Hypoxia-inducible factors regulate tumorigenic capacity of glioma stem cells. Cancer Cell 2009;15:501-13.

(36) Yeung TM, Gandhi SC, Bodmer WF. Hypoxia and lineage specification of cell line-derived colorectal cancer stem cells. Proc Natl Acad Sci U S A 2011;108:4382-7.

(37) Keith B, Simon MC. Hypoxia-inducible factors, stem cells, and cancer. Cell 2007;129:465-72.

(38) Calabrese C, Poppleton H, Kocak M, Hogg TL, Fuller C, Hamner B, et al. A perivascular niche for brain tumor stem cells. Cancer Cell 2007;11:69-82.

(39) Gilbertson RJ, Rich JN. Making a tumour's bed: glioblastoma stem cells and the vascular niche. Nat Rev Cancer 2007;7:733-6.

(40) Bigarella CL, Liang R, Ghaffari S. Stem cells and the impact of ROS signaling. Development 2014;141:4206-18.

(41) Holmstrom KM, Finkel T. Cellular mechanisms and physiological consequences of redox-dependent signalling. Nat Rev Mol Cell Biol 2014;15:411-21.

(42) Ito K, Hirao A, Arai F, Matsuoka S, Takubo K, Hamaguchi I, et al. Regulation of oxidative stress by ATM is required for self-renewal of haematopoietic stem cells. Nature 2004;431:997-1002.

(43) Tothova Z, Kollipara R, Huntly BJ, Lee BH, Castrillon DH, Cullen DE, et al. FoxOs are critical mediators of hematopoietic stem cell resistance to physiologic oxidative stress. Cell 2007;128:325-39.

(44) Miyamoto K, Araki KY, Naka K, Arai F, Takubo K, Yamazaki S, et al. Foxo3a is essential for maintenance of the hematopoietic stem cell pool. Cell Stem Cell 2007;1:101-12.

(45) Phillips TM, McBride WH, Pajonk F. The response of CD24(-/low)/CD44+ breast cancer-initiating cells to radiation. J Natl Cancer Inst 2006;98:1777-85.

(46) Bao S, Wu Q, McLendon RE, Hao Y, Shi Q, Hjelmeland AB, et al. Glioma stem cells promote radioresistance by preferential activation of the DNA damage response. Nature 2006;444:756-60.

(47) Kobayashi CI, Suda T. Regulation of reactive oxygen species in stem cells and cancer stem cells. J Cell Physiol 2012;227:421-30.

(48) Catalano V, Turdo A, Di FS, Dieli F, Todaro M, Stassi G. Tumor and its microenvironment: a synergistic interplay. Semin Cancer Biol 2013;23:522-32.

(49) Diehn M, Cho RW, Lobo NA, Kalisky T, Dorie MJ, Kulp AN, et al. Association of reactive oxygen species levels and radioresistance in cancer stem cells. Nature 2009;458:780-3.

(50) Ishimoto T, Nagano O, Yae T, Tamada M, Motohara T, Oshima H, et al. CD44 variant regulates redox status in cancer cells by stabilizing the xCT subunit of system xc(-) and thereby promotes tumor growth. Cancer Cell 2011;19:387-400.

(51) Miller AC, Henderson BW. The influence of cellular glutathione content on cell survival following photodynamic treatment in vitro. Radiat Res 1986;107:83-94.

(52) Bachor R, Scholz M, Shea CR, Hasan T. Mechanism of photosensitization by microsphere-bound chlorin e6 in human bladder carcinoma cells. Cancer Res 1991;51:4410-4.

(53) Kiesslich T, Plaetzer K, Oberdanner CB, Berlanda J, Obermair FJ, Krammer B. Differential effects of glucose deprivation on the cellular sensitivity towards photodynamic treatment-based production of reactive oxygen species and apoptosis-induction. FEBS Lett 2005;579:185-90.

(54) Moitra K, Lou H, Dean M. Multidrug efflux pumps and cancer stem cells: insights into multidrug resistance and therapeutic development. Clin Pharmacol Ther 2011;89:491-502.

(55) Robey RW, To KK, Polgar O, Dohse M, Fetsch P, Dean M, et al. ABCG2: a perspective. Adv Drug Deliv Rev 2009;61:3-13.

(56) Jonker JW, Merino G, Musters S, van Herwaarden AE, Bolscher E, Wagenaar E, et al. The breast cancer resistance protein BCRP (ABCG2) concentrates drugs and carcinogenic xenotoxins into milk. Nat Med 2005;11:127-9.

(57) Krishnamurthy P, Schuetz JD. Role of ABCG2/BCRP in biology and medicine. Annu Rev Pharmacol Toxicol 2006;46:381-410.

(58) Krishnamurthy P, Ross DD, Nakanishi T, Bailey-Dell K, Zhou S, Mercer KE, et al. The stem cell marker Bcrp/ABCG2 enhances hypoxic cell survival through interactions with heme. J Biol Chem 2004;279:24218-25.

(59) Zhou S, Schuetz JD, Bunting KD, Colapietro AM, Sampath J, Morris JJ, et al. The ABC transporter Bcrp1/ABCG2 is expressed in a wide variety of stem cells and is a molecular determinant of the side-population phenotype. Nat Med 2001;7:1028-34.

(60) Golebiewska A, Brons NH, Bjerkvig R, Niclou SP. Critical appraisal of the side population assay in stem cell and cancer stem cell research. Cell Stem Cell 2011;8:136-47.

(61) Hirschmann-Jax C, Foster AE, Wulf GG, Nuchtern JG, Jax TW, Gobel U, et al. A distinct "side population" of cells with high drug efflux capacity in human tumor cells. Proc Natl Acad Sci U S A 2004;101:14228-33.

(62) Szotek PP, Pieretti-Vanmarcke R, Masiakos PT, Dinulescu DM, Connolly D, Foster R, et al. Ovarian cancer side population defines cells with stem cell-like characteristics and Mullerian Inhibiting Substance responsiveness. Proc Natl Acad Sci U S A 2006;103:11154-9.

(63) Patrawala L, Calhoun T, Schneider-Broussard R, Zhou J, Claypool K, Tang DG. Side population is enriched in tumorigenic, stem-like cancer cells, whereas ABCG2+ and ABCG2- cancer cells are similarly tumorigenic. Cancer Res 2005;65:6207-19.

(64) Kruger JA, Kaplan CD, Luo Y, Zhou H, Markowitz D, Xiang R, et al. Characterization of stem cell-like cancer cells in immune-competent mice. Blood 2006;108:3906-12.

(65) Harris MA, Yang H, Low BE, Mukherjee J, Guha A, Bronson RT, et al. Cancer stem cells are enriched in the side population cells in a mouse model of glioma. Cancer Res 2008;68:10051-9.

(66) Kondo T, Setoguchi T, Taga T. Persistence of a small subpopulation of cancer stem-like cells in the C6 glioma cell line. Proc Natl Acad Sci U S A 2004;101:781-6.

(67) Dean M, Fojo T, Bates S. Tumour stem cells and drug resistance. Nat Rev Cancer 2005;5:275-84.

(68) Purkiss SF, Grahn MF, Turkish M, Macey MG, Williams NS. In vitro modulation of haematoporphyrin derivative photodynamic therapy on colorectal carcinoma multicellular spheroids by verapamil. Br J Surg 1992;79:120-5.

(69) Kessel D, Erickson C. Porphyrin photosensitization of multi-drug resistant cell types. Photochem Photobiol 1992;55:397-9.

(70) Frazier DL, Barnhill MA, Lu X, Jones E, Niemeyer G, Mishu L, et al. Effect of multidrug-resistant P-glycoprotein gene expression on chloroaluminum tetrasulfonate phthalocyanine concentration. Lasers Surg Med 1993;13:511-6.

(71) Teiten MH, Bezdetnaya L, Merlin JL, Bour-Dill C, Pauly ME, Dicato M, et al. Effect of meta-tetra(hydroxyphenyl)chlorin (mTHPC)-mediated photodynamic therapy on sensitive and multidrug-resistant human breast cancer cells. J Photochem Photobiol B 2001;62:146-52.

(72) Jonker JW, Buitelaar M, Wagenaar E, van d, V, Scheffer GL, Scheper RJ, et al. The breast cancer resistance protein protects against a major chlorophyll-derived dietary phototoxin and protoporphyria. Proc Natl Acad Sci U S A 2002;99:15649-54.

(73) Robey RW, Steadman K, Polgar O, Bates SE. ABCG2-mediated transport of photosensitizers: potential impact on photodynamic therapy. Cancer Biol Ther 2005;4:187-94.

(74) Robey RW, Steadman K, Polgar O, Morisaki K, Blayney M, Mistry P, et al. Pheophorbide a is a specific probe for ABCG2 function and inhibition. Cancer Res 2004;64:1242-6.

(75) Liu W, Baer MR, Bowman MJ, Pera P, Zheng X, Morgan J, et al. The tyrosine kinase inhibitor imatinib mesylate enhances the efficacy of photodynamic therapy by inhibiting ABCG2. Clin Cancer Res 2007;13:2463-70.

(76) Morgan J, Jackson JD, Zheng X, Pandey SK, Pandey RK. Substrate Affinity of Photosensitizers Derived from Chlorophyll-a: The ABCG2 Transporter Affects the Phototoxic Response of Side Population Stem Cell-like Cancer Cells to Photodynamic Therapy. Mol Pharm 2010;7:1789-804.

(77) Usuda J, Tsunoda Y, Ichinose S, Ishizumi T, Ohtani K, Maehara S, et al. Breast cancer resistant protein (BCRP) is a molecular determinant of the outcome of photodynamic therapy (PDT) for centrally located early lung cancer. Lung Cancer 2010;67:198-204.

(78) Jendzelovsky R, Mikes J, Koval' J, Soucek K, Prochazkova J, Kello M, et al. Drug efflux transporters, MRP1 and BCRP, affect the outcome of hypericin-mediated photodynamic therapy in HT-29 adenocarcinoma cells. Photochem Photobiol Sci 2009;8:1716-23.

(79) Eyler CE, Rich JN. Survival of the fittest: cancer stem cells in therapeutic resistance and angiogenesis. J Clin Oncol 2008;26:2839-45.

(80) Maugeri-Sacca M, Bartucci M, De MR. DNA damage repair pathways in cancer stem cells. Mol Cancer Ther 2012;11:1627-36.

(81) Agostinis P, Berg K, Cengel KA, Foster TH, Girotti AW, Gollnick SO, et al. Photodynamic therapy of cancer: An update. CA Cancer J Clin 2011;61:250-81.

(82) Lessard J, Sauvageau G. Bmi-1 determines the proliferative capacity of normal and leukaemic stem cells. Nature 2003;423:255-60.

(83) Ruiz i Altaba A. Hedgehog signaling and the Gli code in stem cells, cancer, and metastases. Sci Signal 2011;4:t9.

(84) Hoffmeyer K, Raggioli A, Rudloff S, Anton R, Hierholzer A, Del V, I, et al. Wnt/beta-catenin signaling regulates telomerase in stem cells and cancer cells. Science 2012;336:1549-54.

(85) Wang J, Sullenger BA, Rich JN. Notch signaling in cancer stem cells. Adv Exp Med Biol 2012;727:174-85.

(86) Kim E, Kim M, Woo DH, Shin Y, Shin J, Chang N, et al. Phosphorylation of EZH2 activates STAT3 signaling via STAT3 methylation and promotes tumorigenicity of glioblastoma stem-like cells. Cancer Cell 2013;23:839-52.

(87) Vanner RJ, Remke M, Gallo M, Selvadurai HJ, Coutinho F, Lee L, et al. Quiescent sox2(+) cells drive hierarchical growth and relapse in sonic hedgehog subgroup medulloblastoma. Cancer Cell 2014;26:33-47.

(88) Korkaya H, Paulson A, Iovino F, Wicha MS. HER2 regulates the mammary stem/progenitor cell population driving tumorigenesis and invasion. Oncogene 2008;27:6120-30.

(89) Boccaccio C, Luraghi P, Comoglio PM. MET-mediated resistance to EGFR inhibitors: an old liaison rooted in colorectal cancer stem cells. Cancer Res 2014;74:3647-51.

(90) Weyergang A, Selbo PK, Berg K. Photochemically stimulated drug delivery increases the cytotoxicity and specificity of EGF–saporin. J Control Release 2006;111:165-73.

(91) Yip WL, Weyergang A, Berg K, Tonnesen HH, Selbo PK. Targeted Delivery and Enhanced Cytotoxicity of Cetuximab-Saporin by Photochemical Internalization in EGFR-Positive Cancer Cells. Mol Pharmaceutics 2007;4:241-51.

(92) Weyergang A, Selbo PK, Berg K. Sustained ERK inhibition by EGFR targeting therapies is a predictive factor for synergistic cytotoxicity with PDT as neoadjuvant therapy. Biochim Biophys Acta 2012;10.

(93) Berstad MB, Cheung L, Berg K, Peng Q, Fremstedal A, Patzke S, et al. Design of an EGFR-targeting toxin for photochemical delivery; in vitro and in vivo selectivity and efficacy. Oncogene 2015;In press.

(94) Bao S, Wu Q, Sathornsumetee S, Hao Y, Li Z, Hjelmeland AB, et al. Stem cell-like glioma cells promote tumor angiogenesis through vascular endothelial growth factor. Cancer Res 2006;66:7843-8.

(95) Oka N, Soeda A, Inagaki A, Onodera M, Maruyama H, Hara A, et al. VEGF promotes tumorigenesis and angiogenesis of human glioblastoma stem cells. Biochem Biophys Res Commun 2007;360:553-9.

(96) Ferrario A, von Tiehl KF, Rucker N, Schwarz MA, Gill PS, Gomer CJ. Antiangiogenic treatment enhances photodynamic therapy responsiveness in a mouse mammary carcinoma. Cancer Res 2000;60:4066-9.

(97) Solban N, Selbo PK, Sinha AK, Chang SK, Hasan T. Mechanistic Investigation and Implications of PDT-Induction of VEGF in Prostate Cancer . Cancer Res 2006;66:5633-40.

(98) Ferrario A, Fisher AM, Rucker N, Gomer CJ. Celecoxib and NS-398 enhance photodynamic therapy by increasing in vitro apoptosis and decreasing in vivo inflammatory and angiogenic factors. Cancer Res 2005;65:9473-8.

(99) Ferrario A, Gomer CJ. Avastin Enhances Photodynamic Therapy Treatment of Kaposi's Sarcoma in a Mouse Tumor Model. J Environ Pathol Toxicol Oncol 2006;25:251-60.

(100) Weiss A, van Beijnum JR, Bonvin D, Jichlinski P, Dyson PJ, Griffioen AW, et al. Low-dose angiostatic tyrosine kinase inhibitors improve photodynamic therapy for cancer: lack of vascular normalization. J Cell Mol Med 2014;18:480-91.

(101) Piette J, Volanti C, Vantieghem A, Matroule JY, Habraken Y, Agostinis P. Cell death and growth arrest in response to photodynamic therapy with membrane-bound photosensitizers. Biochem Pharmacol 2003;66:1651-9.

(102) Gomer CJ. Induction of prosurvival molecules during treatment: rethinking therapy options for photodynamic therapy. J Natl Compr Canc Netw 2012;10:S35-S39.

(103) Hendrickx N, Volanti C, Moens U, Seternes OM, de WP, Vandenheede JR, et al. Up-regulation of cyclooxygenase-2 and apoptosis resistance by p38 MAPK in hypericin-mediated photodynamic therapy of human cancer cells. J Biol Chem 2003;278:52231-9.

(104) Rapozzi V, Umezawa K, Xodo LE. Role of NF-kappaB/Snail/RKIP loop in the response of tumor cells to photodynamic therapy. Lasers Surg Med 2011;43:575-85.

(105) Bhowmick R, Girotti AW. Cytoprotective signaling associated with nitric oxide upregulation in tumor cells subjected to photodynamic therapy-like oxidative stress. Free Radic Biol Med 2013;57:39-48.

(106) Liu M, Sakamaki T, Casimiro MC, Willmarth NE, Quong AA, Ju X, et al. The canonical NF-kappaB pathway governs mammary tumorigenesis in transgenic mice and tumor stem cell expansion. Cancer Res 2010;70:10464-73.

(107) Kim PJ, Plescia J, Clevers H, Fearon ER, Altieri DC. Survivin and molecular pathogenesis of colorectal cancer. Lancet 2003;362:205-9.

(108) Pishvaian MJ, Byers SW. Biomarkers of WNT signaling. Cancer Biomark 2007;3:263-74.

(109) Weyergang A, Kaalhus O, Berg K. Photodynamic targeting of EGFR does not predict the treatment outcome in combination with the EGFR tyrosine kinase inhibitor Tyrphostin AG1478. Photochem Photobiol Sci 2008;7:1032-40.

(110) Weyergang A, Cheung LH, Rosenblum MG, Mohamedali KA, Peng Q, Waltenberger J, et al. Photochemical internalization augments tumor vascular cytotoxicity and specificity of VEGF(121)/rGel fusion toxin. J Control Release 2014;180:1-9.

(111) Fuchs E. The tortoise and the hair: slow-cycling cells in the stem cell race. Cell 2009;137:811-9.

(112) Li L, Bhatia R. Stem cell quiescence. Clin Cancer Res 2011;17:4936-41.

(113) Essers MA, Trumpp A. Targeting leukemic stem cells by breaking their dormancy. Mol Oncol 2010;4:443-50.

(114) Cheung TH, Rando TA. Molecular regulation of stem cell quiescence. Nat Rev Mol Cell Biol 2013;14:329-40.

(115) Masters JR, Koberle B. Curing metastatic cancer: lessons from testicular germ-cell tumours. Nat Rev Cancer 2003;3:517-25.

(116) Ito K, Suda T. Metabolic requirements for the maintenance of self-renewing stem cells. Nat Rev Mol Cell Biol 2014;15:243-56.

(117) Ortel B, Chen N, Brissette J, Dotto GP, Maytin E, Hasan T. Differentiation-specific increase in ALA-induced protoporphyrin IX accumulation in primary mouse keratinocytes. Br J Cancer 1998;77:1744-51.

(118) Feldman D, Krishnan AV, Swami S, Giovannucci E, Feldman BJ. The role of vitamin D in reducing cancer risk and progression. Nat Rev Cancer 2014;14:342-57.

(119) Bose M, Hao X, Ju J, Husain A, Park S, Lambert JD, et al. Inhibition of tumorigenesis in ApcMin/+ mice by a combination of (-)-epigallocatechin-3-gallate and fish oil. J Agric Food Chem 2007;55:7695-700.

(120) Palmer HG, Gonzalez-Sancho JM, Espada J, Berciano MT, Puig I, Baulida J, et al. Vitamin D(3) promotes the differentiation of colon carcinoma cells by the induction of E-cadherin and the inhibition of beta-catenin signaling. J Cell Biol 2001;154:369-87.

(121) Mellman I. Endocytosis and molecular sorting. Annu Rev Cell Dev Biol 1996;12:575-625.:575-625.

(122) Morel AP, Lievre M, Thomas C, Hinkal G, Ansieau S, Puisieux A. Generation of breast cancer stem cells through epithelial-mesenchymal transition. PLoS ONE 2008;3:e2888.

(123) Mani SA, Guo W, Liao MJ, Eaton EN, Ayyanan A, Zhou AY, et al. The epithelial-mesenchymal transition generates cells with properties of stem cells. Cell 2008;133:704-15.

(124) Yang MH, Hsu DS, Wang HW, Wang HJ, Lan HY, Yang WH, et al. Bmi1 is essential in Twist1-induced epithelial-mesenchymal transition. Nat Cell Biol 2010;12:982-92.

(125) Vermeulen L, de Sousa E Melo, van der Heijden M, Cameron K, de Jong JH, Borovski T, et al. Wnt activity defines colon cancer stem cells and is regulated by the microenvironment. Nat Cell Biol 2010;12:468-76.

(126) Rizvi I, Gurkan UA, Tasoglu S, Alagic N, Celli JP, Mensah LB, et al. Flow induces epithelial-mesenchymal transition, cellular heterogeneity and biomarker modulation in 3D ovarian cancer nodules. Proc Natl Acad Sci U S A 2013;110:E1974-E1983.

(127) Shipitsin M, Campbell LL, Argani P, Weremowicz S, Bloushtain-Qimron N, Yao J, et al. Molecular definition of breast tumor heterogeneity. Cancer Cell 2007;11:259-73.

(128) Park SY, Gonen M, Kim HJ, Michor F, Polyak K. Cellular and genetic diversity in the progression of in situ human breast carcinomas to an invasive phenotype. J Clin Invest 2010;120:636-44.

(129) Lottaz C, Beier D, Meyer K, Kumar P, Hermann A, Schwarz J, et al. Transcriptional profiles of CD133+ and CD133- glioblastoma-derived cancer stem cell lines suggest different cells of origin. Cancer Res 2010;70:2030-40.

(130) Berg K, Selbo PK, Prasmickaite L, Tjelle TE, Sandvig K, Moan J, et al. Photochemical internalization: a novel technology for delivery of macromolecules into cytosol. Cancer Res 1999;59:1180-3.

(131) Høgset A, Prasmickaite L, Selbo PK, Hellum M, Engesaeter BO, Bonsted A, et al. Photochemical internalisation in drug and gene delivery. Adv Drug Deliv Rev 2004;56:95-115.

(132) Selbo PK, Weyergang A, Hogset A, Norum OJ, Berstad MB, Vikdal M, et al. Photochemical internalization provides time- and space-controlled endolysosomal escape of therapeutic molecules. J Control Release 2010;148:2-12.

(133) Selbo PK, Sandvig K, Kirveliene V, Berg K. Release of gelonin from endosomes and lysosomes to cytosol by photochemical internalization. Biochim Biophys Acta 2000;1475:307-13.

(134) Berg K, Nordstrand S, Selbo PK, Tran DT, ngell-Petersen E, Hogset A. Disulfonated tetraphenyl chlorin (TPCS2a), a novel photosensitizer developed for clinical utilization of photochemical internalization. Photochem Photobiol Sci 2011;10:1637-51.

(135) Weyergang A, Selbo PK, Berstad ME, Bostad M, Berg K. Photochemical internalization of tumor-targeted protein toxins. Lasers Surg Med 2011;43:721-33.

(136) Berg K, Dietze A, Kaalhus O, Høgset A. Site-specific drug delivery by photochemical internalization enhances the antitumor effect of bleomycin. Clin Cancer Res 2005;11:8476-85.

(137) Lou PJ, Lai PS, Shieh MJ, MacRobert AJ, Berg K, Bown SG. Reversal of doxorubicin resistance in breast cancer cells by photochemical internalization. Int J Cancer 2006;119:2692-8.

(138) Håkerud M, Waeckerle-Men Y, Selbo PK, Kundig TM, Høgset A, Johansen P. Intradermal photosensitisation facilitates stimulation of MHC class-I restricted CD8 T-cell responses of co-administered antigen. J Control Release 2014;174:143-50.

(139) Håkerud M, Selbo PK, Waeckerle-Men Y, Contassot E, Dziunycz P, Kundig TM, et al. Photosensitisation facilitates cross-priming of adjuvant-free protein vaccines and stimulation of tumour-suppressing CD8 T cells. J Control Release 2014;198:10-7.

(140) Lou H, Dean M. Targeted therapy for cancer stem cells: the patched pathway and ABC transporters. Oncogene 2007;26:1357-60.

(141) Allen JD, van LA, Lakhai JM, van d, V, van TO, Reid G, et al. Potent and specific inhibition of the breast cancer resistance protein multidrug transporter in vitro and in mouse intestine by a novel analogue of fumitremorgin C. Mol Cancer Ther 2002;1:417-25.

(142) Houghton PJ, Germain GS, Harwood FC, Schuetz JD, Stewart CF, Buchdunger E, et al. Imatinib mesylate is a potent inhibitor of the ABCG2 (BCRP) transporter and reverses resistance to topotecan and SN-38 in vitro. Cancer Res 2004;64:2333-7.

(143) Ozvegy-Laczka C, Hegedus T, Varady G, Ujhelly O, Schuetz JD, Varadi A, et al. High-affinity interaction of tyrosine kinase inhibitors with the ABCG2 multidrug transporter. Mol Pharmacol 2004;65:1485-95.

(144) Yanase K, Tsukahara S, Asada S, Ishikawa E, Imai Y, Sugimoto Y. Gefitinib reverses breast cancer resistance protein-mediated drug resistance. Mol Cancer Ther 2004;3:1119-25.

(145) Brendel C, Scharenberg C, Dohse M, Robey RW, Bates SE, Shukla S, et al. Imatinib mesylate and nilotinib (AMN107) exhibit high-affinity interaction with ABCG2 on primitive hematopoietic stem cells. Leukemia 2007;21:1267-75.

(146) Dikic I. Mechanisms controlling EGF receptor endocytosis and degradation. Biochem Soc Trans 2003;31:1178-81.

(147) Chen YJ, Huang WC, Wei YL, Hsu SC, Yuan P, Lin HY, et al. Elevated BCRP/ABCG2 expression confers acquired resistance to gefitinib in wild-type EGFR-expressing cells. PLoS One 2011;6 doi: 10.1371/journal.pone.0021428.:e21428.

(148) Porcelli L, Giovannetti E, Assaraf YG, Jansen G, Scheffer GL, Kathman I, et al. The EGFR Pathway Regulates BCRP Expression in NSCLC Cells: Role of Erlotinib. Curr Drug Targets 2014;15:1322-30.

(149) Wang XK, He JH, Xu JH, Ye S, Wang F, Zhang H, et al. Afatinib enhances the efficacy of conventional chemotherapeutic agents by eradicating cancer stem-like cells. Cancer Res 2014;74:4431-45.

(150) Mazard T, Causse A, Simony J, Leconet W, Vezzio-Vie N, Torro A, et al. Sorafenib overcomes irinotecan resistance in colorectal cancer by inhibiting the ABCG2 drug-efflux pump. Mol Cancer Ther 2013;12:2121-34.

(151) del Carmen MG, Rizvi I, Chang Y, Moor AC, Oliva E, Sherwood M, et al. Synergism of epidermal growth factor receptor-targeted immunotherapy with photodynamic treatment of ovarian cancer in vivo. J Natl Cancer Inst 2005;97:1516-24.

(152) Bhatia P, Bernier M, Sanghvi M, Moaddel R, Schwarting R, Ramamoorthy A, et al. Breast cancer resistance protein (BCRP/ABCG2) localises to the nucleus in glioblastoma multiforme cells. Xenobiotica 2012;42:748-55.

(153) Bostad M, Berg K, Hogset A, Skarpen E, Stenmark H, Selbo PK. Photochemical internalization (PCI) of immunotoxins targeting CD133 is specific and highly potent at femtomolar levels in cells with cancer stem cell properties. J Control Release 2013;168:317-26.

(154) Stratford EW, Bostad M, Castro R, Skarpen E, Berg K, Hogset A, et al. Photochemical internalization of CD133-targeting immunotoxins efficiently depletes sarcoma cells with stem-like properties and reduces tumorigenicity. Biochim Biophys Acta 2013;1830:4235-43.

(155) Bostad M, Kausberg M, Weyergang A, Olsen CE, Berg K, Hogset A, et al. Light-triggered, efficient cytosolic release of IM7-saporin targeting the putative cancer stem cell marker CD44 by photochemical internalization. Mol Pharm 2014;11:2764-76.

(156) Pastan I, Kreitman RJ. Immunotoxins for targeted cancer therapy. Adv Drug Del Rev 1998;31:53-88.

(157) Pastan I, Hassan R, FitzGerald DJ, Kreitman RJ. Immunotoxin therapy of cancer. Nat Rev Cancer 2006;6:559-65.

(158) Natarajan TG, FitzGerald KT. Markers in normal and cancer stem cells. Cancer Biomark 2007;3:211-31.

(159) Tijink BM, Buter J, de BR, Giaccone G, Lang MS, Staab A, et al. A phase I dose escalation study with anti-CD44v6 bivatuzumab mertansine in patients with incurable squamous cell carcinoma of the head and neck or esophagus. Clin Cancer Res 2006;12:6064-72.

(160) Yin AH, Miraglia S, Zanjani ED, meida-Porada G, Ogawa M, Leary AG, et al. AC133, a novel marker for human hematopoietic stem and progenitor cells. Blood 1997;90:5002-12.

(161) Miraglia S, Godfrey W, Yin AH, Atkins K, Warnke R, Holden JT, et al. A novel five-transmembrane hematopoietic stem cell antigen: isolation, characterization, and molecular cloning. Blood 1997;90:5013-21.

(162) Weigmann A, Corbeil D, Hellwig A, Huttner WB. Prominin, a novel microvilli-specific polytopic membrane protein of the apical surface of epithelial cells, is targeted to plasmalemmal protrusions of non-epithelial cells. Proc Natl Acad Sci U S A 1997;94:12425-30.

(163) Singh SK, Hawkins C, Clarke ID, Squire JA, Bayani J, Hide T, et al. Identification of human brain tumour initiating cells. Nature 2004;432:396-401.

(164) Salmaggi A, Boiardi A, Gelati M, Russo A, Calatozzolo C, Ciusani E, et al. Glioblastoma-derived tumorospheres identify a population of tumor stem-like cells with angiogenic potential and enhanced multidrug resistance phenotype. Glia 2006;54:850-60.

(165) Hermann PC, Huber SL, Herrler T, Aicher A, Ellwart JW, Guba M, et al. Distinct populations of cancer stem cells determine tumor growth and metastatic activity in human pancreatic cancer. Cell Stem Cell 2007;1:313-23.

(166) Collins AT, Berry PA, Hyde C, Stower MJ, Maitland NJ. Prospective identification of tumorigenic prostate cancer stem cells. Cancer Res 2005;65:10946-51.

(167) Curley MD, Therrien VA, Cummings CL, Sergent PA, Koulouris CR, Friel AM, et al. CD133 expression defines a tumor initiating cell population in primary human ovarian cancer. Stem Cells 2009;27:2875-83.

(168) Patel GK, Yee CL, Terunuma A, Telford WG, Voong N, Yuspa SH, et al. Identification and characterization of tumor-initiating cells in human primary cutaneous squamous cell carcinoma. J Invest Dermatol 2012;132:401-9.

(169) Suva ML, Riggi N, Stehle JC, Baumer K, Tercier S, Joseph JM, et al. Identification of cancer stem cells in Ewing's sarcoma. Cancer Res 2009;69:1776-81.

(170) Tirino V, Desiderio V, d'Aquino R, De FF, Pirozzi G, Graziano A, et al. Detection and characterization of CD133+ cancer stem cells in human solid tumours. PLoS ONE 2008;3:e3469.

(171) Zeppernick F, Ahmadi R, Campos B, Dictus C, Helmke BM, Becker N, et al. Stem cell marker CD133 affects clinical outcome in glioma patients. Clin Cancer Res 2008;14:123-9.

(172) Horst D, Kriegl L, Engel J, Kirchner T, Jung A. CD133 expression is an independent prognostic marker for low survival in colorectal cancer. Br J Cancer 2008;99:1285-9.

(173) Kemper K, Versloot M, Cameron K, Colak S, Bleakley J, Vermeulen L, et al. Mutations in the Ras-Raf axis underlie the prognostic value of CD133 in colorectal cancer. Clin Cancer Res 2012.

(174) Liu G, Yuan X, Zeng Z, Tunici P, Ng H, Abdulkadir IR, et al. Analysis of gene expression and chemoresistance of CD133+ cancer stem cells in glioblastoma. Mol Cancer 2006;5:67.:67.

(175) Wang J, Sakariassen PO, Tsinkalovsky O, Immervoll H, Boe SO, Svendsen A, et al. CD133 negative glioma cells form tumors in nude rats and give rise to CD133 positive cells. Int J Cancer 2008;122:761-8.

(176) Shmelkov SV, Butler JM, Hooper AT, Hormigo A, Kushner J, Milde T, et al. CD133 expression is not restricted to stem cells, and both CD133+ and CD133- metastatic colon cancer cells initiate tumors. J Clin Invest 2008;118:2111-20.

(177) Kemper K, Sprick MR, de BM, Scopelliti A, Vermeulen L, Hoek M, et al. The AC133 epitope, but not the CD133 protein, is lost upon cancer stem cell differentiation. Cancer Res 2010;70:719-29.

(178) Waldron NN, Kaufman DS, Oh S, Inde Z, Hexum MK, Ohlfest JR, et al. Targeting tumor-initiating cancer cells with dCD133KDEL shows impressive tumor reductions in a xenotransplant model of human head and neck cancer. Mol Cancer Ther 2011;10:1829-38.

(179) Lehnus KS, Donovan LK, Huang X, Zhao N, Warr TJ, Pilkington GJ, et al. CD133 glycosylation is enhanced by hypoxia in cultured glioma stem cells. Int J Oncol 2013;10.

(180) Campos B, Herold-Mende CC. Insight into the complex regulation of CD133 in glioma. Int J Cancer 2011;128:501-10.

(181) Chen TR, Drabkowski D, Hay RJ, Macy M, Peterson W, Jr. WiDr is a derivative of another colon adenocarcinoma cell line, HT-29. Cancer Genet Cytogenet 1987;27:125-34.

(182) Rodrigues NR, Rowan A, Smith ME, Kerr IB, Bodmer WF, Gannon JV, et al. p53 mutations in colorectal cancer. Proc Natl Acad Sci U S A 1990;87:7555-9.

(183) Prahallad A, Sun C, Huang S, Di NF, Salazar R, Zecchin D, et al. Unresponsiveness of colon cancer to BRAF(V600E) inhibition through feedback activation of EGFR. Nature 2012;483:100-3.

(184) Peters GJ, Smitskamp-Wilms E, Smid K, Pinedo HM, Jansen G. Determinants of activity of the antifolate thymidylate synthase inhibitors Tomudex (ZD1694) and GW1843U89 against mono- and multilayered colon cancer cell lines under folate-restricted conditions. Cancer Res 1999;59:5529-35.

(185) Virsik-Kopp P, Hofman-Huther H, Rave-Frank M, Schmidberger H. The effect of wortmannin on radiation-induced chromosome aberration formation in the radioresistant tumor cell line WiDr. Radiat Res 2005;164:148-56.

(186) Dittfeld C, Dietrich A, Peickert S, Hering S, Baumann M, Grade M, et al. CD133 expression is not selective for tumor-initiating or radioresistant cell populations in the CRC cell lines HCT-116. Radiother Oncol 2009;92:353-61.

(187) Real FX, Egea G, Franci C, Schussler MH, Xu M, Welt S. Mucin production by colon cancer cells cultured in serum-free medium. Int J Cancer 1991;49:787-95.

(188) Noguchi P, Wallace R, Johnson J, Earley EM, O'Brien S, Ferrone S, et al. Characterization of the WIDR: a human colon carcinoma cell line. In Vitro 1979;15:401-8.

(189) Yeung TM, Gandhi SC, Wilding JL, Muschel R, Bodmer WF. Cancer stem cells from colorectal cancer-derived cell lines. Proc Natl Acad Sci U S A 2010;107:3722-7.

(190) Sahlberg SH, Spiegelberg D, Glimelius B, Stenerlow B, Nestor M. Evaluation of cancer stem cell markers CD133, CD44, CD24: association with AKT isoforms and radiation resistance in colon cancer cells. PLoS ONE 2014;9:e94621.

(191) Stratford EW, Castro R, Wennerstrom A, Holm R, Munthe E, Lauvrak S, et al. Liposarcoma Cells with Aldefluor and CD133 Activity have a Cancer Stem Cell Potential. Clin Sarcoma Res 2011;1:8.

(192) Dalerba P, Dylla SJ, Park IK, Liu R, Wang X, Cho RW, et al. Phenotypic characterization of human colorectal cancer stem cells. Proc Natl Acad Sci U S A 2007;104:10158-63.

(193) Kemper K, Grandela C, Medema JP. Molecular identification and targeting of colorectal cancer stem cells. Oncotarget 2010;1:387-95.

(194) Ghosh SC, Neslihan AS, Klostergaard J. CD44: a validated target for improved delivery of cancer therapeutics. Expert Opin Ther Targets 2012;16:635-50.

(195) Liu C, Tang DG. MicroRNA regulation of cancer stem cells. Cancer Res 2011;71:5950-4.

(196) Fillmore CM, Kuperwasser C. Human breast cancer cell lines contain stem-like cells that self-renew, give rise to phenotypically diverse progeny and survive chemotherapy. Breast Cancer Res 2008;10:R25.

(197) Dallas NA, Xia L, Fan F, Gray MJ, Gaur P, van BG, et al. Chemoresistant colorectal cancer cells, the cancer stem cell phenotype, and increased sensitivity to insulin-like growth factor-I receptor inhibition. Cancer Res 2009;69:1951-7.

(198) Hwang WL, Yang MH, Tsai ML, Lan HY, Su SH, Chang SC, et al. SNAIL regulates interleukin-8 expression, stem cell-like activity, and tumorigenicity of human colorectal carcinoma cells. Gastroenterology 2011;141:279-91, 291.

(199) Lee CJ, Dosch J, Simeone DM. Pancreatic cancer stem cells. J Clin Oncol 2008;26:2806-12.

(200) Patrawala L, Calhoun T, Schneider-Broussard R, Li H, Bhatia B, Tang S, et al. Highly purified CD44+ prostate cancer cells from xenograft human tumors are enriched in tumorigenic and metastatic progenitor cells. Oncogene 2006;25:1696-708.

(201) Maitland NJ, Collins AT. Prostate cancer stem cells: a new target for therapy. J Clin Oncol 2008;26:2862-70.

(202) Chang L, Graham PH, Hao J, Ni J, Bucci J, Cozzi PJ, et al. Acquisition of epithelial-mesenchymal transition and cancer stem cell phenotypes is associated with activation of the PI3K/Akt/mTOR pathway in prostate cancer radioresistance. Cell Death Dis 2013;4:e875. doi: 10.1038/cddis.2013.407.

(203) Williams K, Motiani K, Giridhar PV, Kasper S. CD44 integrates signaling in normal stem cell, cancer stem cell and (pre)metastatic niches. Exp Biol Med (Maywood) 2013;238:324-38.

(204) Nagano O, Okazaki S, Saya H. Redox regulation in stem-like cancer cells by CD44 variant isoforms. Oncogene 2013;32:5191-8.

(205) Calcagno AM, Salcido CD, Gillet JP, Wu CP, Fostel JM, Mumau MD, et al. Prolonged drug selection of breast cancer cells and enrichment of cancer stem cell characteristics. J Natl Cancer Inst 2010;102:1637-52.

(206) Misra S, Ghatak S, Toole BP. Regulation of MDR1 expression and drug resistance by a positive feedback loop involving hyaluronan, phosphoinositide 3-kinase, and ErbB2. J Biol Chem 2005;280:20310-5.

(207) Trzpis M, McLaughlin PM, de Leij LM, Harmsen MC. Epithelial cell adhesion molecule: more than a carcinoma marker and adhesion molecule. Am J Pathol 2007;171:386-95.

(208) Baeuerle PA, Gires O. EpCAM (CD326) finding its role in cancer. Br J Cancer 2007;96:417-23.

(209) Al-Hajj M, Wicha MS, Benito-Hernandez A, Morrison SJ, Clarke MF. Prospective identification of tumorigenic breast cancer cells. Proc Natl Acad Sci U S A 2003;100:3983-8.

(210) Li C, Heidt DG, Dalerba P, Burant CF, Zhang L, Adsay V, et al. Identification of pancreatic cancer stem cells. Cancer Res 2007;67:1030-7.

(211) Munz M, Kieu C, Mack B, Schmitt B, Zeidler R, Gires O. The carcinoma-associated antigen EpCAM upregulates c-myc and induces cell proliferation. Oncogene 2004;23:5748-58.

(212) Maetzel D, Denzel S, Mack B, Canis M, Went P, Benk M, et al. Nuclear signalling by tumour-associated antigen EpCAM. Nat Cell Biol 2009;11:162-71.

(213) Gunnarsson LDC, Paus D, Karlsson JM, Kiprijanov SM, inventors; Affitech Research AS, assignee. Anti EpCAM antibodies.United States patent US008637017B2. 2014.

(214) Campoli MR, Chang CC, Kageshita T, Wang X, McCarthy JB, Ferrone S. Human high molecular weight-melanoma-associated antigen (HMW-MAA): a melanoma cell surface

chondroitin sulfate proteoglycan (MSCP) with biological and clinical significance. Crit Rev Immunol 2004;24:267-96.

(215) Price MA, Colvin Wanshura LE, Yang J, Carlson J, Xiang B, Li G, et al. CSPG4, a potential therapeutic target, facilitates malignant progression of melanoma. Pigment Cell Melanoma Res 2011;24:1148-57.

(216) Legg J, Jensen UB, Broad S, Leigh I, Watt FM. Role of melanoma chondroitin sulphate proteoglycan in patterning stem cells in human interfollicular epidermis. Development 2003;130:6049-63.

(217) Wang X, Osada T, Wang Y, Yu L, Sakakura K, Katayama A, et al. CSPG4 protein as a new target for the antibody-based immunotherapy of triple-negative breast cancer. J Natl Cancer Inst 2010;102:1496-512.

(218) Weyergang A, Berg K, Kaalhus O, Peng Q, Selbo PK. Photodynamic Therapy Targets the mTOR Signaling Network in Vitro and in Vivo. Mol Pharmaceutics 2009;6:255-64.

(219) He J, Liu Y, Zhu T, Zhu J, DiMeco F, Vescovi AL, et al. CD90 is identified as a candidate marker for cancer stem cells in primary high-grade gliomas using tissue microarrays. Mol Cell Proteomics 2012;11:M111 doi: 10.1074/mcp.M111.010744 .

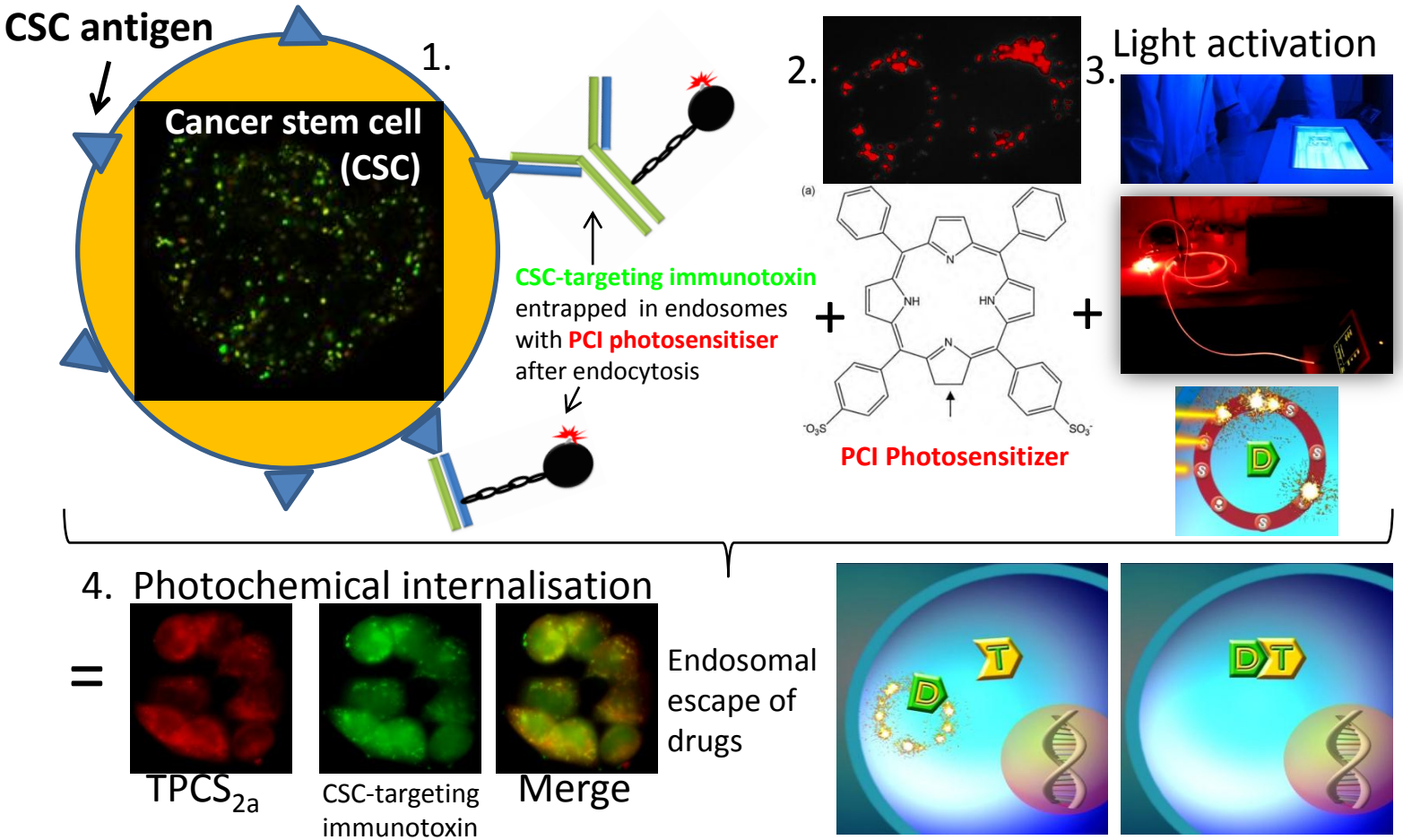
(220) Tehranchi R, Woll PS, Anderson K, Buza-Vidas N, Mizukami T, Mead AJ, et al. Persistent malignant stem cells in del(5q) myelodysplasia in remission. N Engl J Med 2010;363:1025-37.

(221) Donnenberg VS, Donnenberg AD, Zimmerlin L, Landreneau RJ, Bhargava R, Wetzel RA, et al. Localization of CD44 and CD90 positive cells to the invasive front of breast tumors. Cytometry B Clin Cytom 2010;78:287-301.

(222) Yang ZF, Ho DW, Ng MN, Lau CK, Yu WC, Ngai P, et al. Significance of CD90+ cancer stem cells in human liver cancer. Cancer Cell 2008;13:153-66.

(223) Civenni G, Walter A, Kobert N, Mihic-Probst D, Zipser M, Belloni B, et al. Human CD271-positive melanoma stem cells associated with metastasis establish tumor heterogeneity and long-term growth. Cancer Res 2011;71:3098-109.

(224) Boiko AD, Razorenova OV, van de RM, Swetter SM, Johnson DL, Ly DP, et al. Human melanoma-initiating cells express neural crest nerve growth factor receptor CD271. Nature 2010;466:133-7.



Graphical abstract