Mechanisms of cisplatin resistance and targeting of cancer stem cells: Adding glycosylation to the equation

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ABSTRACT

Cisplatin-based chemotherapeutic regimens are the most frequently used (neo)adjuvant treatments for the majority of solid tumors. While platinum-based chemotherapeutic regimens have proven effective against highly proliferative malignant tumors, significant relapse and progression rates as well as decreased overall survival are still observed. Currently, it is known that sub-populations of chemoresistant cells share biological properties with cancer stem cells (CSC), which are believed to be responsible for tumor relapse, invasion and ultimately disease dissemination through acquisition of mesenchymal cell traits. In spite of concentrated efforts devoted to decipher the mechanisms underlying CSC chemoresistance and to design targeted therapeutics to these cells, proteomics has failed to unveil molecular signatures capable of distinguishing between malignant and non-malignant stem cells. This has hampered substantial developments in this complex field. Envisaging a novel rationale for an effective therapy, the current review summarizes the main cellular and molecular mechanisms underlying cisplatin resistance and the impact of chemotherapy challenge in CSC selection and clinical outcome. It further emphasizes the growing amount of data supporting a role for protein glycosylation in drug resistance. The dynamic and context-dependent nature of protein glycosylation is also comprehensively discussed, hence highlighting its potentially important role as a biomarker of CSC. As the paradigm of cancer therapeutics shifts towards precision medicine and patient-tailored therapeutics, we bring into focus the need to introduce glycomics and glycoproteomics in holistic pan-omics models, in order to integrate diverse, multimodal and clinically relevant information towards more effective cancer therapeutics.

1. INTRODUCTION

Cisplatin (cis-diamminedichloridoplatinum(II); cis-[Pt(NH3)2(Cl)2]) was first described by Michele Peyrone in 1845, but its structure was only determined in 1893 (Trzaska, 2005). After several years of investigation, Rosenberg realized its potential to induce tumor cells death (Rosenberg, 1973) and finally in 1978 the drug was approved by the FDA for the treatment of testicular and ovarian cancer (Trzaska, 2005). Nowadays, cisplatin-based regimens are widely used as (neo)adjuvant chemotherapy against a spectrum of solid tumors including gastric, non-small cell lung (NSCLC), head and neck, gallbladder and urinary bladder cancer. However, cisplatin treatment exhibits severe side effects including immunosuppression, renal toxicity, gastrointestinal disorders and ototoxicity (Boussios et al., 2012, Karasawa and Steyger, 2015). It may also cause gonadal suppression resulting in amenorrhea or azoospermia, partial or irreversible infertility and embryo-toxicity (Brennemann et al., 1997, Meistrich, 2009).

Cisplatin is an alkylating agent capable of forming adducts with macromolecules, particularly with N7 atoms of purine nucleobases. This results in inter- and intra-strand DNA cross-links that bring induce cell cycle arrest mainly in the G2/M checkpoint (Yuan et al., 2003). The inability to repair this DNA damage ultimately leads to programed cell death. However, experimental evidence revealed that other mechanisms such as the production of reactive oxygen species (ROS) and the activation of inflammatory pathways, may also contribute to the induction of apoptosis (Casares et al., 2012). Cisplatin has shown significant efficacy against rapidly proliferating tumor cells. However, despite a fairly acceptable intrinsic drug response rate, there is a 95% risk of tumor relapse in NSCLC patients. The 5-year survival rate is approximately 50% for muscle-invasive bladder cancer, (Nadal and

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Bellmunt, 2014) and 15–20% for ovarian cancer patients (Siddik, 2003); similar survival rates have been reported for other solid tumors. It has been hypothesized that chemotherapy may either act as a selective pressure for more aggressive cell phenotypes (Freitas et al., 2014), or that tumor cells which are less drug sensitive may acquire mutations during the course of treatment, that enable them to evade drug-induced cell death (Crea et al., 2011). The failure of cisplatin-based regimens is considered both life-threatening and a major burden to health care systems, as it requires the introduction of more expensive second-line treatments. Therefore, deciphering the mechanisms underlying this treatment failure has been a primary goal of cancer research and, in the past two decades, some of the modalities underlying anticancer drug resistance have been identified; however the implications for improving drug therapy have been limited.

Chemotherapy resistance results from a synergism of events that include tumor cell extrinsic factors (pharmacokinetic resistance and tumor microenvironment) as well as intrinsic factors, namely alterations in drug transport and metabolism, relative dormancy/slow cell cycle kinetics, efficient DNA repair systems and inhibition of apoptosis (Martin et al., 2008, Pommier et al., 2004, Raguz and Yague, 2008). In addition, some chemoresistant tumor cell clones may present self-renewal and pluri/multipotent differentiation capabilities, which are characteristics associated with cancer-stem cells (CSC; Visvader and Lindeman, 2008). Therefore, these cells constitute a small pool of CSC capable of generating more differentiated sub-populations that, during subsequent divisions, form the vast majority of the tumor bulk. The remarkable longevity of CSC also renders them more susceptible to the accumulation of genetic damage and epigenetic alterations that may ultimately promote the proliferation of heterogeneous and aggressive cell phenotypes (Muñoz et al., 2012). Some subsets of CSC can be found in poorly vascularized hypoxic tumor niches, which favor the maintenance of stem-cell characteristics, and are consequently exposed to suboptimal drug concentrations (Lin and Yun, 2010). Furthermore, these cells may undergo epithelial-to-mesenchymal transition (EMT) in response to microenvironmental stimuli, namely prolonged exposure to low oxygen levels, and may acquire the capability to invade and metastasize to regional lymph nodes and distant organs (Jiang et al., 2011). In summary, it became evident that cisplatin and other conventional chemotherapeutic drugs may ultimately contribute to the selection of a pool of slow dividing or quiescent CSC (Wang et al., 2014a, Wang et al., 2014b). These cells are endowed with the capability of recapitulating tumor heterogeneity and undergo EMT, considered as one of the driving forces of cancer dissemination (Frank et al., 2010). As such, patients would greatly benefit from combined therapies including agents capable of selectively eliminating CSC. The ideal therapy should specifically recognize these cells from the tumor bulk, include means to inhibit resistance mechanisms, as well as include CSC-killing agents. However, the majority of membrane-bound CSC biomarkers known to date can also be found in normal stem- and non-malignant cells (Cojoc et al., 2015), which hampers the development of specific targeted therapeutics.

More recently, several studies have demonstrated that profound alterations in protein glycosylation that often accompany malignant transformation may also influence resistance to chemotherapy. This rather neglected mechanism of drug resistance has been often associated with impaired function of membrane-bound glycoproteins, such as ATP-binding cassette efflux transporters, due to specific alterations in their glycosylation patterns (Beers et al., 2013, Nakagawa et al., 2009). However, alterations in cell-surface protein glycosylation have also been shown to favor oncogenic signaling pathways associated with chemoresistance and CSC-like phenotypes (Dall'Olio et al., 2014, Häuselmann and Borsig, 2014, Ju et al., 2008, Pinho et al., 2012). Therefore, cancer-

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associated glycans constitute markers of chemoresistance and bear potential promise for the identification and therapeutic targeting of CSC.

Envisaging a rationale for an effective therapy, the present review discusses the main mechanisms of cisplatin resistance known to date, integrating key insights about the role of cancer-associated glycans. Although the current review focuses mainly on cisplatin, it is proposed here that many of these strategies mediate resistance to other drugs as well. The present paper also provides a comprehensive overview on the impact of the chemotherapeutic challenge in tumor biology, CSC selection and clinical outcome. Moreover, it aims to raise awareness for the fact that CSC harbor distinct glycosylation patterns that should be carefully explored towards the development of highly specific targeted therapeutics.owth, and which can be targets for personalized therapy in gastric cancer patients.

2. Overview on drug resistance mechanisms and CSC selection

Drug resistance is a multifactorial process which is based on both extrinsic and intrinsic factors in tumor cells (Raguz and Yague, 2008). Extrinsic factors such as unfavorable drug pharmacokinetics and abnormal tumor vasculature result in the delivery of suboptimal concentrations of cytotoxic agents to tumor sites (Rohwer and Cramer, 2011). Defective tumor vasculature also results in hypoxic and acidic niches that significantly modulate cell function in manners that favor chemoresistance (Wilson and Hay, 2011). Similarly, alterations in the extracellular matrix architecture and stromal cell paracrine signals have been found to influence chemotherapy outcome (Sherman-Baust et al., 2003, Tripathi et al., 2012). In addition, tumor cells may either present, or develop during the course of treatment, various mechanisms to withstand and overcome chemotherapeutic challenges (Shen et al., 2012). These mechanisms include for example: (i) Alterations in drug transport and metabolism; (ii) Enhanced DNA repair mechanisms; (iii) Alterations in cell cycle regulation; and (iv) Inhibition of apoptosis. Emerging evidences support the notion that chemoresistance, driven by the above-mentioned factors, is associated with CSC-like properties as well as the acquisition of EMT capability, thereby explaining the high relapse and progression rates presented by first-line chemotherapy agents (Cojoc et al., 2015). Based on these considerations, the following sections aim to illustrate the influence of the main tumor-associated extrinsic and intrinsic properties in chemoresistance.

2.1. The impact of the microenvironment on drug resistance

2.1.1. Tumor vasculature and hypoxia

Solid tumors often present tortuous, poorly differentiated and truncated vasculature, resulting in the delivery of suboptimal concentrations of cytotoxic drugs to certain niches (Minchinton and Tannock, 2006). This also accounts for the formation of a hypoxic environment that significantly influences cell metabolism and modulates gene expression, ultimately enhancing chemoresistance and maintenance of CSC (Rohwer and Cramer, 2011); discussed in detail in the following sections (Fig. 1). Furthermore, hypoxia modulates the expression of genes linked to EMT—(addressed in detail in subsequent sections) and drug-resistance phenotypes (Adamaki et al., 2012, Jiang et al., 2011, Polyak and Weinberg, 2009, Ruan et al., 2009, Shannon et al., 2003).

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The primary transcription factor mediating the response to hypoxic challenge is hypoxia-inducible factor-1 (HIF-1; Semenza, 2001). HIF-1 consists of a constitutively expressed subunit HIF-1β and a tightly oxygen-regulated subunit HIF-1α (or its paralogs HIF-2α and HIF-3α) (Brocato et al., 2014). Under normoxia conditions, the HIF-1α protein is constitutively expressed but rapidly marked for proteosomal degradation, resulting in a very short cytoplasmic half-life (5–8 min). Under hypoxic conditions, HIF-1α is translocated to the nucleus where it binds to the co-activators HIF-1β and p300/CBP (Semenza, 2001), inducing an array of responses such as overexpression of angiogenic genes (i.e., VEGF, PDGF-BB and NOS) and growth factors (i.e., IGF-II; Brocato et al., 2014, Denko et al., 2003, Harris, 2002), decreases mitotic and metabolic rates and adapts energy requirements to the hypoxic challenge (Hockel and Vaupel, 2001, Shannon et al., 2003). In particular, HIF-1α contributes to the dramatic shift of intracellular glucose metabolism from aerobic cellular respiration to anaerobic glycolysis through the transactivation of genes encoding glucose transporters (i.e., GLUT-1) and several rate-limiting enzymes of glycolysis (Jose et al., 2011, Mucaj et al., 2012). It also suppresses the tricarboxylic cycle (TCA) via the PDK1 gene, encoding pyruvate dehydrogenase kinase 1, which inactivates pyruvate dehydrogenase, resulting in fueling the TCA cycle with acetyl-CoA (Shirato et al., 2011). Moreover, HIF-1α has been shown to regulate the expression of cytochrome C oxidase (COX) allowing cancer cells to optimize the efficiency of respiration at different oxygen levels (Mucaj et al., 2012). Ultimately, HIF-1α contributes to cellular adaptation to hypoxic stress which can culminate in mitochondrial autophagy (Zhang et al., 2008). By modulating mitochondria activity, HIF-1α influences cell death mechanisms mainly by interfering with apoptotic and necrotic signaling (Greijer and Van der Wall, 2004). Furthermore, HIF-1α has also been shown to act as apoptosis suppressor in cancer cells through the regulation of anti-apoptotic target genes and additional molecular mechanisms that still remain largely elusive (Adamaki et al., 2012, Harris, 2002). For instance, hypoxic regulation of p53 has been proposed to be HIF-dependent; however, controversy remains over this topic. Nevertheless, most reports point to the fact that hypoxia acts as a positive selective pressure for the positive selection of p53 mutant cells and hence inducing diminished apoptotic potential (Ruan et al., 2009), thereby compromising the response to chemotherapy (Gogna et al., 2012, Weisz et al., 2007). HIF-1α also acts as a regulator of drug efflux through the activation of the MDR1 gene encoding the multidrug resistance efflux transporter P-glycoprotein (P-gp; ABCB1; Comerford et al., 2002, Rohwer and Cramer, 2011, Shannon et al., 2003). P-gp belongs to the ATP-binding cassette (ABC) superfamily of transporters; however, it does not recognize cisplatin as a transport substrate (Lockhart et al., 2003, Rohwer and Cramer, 2011), but is capable of markedly decreasing the intracellular concentration of a wide range of structurally and functionally distinct hydrophobic chemotherapeutic agents (Lockhart et al., 2003, Shapira et al., 2011).

Under hypoxic conditions an association between high levels of HIF-1α and cisplatin resistance has been widely reported for several representative tumor cell lines, including ovarian cancer (Su et al., 2011), NSCLC (Fischer et al., 2015), hepatocellular carcinoma and hepatic progenitor cell lines (Jiao and Nan, 2012). Supporting a role for HIF-1α in chemoresistance, elevated levels of this key transcription factor have been observed in tumors of different tissue origin and linked to more resilient tumor cells, poor prognosis and resistance to radiotherapy and chemotherapy (Huang et al., 2007, Shannon et al., 2003, Wilson and Hay, 2011). Nevertheless, it should be noticed that, despite the pivotal role of HIF-1α, other independent mechanisms have been found to mediate hypoxia-related chemoresistance (Adamaki et al., 2012, Scholten et al., 2014). More detailed insights about the role of HIF in chemoresistance may be found in previous reviews by Rohwer and Cramer, (2011) and Raguz and Yague, (2008).

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2.1.2. The tumor stroma

The tumor stroma, mainly formed by the basement membrane, extracellular matrix, cancer-associated fibroblasts (CAFs), immune cells, and vasculature, is a complex structure whose interactions markedly affect tumor growth, invasion and metastasis during the course of disease (Bremnes et al., 2011, Pietras and ÿstman, 2010; Fig. 1). Over the recent years, the crosstalk between cancer cells and the tumor stroma has been progressively unveiled (see review Bremnes et al., 2011, Tripathi et al., 2012). However, the dynamics of cancer cell death in response to cisplatin and the tumor microenvironment has yet to be fully characterized. Noteworthy, it has been shown that the presence of stroma in 574 breast cancer specimens from patients who underwent surgery combined or not with adjuvant radiotherapy, adjuvant chemotherapy or adjuvant endocrine therapy, was an independent prognostic factor for relapse-free period, particularly in the triple-negative subpopulation (de Kruijf et al., 2011). However, extracellular matrix components mediating resistance to chemotherapy are still poorly understood. Several in vitro studies have demonstrated that the extracellular matrix not only constitutes a physical barrier to drug dissemination (Choi et al., 2013), but may also exert a protective effect against apoptosis induced by various anticancer drugs (Chen et al., 2010, Kouniavsky et al., 2002, Sethi et al., 1999). Miyamoto et al. (2004) have further found that the grade of differentiation of pancreatic tumor cells affects the interaction with different ECM macromolecules (fibronectin, collagen I and collagen IV) as well as the matrix-driven sensitivity to chemotherapeutic drugs, including cisplatin. The presence of fibronectin, type IV collagen and laminin have also been linked to cisplatin-resistance and local recurrence of uveal melanomas (Bérubé et al., 2005). The authors observed that apoptosis was less frequent after cisplatin administration in the presence of ECM when compared with cells cultured on a non-permissive matrix (Bérubé et al., 2005). Several studies also found that the overexpression of ECM-associated genes correlates with increased cisplatin resistance (Januchowski et al., 2014, Sherman-Baust et al., 2011, Sherman-Baust et al., 2003). In this respect, the gene expression profile of a subpopulation of the human ovarian carcinoma cell line A2780 displaying cisplatin resistance revealed a significant upregulation of the COL6A3 gene, encoding for collagen VI (Varma et al., 2005). These findings suggest that chemoresistance may modulate ECM composition through altered regulation of gene expression in cancer cells. Moreover, cisplatin-sensitive cells cultured in the presence of collagen VI showed enhanced resistance in vitro and immunohistochemistry studies revealed an association between collagen VI overexpression, tumor grade and resistance to chemotherapy (Sherman-Baust et al., 2003). Furthermore, ovarian tumors with pronounced stromal/mesenchymal gene signatures exhibited the worst outcome when compared to groups with non-stroma-associated gene signatures (Verhaak et al., 2012). In addition, ECM gene signatures were associated with chemotherapy resistance (Mintz et al., 2005). Altogether, these findings suggest that tumor cells may remodel their microenvironment in order to increase survival to chemotherapeutic challenges (Sherman-Baust et al., 2003). Recent studies point towards the pivotal role of β1-integrins in ECM-cancer cells interactions and chemoresistance (Aoudjit and Vuori, 2012). According to Hodkinson et al. (2007), β1-integrin-mediated PI3K activation prevents caspase-3 activation, thereby protecting small cell lung cancer cell lines against chemotherapy-induced cell cycle arrest and apoptosis. A more recent report on oral carcinoma cell lines has shown that adhesions within the carcinoma matrix create an environment in which exposure to cisplatin induces proliferation through the function of β1-integrin, talin and FAK pathways that regulate nuclear activity of NF-kB (Eberle et al., 2011). Based on these observations, it has been suggested that the inhibition of ECM-integrin interactions in combination with chemotherapy could have positive therapeutic implications (Mahadevan and Von Hoff, 2007). Impaired binding of hyaluronan

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to transmembrane receptor CD44 has also been shown to influence multiple cell signaling pathways that stimulate tumor cell proliferation, migration and matrix metalloproteinase secretion, and to promote CSC properties as well as cisplatin resistance (Bourguignon et al., 2012, Bourguignon et al., 2014, Ohashi et al., 2007, Torre et al., 2010). Several metalloproteinases that are responsible for the proteolysis of ECM components during biological processes such as carcinogenesis, differentiation, apoptosis, migration and invasion, tumor angiogenesis and immune surveillance may also contribute to modulate response to treatment (Blons et al., 2004, Ertan et al., 2011, Mahadevan and Von Hoff, 2007). The balance between matrix metalloproteinases (MMP) and tissue inhibitors of metalloproteinases (TIMPs) is crucial for ECM stability (Nagase et al., 2006) and acts in a coordinated manner to drive cancer progression and metastasis (Hara et al., 2001, Rodriguez et al., 2012). Overexpression of TIMP-2, an inhibitor of MMP-2, in ovarian cancer stromal areas was found to be associated with better response to chemotherapy (Hašož et al., 2012). Furthermore, in vitro studies of squamous cell carcinoma of the head and neck have shown significant correlations between resistance to cisplatin and the expression of TIMP-2, suggesting that TIMP-2-mediated chemoresistance is dependent on the physiological environment (Akervall et al., 2004). However, studies using ovarian cancer cell lines failed to show any association between this MMP-2 inhibitor and cisplatin resistance (Hašož et al., 2012). Also, expression of MMP-7 and -13 was found to be associated with cisplatin resistance in head and neck cancer cell lines (Ansell et al., 2009). Again, these observations suggest that the crosstalk between the microenvironment and cancer cells is essential in order to influence intrinsic cell characteristics and chemotherapy resistance. These findings imply that disruption of cell-matrix interactions may provide ways to block ECM-mediated upstream or downstream intracellular signaling cascades, which could result in the overcoming of cisplatin resistance. Taking into account the encouraging results from studies using matrix modulating agents in combination with chemotherapy (Hussain et al., 2012, Kamaraj et al., 2010, Pisano et al., 2014), it is now important to fully clarify the role of ECM components in platinum resistance in order to design rational therapeutic approaches.

Stromal changes also include the development of carcinoma-associated fibroblasts (CAFs) that play a crucial role in disease (Tripathi et al., 2012). In the recent years, the crosstalk between cancer cells and CAFs has been unveiled and comprehensively reviewed by several groups (Brennen et al., 2012, Liu et al., 2012, Puré, 2009). CAFs have been found to directly drive cancer development through multiple mechanisms, which include the promotion of angiogenesis, cellular proliferation, invasion and inhibition of apoptosis in cancer cells (Brennen et al., 2012, Hale et al., 2013). Numerous growth factors, cytokines, proteases, and extracellular matrix proteins, such as SDF-1, FGF2, VEGF, TGF-β, HGF, tenascin-c, LOX, and MMPs are among the key molecules mediating these processes. Growth factors and other proteins produced by activated fibroblasts not only act on cancer cells but also on other components of the stroma, including adipocytes, inflammatory and immune cells as well as in the remodeling of the ECM, hence creating intricate feedback loops mediated by paracrine and autocrine signaling that drives CAFs development, disease progression and dissemination (Brennen et al., 2012, Hale et al., 2013). Nevertheless, only limited data on the response of CAFs to chemotherapy and their potential impact on therapy outcome are available. Namely, Sonnenberg et al. (2008) addressed the influence of CAFs in lung and breast tumors as well as in primary cultures hence revealing that, similarly to cancer cells, the sensitivity of CAFs to cisplatin is highly variable and dependent on the cancer type and its microenvironment. Moreover, studies have demonstrated that the presence of CAFs contributes to the decreased response to chemotherapy in prostate (Franco and Hayward, 2012), breast (Rong et al., 2013) and lung (Sonnenberg et al., 2008) cancers. Furthermore, strategies targeting CAFs components have proven capable of improving the

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response to chemotherapy (Elenbaas and Weinberg, 2001, Loeffler et al., 2006), suggesting a role for these cells in treatment outcome. Also, a recent systematic review by Hale et al. (2013), has highlighted the fact that stroma-derived biomarkers, with emphasis on molecules produced by CAFs, are useful biomarkers to predict response to therapy. Several studies also support the notion that CSC, known for their intrinsic resistance to chemotherapy, also receive critical maintenance cues from supportive stromal elements, including CAFs (Hasegawa et al., 2014, Li et al., 2013a, Li et al., 2013b, Liao et al., 2010). In this respect, the interplay between CSC and CAFs towards increased chemoresistance and CSC self-renewal and invasion, have been recently demonstrated for colorectal cancer; according to this study, chemotherapy induces the remodeling of the tumor microenvironment to support cellular hierarchy through secreted factors that include IL-17A (Lotti et al., 2013).

Overall, there is a growing amount of evidence supporting the notion that stromal components promote chemoresistance; hence, the mechanisms underlying this stromal based chemoresistance should be critically evaluated hence paving the way towards the overcoming of this modality of drug resistance. Furthermore, the incorporation of therapeutics which target the tumor microenvironment may be a determinant for disrupting CSC maintenance mechanisms.

2.2. Alterations in intracellular drug concentrations

Cisplatin-resistant tumor cells often present reduced intracellular platinum accumulation when compared to non-resistant cells following drug exposure. This may result either from reduced uptake or increased drug export (Stewart, 2007).

2.2.1. Cisplatin uptake

Cisplatin uptake is mainly mediated by high affinity copper transporter protein 1 (hCtr1) and its down-regulation has been associated with resistance to platinum-based drugs in vitro (Holzer et al., 2004, Liang et al., 2014, Liang et al., 2012) and in vivo (Fu et al., 2012; Fig. 2). An association between hCtr1 rs7851395 and rs12686377 polymorphisms and platinum resistance in NSCLC patients has also been described (Xu et al., 2012). Nevertheless, more clinical studies are required to determine the prognostic value of hCtr1 and its genetic variants in the context of cisplatin treatment. The hCtr1 transporter protein is responsible by regulating intracellular copper homeostasis. In turn, the expression of hCTR1 is regulated at the transcriptional level by copper via the transcription factor Sp1 and at the post-translational level by a mechanism involving copper-stimulated endocytosis and degradation of the transporter (Howell et al., 2010, Petris et al., 2003). The exposure to copper-chelating agents has been proven to increase the expression of hCtr1 and sensitize tumor cells to platinum (Galluzzi et al., 2012, Ishida et al., 2010, Liang et al., 2012). Furthermore, a pilot clinical trial has shown that administration of a copper-lowering agent can partially resensitize platinum-resistant high-grade epithelial ovarian cancer patients to platinum chemotherapy (Fu et al., 2012). Altogether, these observations suggest a role for hCtr1 in cisplatin resistance and support larger studies to assess the efficacy of this approach in platinum-resistant cancers treatment. Other transporters such as Solute Carrier Family 22 Members 1 and 2 (Li and Shu, 2014, Yonezawa et al., 2006) have also been implicated in cisplatin uptake; nevertheless, more studies are required to delineate their role in cisplatin resistance as well as their potential use as biomarkers for resistance to platinum cytotoxic agents.

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2.2.2. Cisplatin efflux

The efflux of platinum-based drugs is believed to be mediated by Transporting P-type Adenosine Triphosphatases ATP7A and ATP7B (Drayton and Catto, 2012, Stewart, 2007, Tadini-Buoninsegni et al., 2014, Safaei et al., 2008; Fig. 2). Unsurprisingly, the overexpression of these transmembrane carriers have been implicated in cisplatin resistance and poor patient survival in several cancers (Konkimalla et al., 2008, Tadini-Buoninsegni et al., 2014, Yoshizawa et al., 2007). Silencing ATP7A in cisplatin-resistant tumor cells restored cisplatin sensitivity to a certain extent and enhanced apoptosis, suggesting that ATP7A may also be a target to sensitize cancer cells to cisplatin (Li et al., 2012). Resistance to cisplatin has also been associated with the overexpression of Multidrug resistance-associated proteins (MRP) 1, 2, 3 and 5, members of the superfamily of ABC transporters (Borst et al., 2000, Guminski et al., 2006, Konkimalla et al., 2008, Song et al., 2015; Fig. 2). In particular, MRP2 expression seems to determine the efficacy of cisplatin-based chemotherapy in patients with hepatocellular carcinoma (HCC; Korita et al., 2010). Furthermore, a reduction in MRP2 expression using ribozymes has been shown to restore cisplatin sensitivity in cisplatin-resistant adrenocortical carcinoma and melanoma cell lines (Materna et al., 2005), further suggesting a role for this molecule in cisplatin resistance. Nevertheless, it has been demonstrated that cisplatin is not a substrate for MRP transporters and that its ability to transport the drug across the cell membrane requires the formation of glutathione- or metallothioneins-cisplatin conjugates (discussed in detail in the following section; Chen et al., 1998, Fujiwara et al., 1990, Kelland, 2007; Fig. 2).

2.2.3. Detoxification by intracellular molecules

Although purine nucleobases are the main therapeutic target for cisplatin, a vast majority of cisplatin molecules will react with other ligands before reaching the nucleus. In particular, cisplatin shows much higher affinity for thiols than for nitrogen groups, hence making intracellular cisplatin detoxification via these functional groups one of the main mechanisms of cisplatin scavenging (Dabrowiak et al., 2002). As such, resistance to cisplatin has been associated with the overexpression of metallothioneins, a class of low-molecular weight cysteine-rich proteins that promote intracellular homeostasis of physiological metals, providing protection against metal toxicity and oxidative stress (Knipp, 2009; Fig. 2). This has been observed in different cancer models (Gansukh et al., 2013, Peng et al., 2012) as well as in bladder (Siu et al., 1998, Wülfing et al., 2007), colorectal (Hishikawa et al., 2001), esophageal (Yamamoto et al., 1999), and breast (Bay et al., 2006, Lai et al., 2011) carcinomas, among other neoplasia.

Glutathione (GSH), a thiol containing tripeptide (Glu-Cys-Gly), highly expressed in mammalian cells, also plays a key role in cisplatin scavenging (Rocha et al., 2014; Fig. 2). Glutathione-S-transferase 1 (GSTP1-1) conjugates the reduced form of glutathione (GSH) to xenobiotic substrates for the purpose of detoxification and catalyzes the formation of cisplatin-GSH conjugates (Peklak-Scott et al., 2008, Sawers et al., 2014). As such, increased expression of enzymes involved in GSH synthesis and GSH conjugation have been implicated in the generation of cisplatin resistance (Galluzzi et al., 2012, Rocha et al., 2014). Also, elevated levels of glutamate/cysteine transport system (system Xc) were linked with higher intracellular levels of GSH and resistance to cisplatin (Okuno et al., 2003). The efflux of intracellular glutathione S-cisplatin conjugates is an ATP-dependent process mediated by integral membrane glycoproteins belonging to the MRP family (Ishikawa and Ali-Osman, 1993, Keppler, 1999). Furthermore, MRP transporters are overexpressed in cisplatin-resistant human

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cancer cells whose GSH levels are substantially enhanced (Chen and Kuo, 2010, Ishikawa et al., 1994), suggesting that GSH and MRP proteins work in synergy for detoxification purposes.

2.3. Alterations in DNA repair systems

Several DNA repair pathways interact to maintain accurate DNA replication, genomic integrity and normal cell functioning. These include: (i) nucleotide excision repair (NER); (ii) base excision repair (BER); (iii) homologous recombination repair (HR); (iv) mismatch repair (MMR); (v) non-homologous DNA end-joining repair (NHEJ); (vi) O6-alkylguanine DNA alkyl transferase (AGT) mediated repair. Increased efficiency of DNA repair systems is considered essential for the maintenance and longevity of stem cell phenotypes and plays a key role in cytotoxic drugs resistance (Martin et al., 2008). This review will discuss the most studied DNA repair pathways involved in platinum response, i.e., nucleotide excision repair (NER) and mismatch repair (MMR), and will briefly refer the role of BRCA in DNA repair (Fig. 3).

Several reports describe associations between the alterations in the XPF-ERCC1 protein complex of the NER system and platinum-based treatment outcome (Kirschner and Melton, 2010; Fig. 3). This complex is a structure-specific endonuclease involved in recombination, double-strand break and inter-strand crosslink repair (Ahmad et al., 2008). The impossibility to replace damaged nucleotide sequences promotes downstream activation of the mitochondrial BAX protein, inducing apoptosis (Pawlowski and Kraft, 2000; Fig. 4). As such, XPF-ERCC1 overexpression has been found to promote resistance to platinum-based compounds in vitro (Arora et al., 2010, Kirschner and Melton, 2010, McNeil and Melton, 2012), most likely due to overefficient DNA repair. In agreement with these observations, retrospective studies involving patient's histological samples have highlighted an association between the overexpression of ERCC1, resistance to chemotherapy and poor outcome in squamous cell head and neck (Bauman et al., 2013) and bladder carcinomas (Sun et al., 2012). A recent clinical trial has also demonstrated that elevated expression of the ERCC1 gene is an independent prognostic factor for overall survival in first line treatment of advanced gastric cancer (Kwon et al., 2007). Furthermore, a phase II trial showed that single nucleotide polymorphisms of ERCC1 gene (118T/C and 8092C/A) could be used to determine the response of NSCLC patients to cisplatin (Mazzoni et al., 2013). Another study has demonstrated that 8092C/A could be used as an independent predictive biomarker of better response in esophageal cancer patients treated with cisplatin-based regimens (Bradbury et al., 2009). An enhanced expression of ERCC1 has also been observed in esophageal (Zhao et al., 2014a, Zhao et al., 2014b) and ovarian (Abubaker et al., 2013) platinum-resistant cancer-stem cell sub-populations.

Cisplatin resistance has also been associated with somatic mutations in Mismatch Repair system-associated proteins (MMR), namely in MSH_2 and MSH_3 proteins (Clodfelter et al., 2005, Karran et al., 2003, Park et al., 2013; Fig. 3). MSH_2, together with MSH_3, form the MutSβ heteroduplex which interacts with interstrand crosslinks induced by drugs such as cisplatin (Muniandy et al., 2010). Recognition of mismatched base pairs by MMR proteins may directly activate apoptotic pathways, thereby leading to cell death. As such, cisplatin-resistance is often accompanied by loss of expression of MMR proteins (Topping et al., 2009). Takahashi et al. (2011) have further demonstrated that MSH_3 deficiency contributes to the cytotoxicity of platinum drugs through deficient repair of double-strand breaks in the established colorectal cancer cell line HCT116. Moreover, Velasco et al. (2008) demonstrated that decreased MMR expression in testicular tumors is associated with a shorter time to recurrence and death despite chemotherapy.

Likewise, disruption of the homologous recombination (HR) system, responsible for DNA double-strand break repair during the S-phase of the cell cycle, has been shown to modulate response to

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cisplatin (Wang et al., 2011, Wiedemeyer et al., 2014; Fig. 3). In particular, breast cancer susceptibility proteins 1 and 2 (BRCA1/2) play an important role in DNA repair by interacting with components of DNA repair systems and through gene expression regulation of homologous recombination, non-homologous end joining and nucleotide excision repair intermediates. As such, BRCA proteins affect transcriptional regulation, cell cycle control, apoptosis and ubiquitination (Deng and Wang, 2003, Mullan et al., 2006, Wu et al., 2008). Normally, cancer cells with BRCA1/2 deficiency present defective DNA repair by homologous recombination and are sensitive to inter-strand DNA crosslinking agents, such as cisplatin. Therefore, these agents are natural choices for the treatment for BRCA1/2-deficient tumors and were shown to be clinically effective (Shah, 2008). In contrast, during tumor expansion or as a result of chemotherapy, genetic reversion of BRCA1 or BRCA2 mutations can occur mainly due to secondary mutations and restoration of function, leading to cisplatin resistance (Dhillon et al., 2011, Peng and Lin, 2011). Loss of function mutations in genes encoding HR system proteins, BRCA1/2 were associated with increased response to cisplatin in breast (Turner and Tutt, 2012) and ovarian tumors (Kwa et al., 2014). Also, overexpression of BRCA1 in cisplatin-resistant human breast and ovarian carcinoma cell lines (MCF-7 CDDP/R and SKOV-3 CDDP/R, respectively), resulted in increased resistance to cisplatin. In turn, antisense inhibition of BRCA1 expression enhanced cisplatin sensitivity associated with decreased DNA repair by NER and increased apoptosis in the ovarian carcinoma cell line (Husain et al., 1998). Furthermore, BRCA1 mRNA expression levels were also inversely correlated with sensitivity to cisplatin in malignant pleural effusions of NSCLC patients and in ascites of gastric patients (Wang et al., 2008). In addition, several retrospective clinical studies have demonstrated that low BRCA1 mRNA expression was associated with longer survival of breast, ovarian, small cell lung cancer and esophageal squamous cell carcinoma (James et al., 2007, Margeli et al., 2010, Quinn et al., 2007) subjected to cisplatin-based therapeutic regimens, and could be therefore used as predictive and prognostic marker. Moreover, several studies, recently comprehensively reviewed by Buckley and Mullan (2012) suggest a role for BRCA1 in stem cell regulation through activation of the p63 and Notch pathways. Collectively, these studies suggest that functional mutations in BRCA1 as well as reduced BRCA1 mRNA levels may predict a benefit from DNA-damage-based chemotherapy. Furthermore, they highlight a link between BRCA1, stem cell regulation and response to chemotherapy. More detailed information regarding the role of DNA repair systems in resistance to platinum-based treatment can be found in recent reviews by Martin et al., (2008).

Some of the above-described DNA repair-associated effectors were found overexpressed in CSC, when compared with differentiated tumor cells (Maugeri-Saccà et al., 2012). For instance, a significant increase in gene copy number of BRCA1 and RAD51 has been observed in prostatic CSCs compared with the adherent population isolated from the primary site (Maugeri-Saccà et al., 2012). Therefore, it has been hypothesized that CSC are endowed with proficient DNA repair mechanisms which allow them to resist conventional chemotherapy and act as tumor initiators (Mathews et al., 2011), urging CSC-targeted therapeutics aimed at the elimination of these residual tumorigenic cell sub-populations.

2.4. Alterations in apoptosis-regulatory pathways

The cytotoxic effect of cisplatin is primarily mediated by the activation of a multibranched pro-apoptotic signaling cascade in response to the impossibility to repair DNA damage. Details on these mechanisms have been extensively and comprehensively reviewed by several groups (Basu and Krishnamurthy, 2010, Siddik, 2003). As such, the response to cisplatin is significantly dependent on

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functional apoptotic pathways. Furthermore, genetic and epigenetic alterations in genes encoding key mediators of these processes have been long recognized to be associated with drug resistance (Dasari and Tchounwou, 2014, Galluzzi et al., 2012, Shen et al., 2012). These events may either result in enhanced survival stimuli, diminished death stimuli or diminished sensibility to death signals. Perhaps the most common and well documented apoptosis-related deregulation associated with cisplatin resistance stems from the inactivation of the tumor suppressor protein p53 (Galluzzi et al., 2012, Siddik, 2003; Fig. 4). Furthermore, several studies demonstrated that cancer patients harboring wild-type TP53 have a higher probability to benefit from cisplatin-based chemotherapy than patients with TP53 mutations (Gadducci et al., 2002, Liang et al., 2011). Consistently, mutations occur infrequently (<3%) in germ cell tumors like testicular cancer, even though p53 protein is overexpressed in the vast majority of tumor samples (Guillou et al., 1996), reinforcing the key role of p53 as a mediator of chemotherapy response. Other p53-related nuclear transcription factors including p63 and p73, are also part of a network that together with p53, contribute to regulation of apoptosis and tumorigenesis (Flores et al., 2005, Flores et al., 2002) as well as to chemotherapy-induced DNA damage response (Leong et al., 2007, Müller et al., 2006, Rocco et al., 2006, Yuan et al., 2010). Multiple promoters and alternative splicing events result in the expression of several isoforms of these transcription factors, which include full-length isoforms with transactivation domains (TA) homologous to that of full-length p53, and amino-terminally truncated (DeltaN) isoforms, which lack the TA domains. The TA isoforms of p63 and p73 are able to activate downstream target genes and promote apoptosis. Conversely, the DeltaN isoforms may act as dominant inhibitors of the full-length forms of p53, p63 and p73, hence impairing the activation of target genes and inducing apoptosis upon chemotherapy challenge (Courtois et al., 2002, Marcel et al., 2012, Takahashi et al., 2014; Fig. 4). Several studies have further demonstrated that interfering with the expression or function of DeltaNp63 and/or DeltaNp73 and/or mutant p53 in tumor cells contributes to the improvement of tumor response to chemotherapy (Müller et al., 2005, Müller et al., 2006).

Alterations in other proteins mediating apoptosis triggered either by DNA damage or oxidative stress, via the mitochondrial pathway or by the extrinsic route, may also influence sensitivity to cisplatin (Henkels and Turchi, 1999, Tanida et al., 2012). Several death receptors, cytoplasmic adaptors, pro- and anti-apoptotic members of the Bcl-2 protein family, caspases, calpains, and mitochondrial intermembrane proteins are among the factors shown to modulate the response to cisplatin in vitro (Henkels and Turchi, 1999, Park et al., 2002, van Oosterwijk et al., 2012). Moreover, some have been suggested to modulate clinical response (Aggarwal et al., 2007, Muris et al., 2005). Namely, high endogenous expression of anti-apoptotic members of the Bcl-2 family, such as Bcl-2 and Bcl-XL, were associated with increased cisplatin resistance (Michaud et al., 2009). Likewise, loss of Bax and Bak causes complete resistance to cisplatin (Qian et al., 2014; Fig. 4). Moreover, studies involving the combination of cisplatin with the Bcl-2-inhibitor ABT-737 have shown an improvement in the response to cisplatin, through a synergistic effect mediated by Noxa, to promote cell death and loss of clonogenic survival (Li et al., 2009). Overexpression of survivin, a caspase-inhibitory protein often upregulated in response to cisplatin by phosphoinositide-3-kinase (PI3K)/AKT1-dependent mechanisms (Belyanskaya et al., 2005), has also been associated with lack of response to cisplatin and unfavorable outcome in gastric (Sun et al., 2014), esophageal (Kato et al., 2001), ovarian (Jiang et al., 2013) and NSCLC tumors (Wang et al., 2014a, Wang et al., 2014b). Moreover, the administration of survivin inhibitors (for example, YM155, LY2181308) have also been found to improve the outcome of several malignancies (Church and Talbot, 2012).

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Several of the abovementioned mechanisms have been found to endow CSC with the capability to evade death signals, hence compromising chemosensitivity (Fulda, 2013, Signore et al., 2013). More detailed insights on the mechanisms regulating apoptosis in CSC can be found in recent reviews (He et al., 2014, Medina et al., 2009). As recently revised by Signore et al. (2013) several strategies exploiting signaling pathways that govern self-renewal and survival of CSC are currently approved or are being tested in clinical trials. Strategies to sensitize CSC to chemotherapy include the stimulation of death receptors using Apo2L/TRAIL inducers (Plasilova et al., 2002, Ravi et al., 2004). By promoting the activation of the apoptotic extrinsic pathway, this strategy has the potential to overcome drug resistance in many tumors harboring p53-inactivating mutations (Ashkenazi et al., 2008). Several antibodies and small molecules targeting the mitochondria-associated apoptosis machinery have also been developed including the Bcl-2 inhibitor ABT-727 that was able to sensitize glioma CSC to TRAIL treatment both in vitro and in vivo (Tagscherer et al., 2008). The inhibition of EGF-R/Akt pro-survival signaling is also among the most explored strategies to indirectly induce apoptosis of CSC (Gallia et al., 2009, Signore et al., 2013). Despite the significant success presented by these strategies, it is now consensual that sub-populations of CSC with distinct genetic features may co-exist within the same tumor (He et al., 2014, Wang et al., 2014a, Wang et al., 2014b) and significant molecular variations were also observed between CSC isolated from tumors of the same organ and different organs (Frank et al., 2010). This phenotypic heterogeneity accounts for significant variations in response to targeted strategies and highlights the need for a careful evaluation of the signal transduction pathways that drive each sub-population of CSC to evade apoptosis, towards personalized cancer treatments. Furthermore, despite the encouraging reports with patient-derived xenografts capable of recapitulating CSC expression and tumor heterogeneity in immune deficient animals (Dobbin et al., 2014, Whittle et al., 2015, Williams et al., 2013), more efforts should be devoted to the development of reliable clinical models able to support the development of new anti-cancer strategies.

3. Protein glycosylation in cancer: implications for chemotherapy response and CSC targeting

3.1. Protein glycosylation: structural diversity in cancer

Glycosylation is the most frequent, complex and plastic posttranslational modification of membrane-bound and secreted proteins and results from a coordinated action of nucleotide sugar transporters and biosynthesis pathways, glycosyltransferases and glycosidases in the endoplasmic reticulum (ER) and the Golgi apparatus (GA; Pinho and Reis, 2015, Spiro, 2002). Glycans play a key role in protein folding, trafficking, stability, activity and act as mediators of cell-cell adhesion, cell differentiation, migration, modulation of cell signaling pathways, immune recognition and host-pathogen interactions (Haltiwanger and Lowe, 2004, Ohtsubo and Marth, 2006, Pinho and Reis, 2015, Shental-Bechor and Levy, 2009). Two main classes of glycans can be found at the cell-surface: (i) O-glycans, initiated in the Golgi apparatus by the initial attachment of GalNAc moieties to the hydroxyl groups of Ser or Thr residues of a given polypeptide chain (forming the Tn antigen GalNAcα-Ser/Thr, the simplest form of O-glycosylation); (ii) N-glycans, whose biosynthesis initiates in the ER by the addition of an oligosaccharide chain to an Asn residue within consensus peptide sequences of Asn-X-Ser/Thr (X denotes any amino acid except proline). Less abundant forms of protein glycosylation include O-Fucosylation, O-Glucosylation and C-mannosylation of Thr residues (Pinho and Reis, 2015, Spiro, 2002). Protein glycan chains are often branched or elongated and may

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present sialic acids in blood group related antigens or ABO(H) blood group determinants as terminal structures (Dall'Olio et al., 2012). Other modifications may include phosphorylation, O-acetylation of sialic acid and O-sulfation of galactose and N-acetylglucosamine residues, thereby increasing the structural complexity of the glycophenotype (Muthana et al., 2012). Furthermore, protein glycosylation patterns do not obey a predefined template (Lazar et al., 2011), as it is regulated at the tissue level, and promptly responds to physiological changes and cues (Ohtsubo and Marth, 2006, Palorini et al., 2013, Shirato et al., 2011, Testa et al., 2015). In fact, variations in glucose and oxygen levels, among other microenviromental stimuli and signaling molecules, have been shown to influence the transcription and activity of glycosyltransferases and glycosidases, the trafficking of these enzymes to the ER and the Golgi apparatus and the availability of sugar donors (Carvalho et al., 2010, Dall'Olio et al., 2012, Gill et al., 2013, Pinho and Reis, 2015, Shirato et al., 2011). Recent studies have challenged the classical view of protein glycosylation as an intracellular event by demonstrating that glycans may experience further structural remodeling by extracellular enzymes (Lee et al., 2014, Nasirikenari et al., 2014). This makes the glycome a highly dynamic molecular entity that mirrors a particular biological milieu and the glycomic/glycoproteomic characterization, a challenging analytical enterprise. Detailed insights on the structure of these glycans and their biosynthesis are available in several reviews and textbooks on the subject (Brockhausen et al., 2009, Stanley et al., 2009) and will not, therefore, be discussed in detail.

Malignant transformation is often accompanied by the expression of glycosylated structures that promote tumor growth, cell survival, cell-to-cell detachment, migration, immune evasion and metastasis (Fig. 5; Christiansen et al., 2014, Dall'Olio et al., 2014, Dall'Olio et al., 2012, Gomes et al., 2013a, Gomes et al., 2013b, Pinho et al., 2007, Radhakrishnan et al., 2014). In some cases, malignant tissues recapitulate the glycosylated antigens expressed during fetal life (Feizi, 1985, Hakomori, 1989, Varki et al., 2009). Perhaps the most studied cancer-associated glycans include variants resulting from a premature stop in protein O-glycosylation, namely the Tn antigen (the simplest O-glycan), its sialylated counterpart sialyl-Tn (sTn; Neu5Acα2-6GalNAcα-O-Ser/Thr) and the T or core 1 antigen that results from the addition of a Gal residue to the Tn antigen (Galβ1-3GalNAc-Ser/Thr; Cazet et al., 2010, Ju et al., 2008, Julien et al., 2012, Marcos et al., 2004). These antigens have been classically termed simple mucin-type O-glycans reflecting the abundance of O-glycosylation sites in mucins and stem from the incapability of the cellular glycosylation machinery to produce more elongated glycans. Recently, a precision mapping of the human O-GalNAc glycoproteome has revealed over 6000 glycosites in more than 600 O-glycoproteins, the majority of which were found at the cell surface (Campos et al., 2015a, Campos et al., 2015b, Schjoldager and Clausen, 2012, Steentoft et al., 2013, Steentoft et al., 2011), thereby greatly expanding the view of the O-glycoproteome and its functional role. Of note, several intracellular proteins, including cytoplasmic, mitochondrial and nuclear proteins, have also been found expressing this type of posttranslational modification (Steentoft et al., 2013). Despite the need for further analytical validation, these observations may suggest a currently neglected role for O-GalNAc glycosylation in intracellular physiological and pathological events. Similar subsequent studies have contributed to the notion that cells of different origins express a minor and unique O-glycoproteome that should be carefully explored when envisaging highly specific cancer biomarkers (Campos et al., 2015a, Campos et al., 2015b)

While hindered by extended glycosylation in healthy and benign tissues, simple mucin-type O-GalNAc glycans are uncovered in the majority of human carcinomas, particularly in advanced stages of the disease (Fig. 5; Dall'Olio et al., 2012, Freire-de-Lima, 2014, Julien et al., 2012, Marcos et al.,

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2004). The Tn, sTn and T antigens have been associated with malignant cell phenotypes, disease progression, metastasis and poor prognosis in clinical settings for a variety of different solid tumors (Cazet et al., 2010, Julien et al., 2012, Marcos et al., 2011, Reis et al., 2010). Recently, we have reported the association between sTn, high-grade bladder cancer and muscle-invasion (Bernardo et al., 2014, Ferreira et al., 2013, Lima et al., 2013) as well as the capability of sTn+-bladder cancer cells to promote a tolerogenic immune response of dendritic cells (Carrascal et al., 2014), demonstrating the involvement of glycans in the mediation of cancer immune responses. The fact that these simple glycans are absent, significantly underexpressed or restricted to some cell types in healthy tissues, renders them potential diagnostic and therapeutic targets. Proteins carrying abnormal glycosylation are often released from the cell-surface, therefore increasing sTn concentrations in the serum (CA72-4 test) of gastric, colorectal and pancreatic carcinoma patients (Carpelan-Holmström et al., 2004, Reis et al., 2010, Ychou et al., 2000) as well as in pre-cancerous gastric lesions (Gomes et al., 2013a, Gomes et al., 2013b). The elevation of CA72-4 was shown to be an independent prognostic factor in gastric cancer (Louhimo et al., 2004a, Louhimo et al., 2004b) and predictive of tumor recurrence in gastric (Ychou et al., 2000) as well as pancreatic cancers (Louhimo et al., 2004a, Louhimo et al., 2004b). Moreover, monoclonal antibodies have been developed for sTn-MUC1 glycopeptides (Sørensen et al., 2006) as well as the therapeutic vaccine Theratope that, despite promising initial results in pre-clinical and in early stage clinical trials for advanced breast tumors (Holmberg et al., 2000, Holmberg and Sandmaier, 2004), it remains to be thoroughly assessed in other solid tumors known to overexpress sTn.

Several evidences also point to an increase in the complexity of N-linked glycans and glycolipids through β-1,6 branching, mediated by β-1,6-N-acetylglucosaminyltransferase V (Mgat5) in cancer (Fig. 5; Fortuna-Costa et al., 2014, Liu et al., 2015a, Liu et al., 2015b, Pinho et al., 2013, Pinho et al., 2011, Taniguchi and Kizuka, 2015). The β-1,6-N-acetylglucosamine-branched glycans are responsible to the high-affinity binding of Galectin-3 to several membrane glycoproteins and glycolipids, with implications to angiogenesis, metastasis, and other tumor progression events (Fortuna-Costa et al., 2014, Funasaka et al., 2014). For instance, lysosomal membrane-associated glycoproteins such as (LAMPs)-1and -2, Mac-1 and Mac-3, CD-98, CD-45, and CD-7 as well as EGF, TGF-β, and VEGF are known ligands for Galectin-3. Galectin-3 also binds CEA and MUC-1, among other relevant cancer-associated proteins (Fortuna-Costa et al., 2014). Galectin-3 crosslinks cell surface glycoprotein receptors resulting in functional microdomains that regulate extracellular signal transduction (Boscher et al., 2011, Lajoie et al., 2009), glycoprotein receptor turnover and endocytosis (Lakshminarayan et al., 2014). As recently highlighted by several reports (Fortuna-Costa et al., 2014, Liu and Rabinovich, 2005, Rabinovich and Toscano, 2009) galectin-3 mediates several hallmarks of cancer, including tumor growth (Peng et al., 2008), anoikis resistance (Kim et al., 1999), inhibition of apoptosis (Takenaka et al., 2004), angiogenesis (Nangia-Makker et al., 2010), cell adhesion (Khaldoyanidi et al., 2003), cell motility (Boscher and Nabi, 2013), cell invasion (Tsuboi et al., 2007) as well as microenvironment modulation (Nangia-Makker et al., 2008, Reticker-Flynn and Bhatia, 2015). Increased β-1,6-branching of cadherins, integrins and other cytokine/growth factor receptors has also been found to enhance and promote tumor growth and metastasis (Carvalho et al., 2015, Granovsky et al., 2000, Pinho et al., 2013, Zhao et al., 2008) while the Mgat5 knockout inhibited these phenomena in vitro and in vivo in different experimental cancer models (Carvalho et al., 2015, Dall'Olio et al., 2012, Fortuna-Costa et al., 2014, Lau and Dennis, 2008).

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Two other common cancer-associated antigens are the α-2,3-sialylated forms of type 1 and type 2 Lewis blood group determinants Lea and Lex, sLea and sLex, that may be found as terminal epitopes of glycan chains in both glycoproteins and glycolipids (Carvalho et al., 2010, Dall'Olio et al., 2012, Julien et al., 2011, Kannagi, 2007, Tozawa et al., 2005). These glycans are specific ligands for E- and P-selectins in endothelial cells, two proteins that mediate leukocyte extravasation at sites of tissue injury in a sLea/sLex-dependent manner (Dall'Olio et al., 2012). Similarly, these sialylated glycans are thought to act as regulators of the metastatic cascade by promoting endothelial adhesion (Kannagi, 1997). In addition to a role in metastasis, selectin ligands are also thought to play a role in tumor growth and angiogenesis (Gomes et al., 2013a, Gomes et al., 2013b, Terraneo et al., 2013). In particular, several reports associate the overexpression of sLea with decreased overall survival in digestive tract tumors (Kannagi, 2007, Matsui et al., 2004, Portela et al., 2011). Furthermore, serological detection of sLea on glycolipids and glycoproteins by the CA19-9 assay is used as a diagnostic tool to monitor clinical response to therapy for these tumors (Humphris et al., 2012, Kannagi, 2007, Yang et al., 2013, Kim et al., 2011). Furthermore, the 2,6-oversialylation of lactosamine chains and overfucosylation of terminal motifs are also among some of the commonly observed structural alterations present in cancer cells (Christiansen et al., 2014). An association between α-2,6-sialylation and invasive growth has been suggested by several studies (Dall'Olio et al., 2012, Dall'Olio et al., 2014) and the oversialylation of β-integrins has been found to modulate intracellular signaling pathways towards increased cell survival (Dall'Olio et al., 2014). Different mechanisms may account for the above described structural changes, namely alterations in transcription of biosynthetic and degradation enzymes, derangement of secreting organelles and availability of sugar nucleotides, promoted either by microenvironmental stimuli and/or dysfunctional glycogenes (Dall'Olio et al., 2012, Itzkowitz et al., 1989, Reis et al., 2010). Altogether, abnormal glycosylation is considered a typical hallmark of the transition from healthy to neoplastic tissues with direct influence on cell behavior and clinical outcome. More detailed information about the role of glycosylation in cancer may be found in a comprehensive recent review by Pinho and Reis (2015). Since alterations in glycosylation take place at the cell-surface resulting in highly distinct protein glycovariants, these present an opportunity for targeted therapeutics. However, as highlighted by recent publications, both similarities and differences exist in the glycosylation patterns of different tumors, mirroring the dynamic nature of the glycome (Christiansen et al., 2014). Furthermore, the same glycans may present opposite biological roles in different tumors, denoting the need for a comprehensive analysis of the glycoproteome in a clinical context, towards personalized cancer treatment.

3.2. Protein glycosylation: contribution to chemoresistance

Several studies have described that subpopulations of multidrug resistant (MDR) cancer cell lines of distinct tissue origin present altered glycosylation patterns, particularly at the N-glycosylation level, when compared with the parental cell lines (Beretta et al., 2010, Kudo et al., 2007, Noda et al., 1999, Schultz et al., 2013, Zhang et al., 2012a, Zhang et al., 2012b, Zhao et al., 2014a, Zhao et al., 2014b). Alterations in glycogene expression were also reported and, in most cases, have been found in agreement with the observed glycosylation patterns (Schultz et al., 2013, Zhao et al., 2014a, Zhao et al., 2014b). In particular, some studies have further associated the overexpression of cell-surface ATP binding cassette transporters (MRP1 and MRP4) and CD147 showing abnormal N-glycosylation patterns with drug resistance (Afonso et al., 2015, Beretta et al., 2010). Furthermore, the exposure of chemoresistant cancer cells to tunicamycin, an N-glycosylation biosynthesis inhibitor, has been shown to sensitize these cells to anti-cancer agents (Kramer et al., 1995, Noda et al., 1999).

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Although these observations suggest that alterations in N-glycosylation may alter the functional properties of cell-surface transporters involved in multidrug resistance, it should be noticed that tunicamycin is responsible by inducing significant ER stress leading to apoptosis (Han et al., 2008). Several structural alterations, namely α-2,6-oversialylation of cell-surface molecules, have also been shown to significantly modulate downstream oncogenic signaling pathways towards enhanced cell survival, tumor growth and migration (reviewed in detail by Dall'Olio et al., 2012, Dall'Olio et al., 2014 and Park and Lee (2013)). Yet, the contribution of these events to chemotherapy resistance remains to be determined. Adding to these findings, several studies associated elevation of serum sTn antigen (detected by the CA72-4 test) and sLea (detected by the CA19-9 test) levels in cancer patients with both recurrence after chemotherapy, metastasis and poor overall survival (Kim et al., 2015, Yang et al., 2013, Ziske et al., 2003), suggesting that the presence of these glycans may be associated with drug resistance. However, this hypothesis warrants confirmation in future studies. Altogether, these findings support the notion that glycosylation may constitute a currently neglected posttranslational modification of proteins which may be associated with cancer chemoresistance. Given its cell-surface nature, in-depth studies of these glycans may allow the selective targeting of these aggressive malignant clones and glycosylation remodeling may constitute a novel approach to overcome chemoresistance.

4. Chemotherapy challenge and CSC selection: exploring the role of glycosylation for guided therapeutics

During the last decade, CSC populations were described in various types of solid tumors, albeit their phenotypic and functional characteristics are still under intensive investigation (Tirino et al., 2013, Visvader and Lindeman, 2008). As a result, specific functional features such as self-renewal and long-term repopulation potential were identified in CSC and these characteristics were predominantly related to tumor initiation and maintenance of tumor growth (Al-Hajj and Clarke, 2004). In addition, CSC exhibit a number of genetic and cellular adaptations that confer resistance to conventional chemotherapeutics such as relative dormancy/slow cell cycle kinetics, efficient DNA repair, overexpression of multidrug resistance efflux transporters and resistance to apoptosis (Abdullah and Chow, 2013, Cojoc et al., 2015), as outlined in detail in the previous sections. Furthermore, several reports suggest that conventional therapy often results in enrichment and maintenance of CSC pools that form the tumor after chemotherapy, triggering relapse as chemoresistant tumors. In particular, Levina et al. (2008) suggested that cisplatin-containing chemotherapy leads to propagation of human lung cancer stem cells and inhibition of cell differentiation. Moreover, these cells appear to activate an efficient cytokine network that favors their tumorigenic and metastatic potential (Levina et al., 2008). Other research groups related lung cancer recurrence with maintenance of treatment-selected CSC (Barr et al., 2013, Leung et al., 2010). For instance, using whole genome expression analysis, Hamilton and Olszewski (2013) demonstrated that small cell lung cancer (SCLC) cells express CSC markers (CD44, CD133, CD47, ALDH1A1, AKR1C), as well as WNT and Notch pathway intermediates after chemo-radiotherapy, compared to treatment-naive cells. Bertolini et al. (2009) also reported that cisplatin treatment spares highly tumorigenic CD133+ NSCLC cells that display stem-like features. Zhang et al., 2012a, Zhang et al., 2012b found similar correlations in cisplatin-resistant bladder cancer cells, which displayed enhanced self-renewal and tumorigenicity as well as higher levels of sphere formation, a larger proportion of side population cells and stem cell marker expression. Regarding ovarian

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cancer, cisplatin-based chemotherapy also appears to preserve CSC-like cells, resulting in increased tumor burden (Abubaker et al., 2013, Yasuda et al., 2013).

These findings support the notion that the combination of conventional chemotherapy directed to bulk tumor cells and targeted strategies against CSC may hold promise to improve cancer management compared to monotherapies. Therefore, over the past decade many concentrated efforts have been put on the isolation and molecular characterization of CSC using chemotherapy selection approaches. In fact, the chronic or acute exposure of tumor cell lines, xenografts and patient samples to anticancer drugs has provided a simple strategy for CSC enrichment, as recently reviewed (Freitas et al., 2014). This has allowed the establishment of CSC-biomarker panels, which include, for example, the following proteins: ALDH1, CD29, CD24, CD44, CD90, and CD133 (de Beça et al., 2013, Langan et al., 2013, Yang et al., 2014, Zöller, 2015). Nevertheless, these biomarker molecules fall short to discern cancer from normal adult stem cells, as previously reviewed in detail (Karsten and Goletz, 2013), hence prompting the introduction of more specific molecules for targeted therapeutics. Several authors now suggest that targeting oncofetal stem cell markers, normally not present in adult stem cells, may constitute a novel and more effective treatment strategy.

Exploring cancer-associated glycans, which are often of oncofetal nature, presents a unique and highly specific opportunity to target CSC. In fact, several studies reported alterations in the glycopatterns of CSC of different tissue origins compared to other cancer cells. Many of these findings concern the overexpression of glycans commonly found in glycolipids (CD60a, CD77, GD2, Gb4) and proteins (CD147 or Ley, CD15 or Lex) (Karsten and Goletz, 2013). More recently cumulative evidences confirm that CSC resistant to gemcitabine (Terao et al., 2015) and doxorubicin (Azuma et al., 2014) present distinct glycosylation patterns when compared to drug sensitive parental cell lines. Namely, Terao et al. (2015) described that CSC derived from the gemcitabine chemoresistant pancreatic cancer cell line Panc1 display significant overfucosylation and upregulation of fucosyltransferases, GDP-fucose synthetic enzymes, and GDP-fucose transporters. However, knockdown of GDP-fucose transporters did not improve gemcitabine response, suggesting that overfucosylation is a result of CSC transformation with little influence on chemoresistance. On the other hand, Azuma et al. (2014) explored the oversialylation of doxorubicin-resistant hepatocellular CSC towards the identification of highly specific glycoprotein species. These approaches have contributed to highlight the importance of an in-depth assessment of the glycome and glycoproteome of different CSC populations towards more specific biomarkers.

In this review, we emphasize the growing amount of evidence supporting an association between the protein-specific cancer-associated O-glycans sTn and T and CSC proteins (Fig. 6). The sTn and sialylated T antigens, which result from a premature arrest in protein glycosylation (as addressed in detail in the previous section), are absent from the majority of healthy tissues, but expressed in several solid tumors (Julien et al., 2012, Marcos et al., 2011, Videira et al., 2009). Furthermore, many studies report an increased expression of these proteins in advanced stages of the disease (Julien et al., 2012). Perhaps the most studied is the sTn antigen (CD72-4), whose presence in adult healthy tissues has been highly restricted to specific cells (Ferreira et al., 2006, Julien et al., 2012, Marcos et al., 2011). In contrast, the sTn antigen has been found overexpressed in many advanced stage solid tumors, where it plays a key role in endowing cancer cells with a metastatic potential; furthermore it is also present in the metastatic lesions (Ferreira et al., 2006, Julien et al., 2006, Marcos et al., 2011, Pinho et al., 2007). Although more evidence are required, these findings suggest that sTn-

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expressing cells may be capable of detaching from the primary tumor and colonize distant tissue locations. Moreover, the sTn antigen was also detected in several fetal organs of both genders (e.g., esophagus, stomach, pancreas, colon, lung, mammary gland, gonadal tissue) (Itzkowitz et al., 1989, Julien et al., 2012, Pistolesi et al., 2001, Stanick et al., 1988, Thor et al., 1986). Although little is known about the biological role of sTn during embryonic development, these evidences suggest that cancer cells undergo, to some extent, similar molecular events. In spite of the possibility of sTn being expressed by all proteins presenting O-glycosylation sites, as demonstrated by several studies using engineered cancer cells to express this antigen (Campos et al., 2015a, Campos et al., 2015b, Campos et al., 2015b, Julien et al., 2012), few proteins have emerged from models that naturally express the antigen. Among these proteins are the heavily glycosylated MUC1 (Beatson et al., 2015, Lakshminarayanan et al., 2012) and CD44 (Campos et al., 2015a, Campos et al., 2015b, Carrascal et al., 2014, Irimura et al., 1999, Marcos et al., 2011), whose overexpression has been widely observed in CSC of different tumor models (Bourguignon et al., 2012, Bourguignon et al., 2014, Curry et al., 2013, Nath and Mukherjee, 2014). Likewise, Karsten and Goletz (2013) discussed a similar role for the T antigen (CD176) in their recent review on this topic). Accordingly, this oncofetal antigen has been found in primary tumors and metastases, and detected in CD44 (Lin et al., 2011, Singh et al., 2001), CD45 (Baba et al., 2007), and CD34 (Cao et al., 2008) of CSC from different cancer models.

In summary, while still circumstantial, there is a strong suggestion that the expression of short-chain O-glycans is associated with CSC phenotypes. Furthermore, currently there are no direct links between cancer-associated O-glycans and chemoresistance. We therefore advocate that a careful investigation of the glycoproteome of CSC focusing on the expression of these fetal glycans may provide more specific biomarkers than the currently available panels and provide insights to the possible role of O-glycans in chemoresistance.

5. Concluding remarks

Cancer cells show remarkable capability to resist chemotherapy treatments due to the synergistic effect of intrinsic, acquired as well as microenvironmental factors. Moreover, chemotherapy frequently acts as a positive selective pressure for the emergence of more aggressive cell subpopulations such as CSC, which are responsible for promoting disease relapse and progression. While many molecular mechanisms linking chemoresistance and CSC were already identified, CSC targeting remains a challenge, mostly due to the molecular similarities of CSC to non-malignant stem cells. We now comprehensively propose, for the first time, that alterations in protein glycosylation are amongst the key events accompanying and possibly driving chemoresistance. In fact, chemoresistant cancer cells supported by favorable microenvironments and endowed with intracellular mechanisms to evade cell death, present distinct N- and O-glycosylation patterns when compared to other cancer and non-malignant cells. Moreover, many studies have brought glycosylation into the spotlight by demonstrating that selective inhibition of N-glycosylation pathways may constitute an important therapeutic strategy to overcome chemoresistance. However, we argue that the influence of glycosylation extends beyond these events, given that it plays a pivotal role in the activation of several oncogenic pathways that sustain chemoresistance. Despite these insights, a direct link between protein glycosylation and chemoresistance remains to be established. Furthermore, the specific structural nature of CSC-associated glycans and glycoproteins, envisaging highly specific biomarkers has not yet been fully disclosed. Nevertheless,

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the context-dependent nature of glycans offers tremendous potential for the identification of unique CSC-glycoproteomes and clinically valuable biomarkers for cancer detection and targeted therapeutics.

As cancer treatment is shifting towards precision medicine and patient-tailored strategies (Kohane, 2015), we emphasize the need to integrate the patients' background, clinical history, tumor molecular features and the microenvironment in a single holistic perspective. Furthermore, we conclude that it will be imperative to address chemoresistance as a multifactorial and dynamic feature, considering many of the aspects discussed in the current review. Therefore, we come forward with a novel model (Fig. 7) that incorporates evaluation of glycomics and glycoproteomics in comprehensive pan-omics approaches envisaging more accurate patient stratification, therapy selection, decision and design. Finally, we highlight that effective cancer treatments will likely benefit from multitargeted approaches selected based on a profound knowledge of the molecular nature of the tumor and patient's physiological and pathological status. The choice of treatment schemes and anti-cancer drugs should be dynamic and should Saccount for individual patient's response to treatment towards true precision medicine.

6. Future perspectives

This review has highlighted the lack of specific biomarkers to target CSC and the necessity of more effective multi-target anticancer therapeutics to address chemoresistance. We believe that glycoproteins carrying oncofetal glycans, such as the sTn and Tn antigens, and other cancer-associated carbohydrates may help paving the way towards more specific targeted therapy. Focus should also be put on a careful and comprehensive glycoproteomic evaluation of chemoresistant clones and their microenvironmental context. These studies will greatly benefit from the tremendous advances, over the past two decades, in the development of highly sensitive mass-spectrometry platforms and protocols specifically designed to address the complex structural nature of protein glycosylation (Almeida et al., 2013, Zhang et al., 2013). Moreover, while tumor cell lines are important starting models for proof-of-concept studies, it will be important to expand studies towards models able to more accurately mimic the tumor microenvironment. Future approaches should include direct human cancer xenografts in animal models, the so-called avatar models, which have been shown to preserve the molecular features of human tumors, including tumor glycosylation (Aparicio et al., 2015, Bernardo et al., 2014, Hidalgo et al., 2014). New bioengineering approaches such as 3D Lab-on-a-chip settings may also provide the necessary system complexity to address drug toxicity in cancer cells, as demonstrated in several recent publications (Kim et al., 2015, Ruppen et al., 2014, Su et al., 2014).

As already mentioned, true improvements in patient care and chemoresistance require the introduction of multi-target patient-tailored therapeutics. In this context, altered protein glycosylation may constitute the necessary mean to selectively deliver emerging nanotherapeutic agents to cancer cells, thereby improving the efficacy and reducing the toxicity of the treatments (Fernandes et al., 2015, Livney and Assaraf, 2013). These nanodelivery sytems may include conventional chemotherapy drugs, genetic-based strategies such as the siRNAs against key oncogenic proteins involved in chemoresistance (Ganesh et al., 2013, Navarro et al., 2012, QingShuo et al., 2013) or the administration of genes capable of rescuing the expression of p53 and other cell cycle checkpoint proteins (Chen et al., 2014, Kamal et al., 2014, Kim et al., 2014, Li et al.,

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2013a, Li et al., 2013b). The introduction of antibodies against cancer-associated glycoepitopes may also allow the selective inhibition of oncogenic pathways or the stimulation of immune responses against malignant cells (Azevedo et al., 2015). Glycan-based therapeutic solutions should also be tested, for the first time, in the context of hematological malignancies that also pose significant chemoresistance issues (Assaraf et al., 2014, Assaraf, 2007, Gonen and Assaraf, 2012). Nevertheless, the particular differences between blood and epithelial cells should be taken into consideration when designing such strategies.

In summary, exploiting altered glycosylation for targeted therapeutics is an emerging research topic that holds great potential for overcoming cancer chemoresistance phenomena. However, the roadmap for prototyping glycan-based therapeutics still requires a careful planning addressing the following research topics: (i) comprehensive characterization of glycomic and glycoproteomic chemoresistant cells; (ii) understanding the biological significance of altered glycosylation in chemoresistance; (iii) development of glycan-specific ligands; and (iv) establishment of cancer animal models mimicking human glycosylation.

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FIGURES

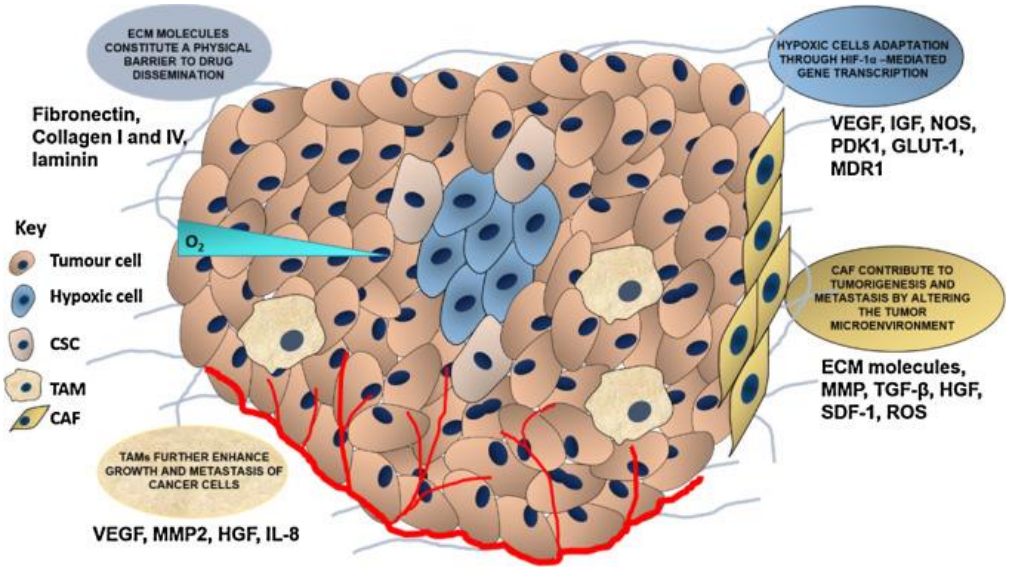

Fig. 1. The microenvironment of solid tumors actively modulates cell metabolism, gene expression and chemotherapy response. Solid tumors frequently present inefficient vasculature, resulting in the delivery of suboptimal concentrations of cytotoxic drugs and highly hypoxic tumor cores. Stromal cells and the ECM strongly mediate tumor growth and stress response, and it constitutes a physical barrier for drug delivery.

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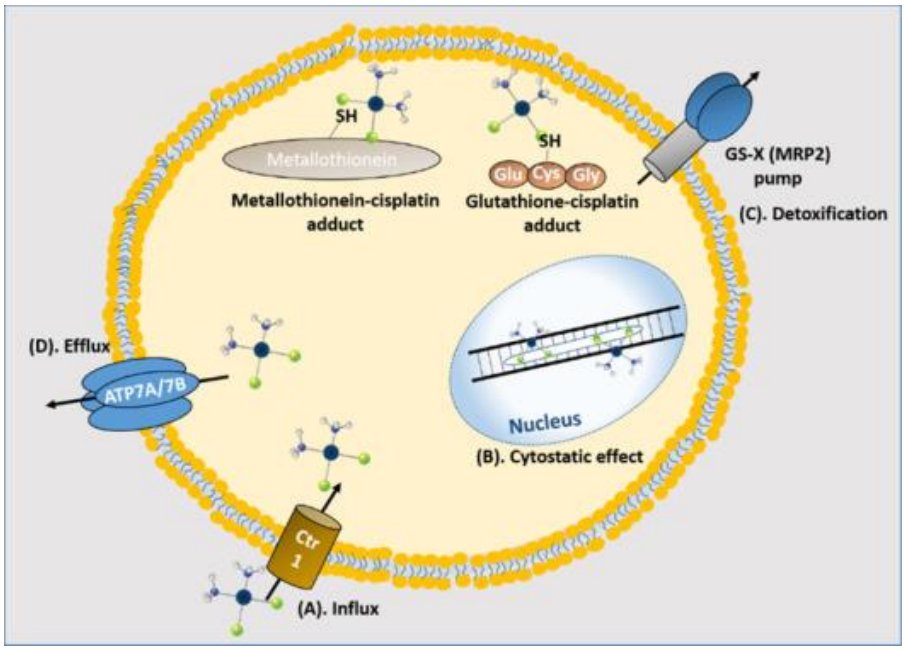

Fig. 2. Intracellular cisplatin concentration depends on several detoxification mechanisms. (A). Cisplatin uptake is mainly mediated by high affinity copper transporter protein 1(Ctr1). Once inside the cell, cisplatin has a number of possible targets: DNA; RNA; sulfur-containing enzymes such as metallothionein and glutathione; as well as mitochondria. (B). The alkylating capability of cisplatin enables the formation of adducts with N7 atoms of DNA purine nucleobases, resulting in inter- and intra-strand DNA cross-links that provoke cell cycle arrest. (C). Glutathione-cisplatin and metallothionein-cisplatin conjugates are excreted by MRPs, hence decreasing intracellular cisplatin accumulation. (D). The efflux of platinum-based drugs is mainly mediated by Transporting P-type Adenosine Triphosphatases ATP7A and ATP7B.

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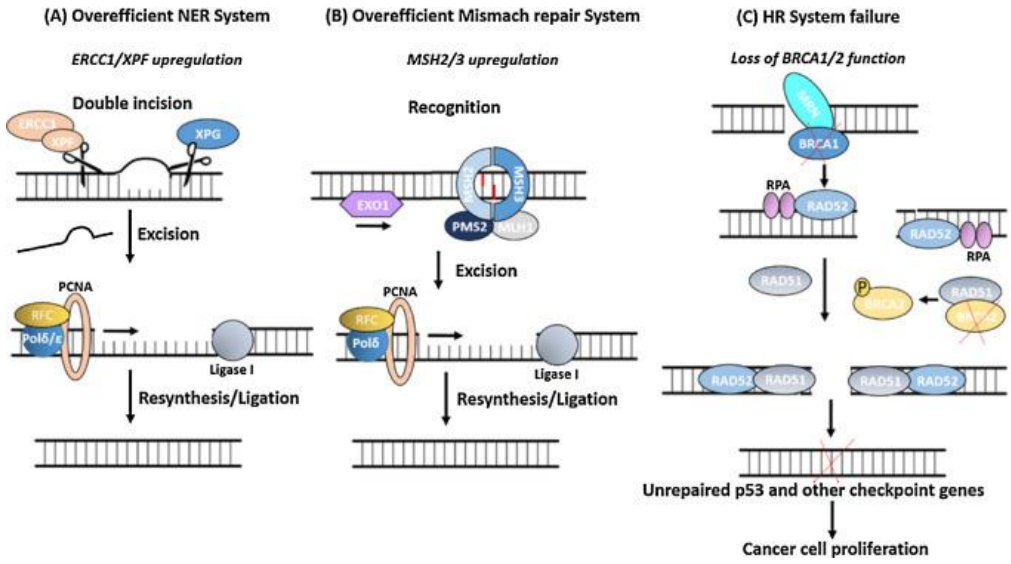

Fig. 3. Alterations of DNA repair mechanisms mediate platinum-resistance. (A) Upregulation of XPF-ERCC1 complex results in resistance to platinum-based regimens by promoting NER over-efficiency. Both XPG and XPF-ERCC1 are specific for junctions between single- and double-stranded DNA. XPG, which is closely related to the FEN-1 nuclease that participates in base excision repair (BER), cuts on the 3′ side of such a junction, while ERCC1/XPF (a heterodimeric protein complex) cuts on the 5′ side. The cut made by XPG is 2–8 nucleotides from the lesion, and the cut made by ERCC1/XPF is 15–24 nucleotides away. Next, the replicative gap-repair proteins, RFC, PCNA, and DNA polymerase delta or epsilon, bind to the 3′ -OH generated by the ERCC1-XPF cut, and they carry out new DNA synthesis that fills the gap. The final nick is sealed by DNA ligase I. (B) Overexpression of MSH DNA-repair proteins leads to cisplatin chemoresistance. The DNA mismatch-repair system includes MSH2, MSH3/6, MLH1 and PMS2. MSH2–MSH3 heterodimers bind to single base-pair mismatches; then heterodimers such as MLH1–MLH3, and PMS2, as well as EXO1, are recruited to this complex. Then, the replicative gap-repair proteins and Ligase I fill and seal the gap, as previously described (Clodfelter et al., 2005, Takahashi et al., 2011). (C) BRCA1/2 deficiency is associated with platinum resistance. After DNA damage is introduced, the MRN complex processes the ends of the DSBs. BRCA1 is phosphorylated by ATM and CHK2 (not shown) and regulates the MRN complex. RPA then associates with the 3′ ssDNA overhangs and becomes phosphorylated. Rad52 binds RPA and displaces it to allow for Rad51 binding. BRCA2 binds to Rad51 until BRCA2 becomes phosphorylated, releasing Rad51 and allowing it to localize to the DSB with Rad52. Rad51 then forms a nucleoprotein filament that invades a homologous sequence and activates strand exchange to generate a crossover between the juxtaposed DNA (not shown). BRCA. BReast CAncer genes; EXO1. Exonuclease 1; MLH. MutL homologues; MRN complex. This protein complex consists of Mre11, Rad50 andNbs1 proteins; MSH. MutS protein homolog; PCNA. Proliferating-cell nuclear antigen; PMS2. Post-meiotic segregation 2; Pol δ/ε. DNA polymerase δ/ε; RAD. Checkpoint protein; RFC. Replication factor C; RPA. Replication protein A.

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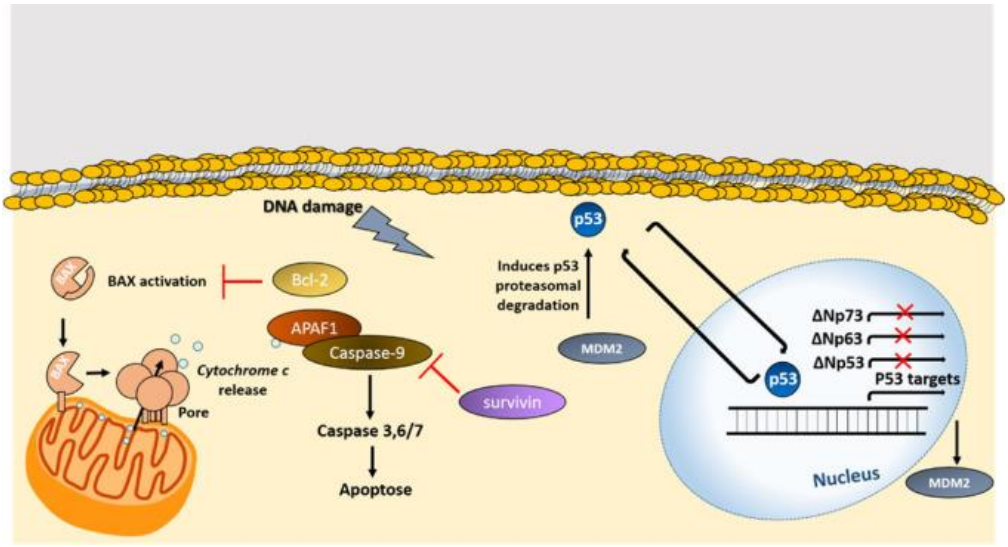

Fig. 4. Anti-apoptotic members of the Bcl-2 family such as Bcl-2, Bcl-XL, and Bcl-w regulate the susceptibility to apoptosis through the intrinsic pathway. (A) BAX (B-cell lymphoma 2 (BCL-2)-associated protein X) is sequestered in an inactive state at the outer mitochondrial membrane by binding to members of the anti-apoptotic Bcl-2 family. Upon various stimuli, anti-apoptotic BCL-2 family members are displaced from BAX and lead to the release of cytochrome c from mitochondria. After being released, cytochrome c binds the apoptotic peptidase activating factor 1 (APAF1), which forms a complex with caspase-9. The complex activates additional caspases leading to cellular death. (B) DeltaN isoforms of p53, p63 and p73 may act as dominant inhibitors of the full-length forms, hence impairing the activation of target genes. MDM2 is a major cellular antagonist of p53 and interferes with the tumor suppressor function of p53.

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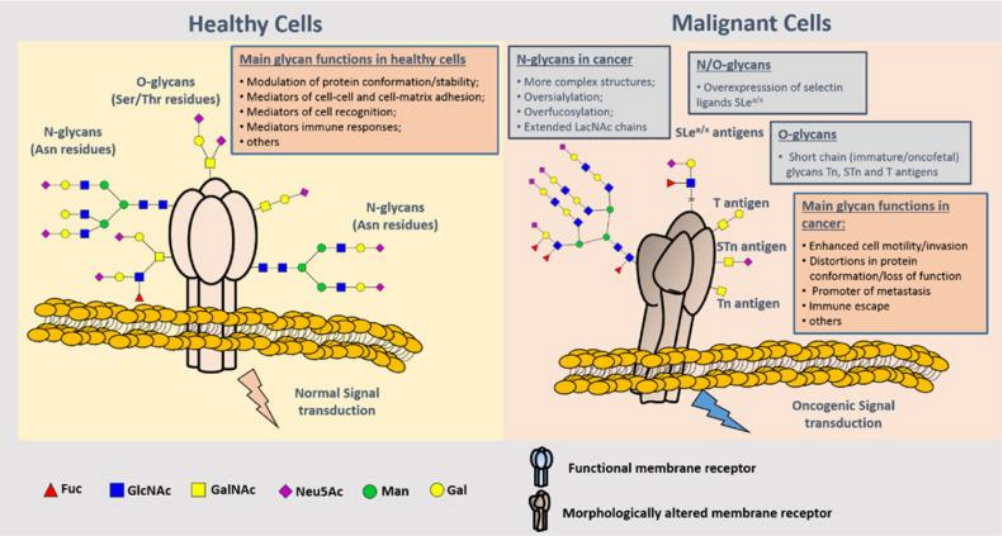

Fig. 5. (A) Representation of protein N- an O-glycosylation in healthy tissues and malignant cells. Protein glycosylation plays a key role in the definition of protein folding and physiological functioning. Glycans contribute to cell-cell and cell-extracellular matrix adhesion, immune cell recognition, among other key biologic processes. Glycosylation is a highly dynamic posttranslational modification resulting from the concerted and highly regulated action of several glycosyltransferases in secretory organelles that rapidly changes in response to physiological stimuli. Several N- (Asn residues) and O- (Ser/Thr residues) glycans may coexist in the same protein backbone, depending on available glycosites and conformational constrains. In comparison to healthy cells, malignant cells tend to present more complex and branched oversialylated and/or fucosylated N-glycans. N-glycans may also be more extended by LacNAc chains. Conversely, more malignant clones present less complex and immature O-glycans, namely the Tn, sTn and T antigens that may be also found in oncofetal tissues. Some cancer cells may also express selectin ligands sLea/x as terminal structures of both N- and O-glycans. These structural alterations at the cell-surface favor more motile and plastic cell phenotypes, invasion, lymphatic and hematogenous dissemination and immune evasion. By impairing normal functions of cell-surface receptors, cancer-associated glycans also interfere with normal intracellular signaling transduction pathways towards the activation of oncogenic features.

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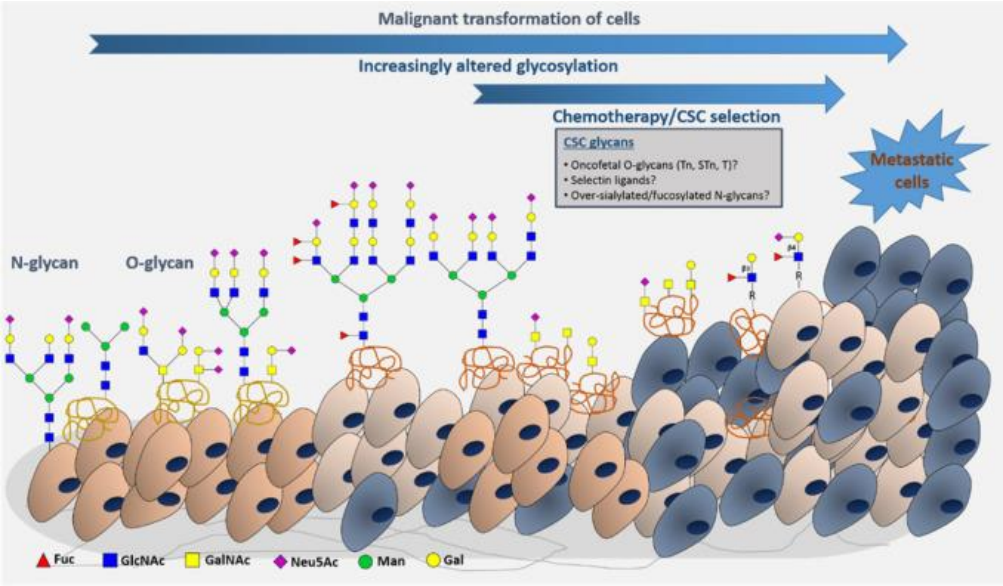

Fig. 6. Representation of CSC selection based on chemotherapy with emphasis on cell-surface glycosylation patterns. It has been demonstrated that chemotherapy, while effective against the tumor bulk may promote the selection/development of highly malignant phenotypes, including CSC, responsible for the recapitulation of tumor heterogeneity and ultimately lead to disease dissemination. Increasing evidences support that CSC endowed with chemoresistant properties present distinct proteomes in comparison to more predominant cell populations. Fig. 6 illustrates different cancer cells with distinct glycosylation patterns, including oversialylation and fucosylation of both N- and O-glycans. The selective pressure of chemotherapy towards CSC phenotypes (in blue) and the differentiated nature of CSC glycosylation is also illustrated. The overexpression of oncofetal immature O-glycans (Tn, STn, T) and alterations in terminal motifs, namely the overrepresentation of selectin ligands, are amongst the emphasized alterations. A careful glycomic and glycoproteomic evaluation of CSC stemming from chemotherapy challenge may provide unique biomarkers and templates for future drug design.(For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

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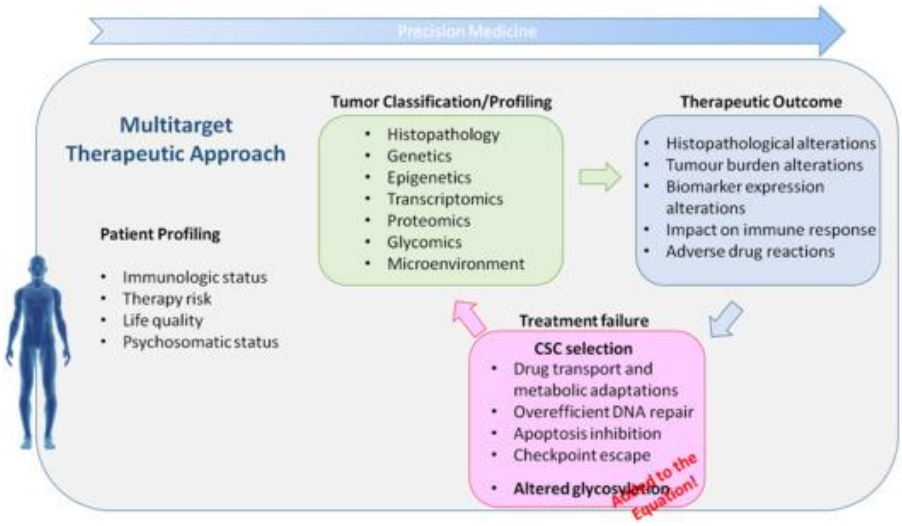

Fig. 7. The concept of precision medicine: adding CSC selection to the equation. Effective cancer therapeutics should be based on a dynamic and adaptive decision-making process relying on patient's profile, tumor classification and patient's therapeutic outcome towards individualized treatments and improved responses. This process should include therapeutics specifically designed and targeted to CSC, which should allow the overcoming of chemoresistance mechanisms. Altered protein glycosylation may hold potential for novel targeted therapeutics approaches.

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