Increased expression of the pathological *O*-glycosylated form of oncofetal fibronectin in the multidrug resistance phenotype of cancer cells

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Abstract

Changes in protein glycosylation are a hallmark of transformed cells and modulate numerous phenomena associated with cancer progression, such as the acquisition of multidrug resistance (MDR) phenotype. Different families of glycosyltransferases and their products have already been described as possible modulators of the MDR phenotype. Among the glycosyltransferases intensively studied in cancer research, UDP-*N*-acetyl-D-galactosamine:polypeptide *N*-acetylgalactosaminyltransferase-6 (pp-GalNAc-T6), which is widely expressed in many organs and tissues, stands out. Its influence in several events associated with kidney, oral, pancreatic, renal, lung, gastric and breast cancer progression has already been described. However, its participation in the MDR phenotype has never been studied. Here, we demonstrate that human breast adenocarcinoma MCF-7 MDR cell lines, generated by chronic exposure to doxorubicin, in addition to exhibiting increased expression of proteins belonging to the ABC superfamily (ABCC1 and ABCG2), and anti-apoptotic proteins (Blcl-2 and Bcl-xL), also present high expression of pp-GalNAc-T6, the enzyme currently proposed as the main responsible for the biosynthesis of oncofetal fibronectin (onf-FN), a major extracellular matrix component expressed by cancer cells and embryonic tissues, but absent in healthy cells. Our results show that onf-FN, which is generated by the addition of a GalNAc unit at a specific threonine residue inside the type III homology connective segment (IIICS) domain of FN, is strongly upregulated during the acquisition of the MDR phenotype. Also, the silencing of pp-GalNAc-T6, not only compromises the expression of the oncofetal glycoprotein, but also made the MDR cells more sensitive to all anticancer drugs tested, partially reversing the MDR phenotype. Taken together, our results demonstrate for the first time the upregulation of the *O*-glycosylated oncofetal fibronectin, as well as the direct participation of pp-GalNAc-T6 during the acquisition of a MDR phenotype in a breast cancer model, giving credence to the hypothesis that in transformed cells, glycosyltransferases and/or their products, such as unusual extracellular matrix glycoproteins can be used as potential therapeutic targets for the treatment of cancer.

Introduction

The development and/ or acquisition of resistance to anticancer drugs by neoplastic cells can be established through different mechanisms [1]. Examples include: modulation in the expression of detoxification mechanisms [2], variations of drug targets [3], increased expression of ATP-binding cassette proteins [4], reduced responsiveness to cell death [5], enhanced DNA repair mechanisms [6,7], and altered

proliferation of transformed cells [8]. Recent finds have demonstrated however, that changes in both expression of extracellular matrix (ECM) components [9,10] and tumor-associated carbohydrate antigens (TACAs) might also facilitate the emergence of chemoresistance in cancer [11]. Along the last ten years, it has been well accepted that the remodeling of ECM components is a hallmark of transformed cells in the tumor microenvironment [10,12]. A very well documented example is the increase in the expression of fibronectin (FN), a high molecular weight glycoprotein, carrying *O*- and *N*-linked glycans in its structure [13,14]. Several papers have evinced that the increased expression of FN in cancer cells promotes proliferation, survival, migration and invasion and resistance to radiotherapy and chemotherapy [15]. However, two important points should be addressed in this context: (i) although there are more than 20 isoforms of FN, most of the studies were performed with the plasma form, which is structurally different of the cellular one [16], and (ii) so far, there is no information about the glycophenotipic characteristics of the cellular FN expressed by multidrug resistant (MDR) cancer cells.

Although recent findings have shown the existence of a direct relationship between chemoresistance acquisition and modifications on the expression of glycoconjugates in cancer cells [17,18], new studies are still needed to better understand how such glycophenotypic alterations may orchestrate the forthcoming of the MDR phenotype. Many enzymes involved in the biosynthesis of cellular glycoconjugates have been studied and identified as promising targets for fighting cancer. Among these, UDP-*N*-acetyl-D-galactosamine: polypeptide *N*-acetylgalactosaminyltransferase 6 (ppGalNAc-T6) stands out, as this glycosyltransferase is capable of modulating several events associated with numerous types of cancer, especially breast cancer [19–25]. Previous works published by ours and others' research groups have pointed that both pp-GalNAc-T6 and UDP-*N*-acetyl-D-galactosamine: polypeptide *N*-acetylgalactosaminyltransferase 3 (pp-GalNAc-T3) may participate in the biosynthesis of an atypical FN isoform, called *O*-glycosylated oncofetal FN (onf-FN) [26–31]. The glycoprotein receives this name for being expressed by both transformed cells and embryonic tissues. However, its expression is absent in healthy adult tissues [32]. Previous studies initiated by Hakomori's group, and continued in our laboratory, revealed that onf-FN modulates events associated with epithelial-to-mesenchymal transition (EMT) process [26,28,29], which has recently been shown to influence the emergence and/or maintenance of drug resistance phenotypes in several types of cancer [33]. However, the expression and/or participation of onf-FN, as well as that of pp-GalNAc-T3 and pp-GalNAc-T6 have never been studied in the context of MDR phenotype in cancer cells. Both glycosyltransferases have been studied in many types of cancer. Examples include pancreatic, ovarian, lung, hepatic and renal cancer [31,34-42]. Unlike pp-GalNAc-T6's, pp-GalNAc-T3's expression and role have been little studied in breast cancer models. In a previous work published in 2007, it was demonstrated that pp-GalNAc-T3 is not detectable in normal/benign breast epithelium, but is expressed in all analyzed malignant cells, including MCF-7 [20], which is known to exhibit an epithelial phenotype [43], and has been used to study both MDR and EMT-related events induced by different stimulus, including anti-cancer drugs, such as doxorubicin (DOX) [44,45]. Based on this set of information, we decided to investigate the expression of both pp-GalNAc-T3 and pp-GalNAc-T6 in breast cancer cell lines, and their possible participation in the onf-FN appearance after acquisition of the MDR phenotype. Here, we demonstrated that DOX treated MCF-7 cells have augmented expression of pp-GalNAc-T6, resulting in high onf-FN levels. These data are corroborated by the knockdown of this glycosyltransfease not only reducing onf-FN expression but also reversing the MDR phenotype, observed by the increased sensitivity to different classes of anti-cancer drugs. On the other hand, pp-GalNAc-T3 showed reduced expression in treated MCF-7 cell line variants, and did not contribute for MDR phenotype in our model. So far, our findings show for the first time, that there is a direct participation of pp-GalNAc-T6 and/or onf-FN in the acquisition and/or maintenance of MDR phenotype, suggesting that they can act as both markers and possible therapeutic targets when dealing with malignant tumors unresponsive to chemotherapeutic agents.

Results

Analysis of the toxic effect of DOX on the MCF-7 cell line and the development of chemoresistant variants

Initially, we evaluated the cytotoxic effect of DOX in MCF-7 cells through MTT assay, determining the IC50 of the drug at 152.7 nM (Fig. 1). From this experiment, we selected the non-lethal DOX concentrations (5, 10, 20 and 40 nM) to induce the emergence of resistance through chronic exposure to the drug. The choice of using low concentrations of DOX was made based on the fact that in clinic, some cancer cells can be exposed to non-toxic drug concentrations due to inefficient drug delivery, which is a result, in part, of tumor aberrant vasculature [46]. The results showed that after six months of exposure to DOX, especially at 40 nM, the IC50

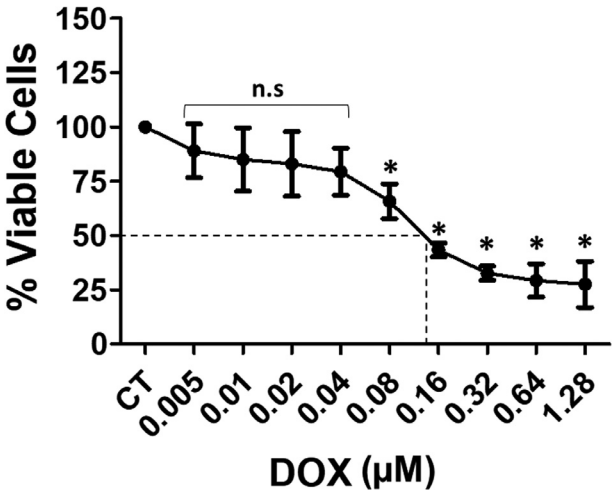

Fig. 1. Evaluation of the toxic effect of doxorubicin on MCF-7 cells. MCF-7 cells were treated with increasing (0.005−1.28 μM) doses of DOX during 48 h. After treatment, cell viability was monitored by MTT assay, allowing us to obtain the IC50 at 152.7 nM. Results are shown as percentage of control ± SD. n = 3; * $p \leq 0.05$ vs. CT (MCF-7 parental cells). n.s. not significant.

presented was higher, not only for DOX (Fig. 2A), but also to other chemotherapeutic agents, such as vincristine (VIN) (Fig. 2B) and cisplatin (CIS) (Fig. 2C), consistent with the emergence of MDR cancer cells. The MDR phenotype adopted by the MCF-7 DOX 40 cell line was monitored through MTT assays carried out using both parental and chemoresistant cells by using different concentrations of DOX (0.005−1.28 μM) (Supp. Fig. 1A, B), VIN (0.125 - 32 μM) (Supp. Fig. 1C, D) and CIS (1.56−400 μM) (Supp. Fig. 1E, F).

Monitoring the acquisition of the multidrug resistance phenotype

It has been well described that the MDR phenotype is established through the modulation of molecular mechanisms that allow cancer cells to evade toxic effects of therapeutic agents [47]. In this way, protein expression related to ABC transporters (ABCG2, ABCC1 and ABCB1), as well as proteins involved in the apoptotic pathway (Bcl-2 and Bcl-xL) were evaluated by Western blot (WB) (Fig. 3) and RT-qPCR (Supp. Fig. 2). The results demonstrated that the expression of ABCG2 (Fig. 3A, B) and ABCC1 (Fig. 3A, C) were upregulated in a dose-dependent fashion in chronically treated cells with DOX, especially at 20 and 40 nM. Regarding ABCB1, no change in protein expression was observed (Fig. 3A, D). Analysis of Bcl-xL (Fig. 3E, F) and Bcl-2 (Fig. 3E, G), also revealed increased protein expression for both anti-apoptotic markers. In addition, results obtained by RT-qPCR corroborated the WB assay for the efflux pumps and anti-apoptotic proteins (Supp. Fig. 2A−E).

Cell glycophenotype analysis

As expected, and corroborating previous published works [18,48-50], the acquisition of resistance promoted significant changes in cell glycophenotype. Those changes were detected through the use of lectins presenting distinct saccharide specificity (Supp. Table 1). Interestingly, we observed that the glycophenotype adopted by MCF-7 cells chronically treated with DOX was similar to the glycophenotype of other resistant cell lines with distinct chemotherapeutic drugs and protocols previously established in our laboratory [50,51]. The changes were more evident in cells chronically treated with DOX 40 nM (Fig. 4A−H). For the lower concentrations (5, 10 and 20 nM), the variations were not as impactful (data not shown). Among the glycophenotypic changes, it was observed high expression of the Tn antigen in MCF-7 DOX 40 cell line (Fig. 4A-B), which can be recognized by both HPA and VVL lectins [52,53]. Pioneering studies carried out in both Hakomori's and Clausen's labs, have demonstrated the appearance of the Tn antigen in the IIICS-FN domain, as the minimal saccharide epitope recognized by the monoclonal antibody (mAb) FDC-6, specific for the *O*-glycosylated onf-FN [32]. After its discovery in the 1980s, *O*-glycosylated onf-FN, initially described in cancer cells and embryonic tissues, remained for over twenty-five years being regarded solely as a glycobiomarker [32,54-56]. Over the past ten years, we have studied the pro-carcinogenic role of onf-FN, demonstrating that this atypical glycoprotein is capable of activating molecular pathways associated with EMT process [26,28-30], an event deeply linked to cancer metastasis [57,58], as well as the acquisition of drug resistance phenotype [17,59,60]. More recently, we have also demonstrated that this unusual glycoprotein is expressed in alternatively activated macrophages (MΦ) [61].

Analysis of fibronectin isoforms in MDR breast cancer cell variants

The increased expression of the Tn antigen, stimulated us to investigate the possible appearance of onf-FN in cells chronically treated with DOX. WB analysis revealed that cells exposed to 10, 20 and 40 nM of the drug, showed high expression for total FN, detected with the commercial mAb, clone EP5, which reacts with all FN isoforms [28] (Fig. 5A, B). Similarly, the chronic treatment with DOX, especially at concentrations of 20 and 40 nM, promoted the upregulation of onf-FN, which was monitored by the labeling with FDC-6 mAb (Fig. 5A, C). RT-qPCR experiments confirmed the WB results, with higher

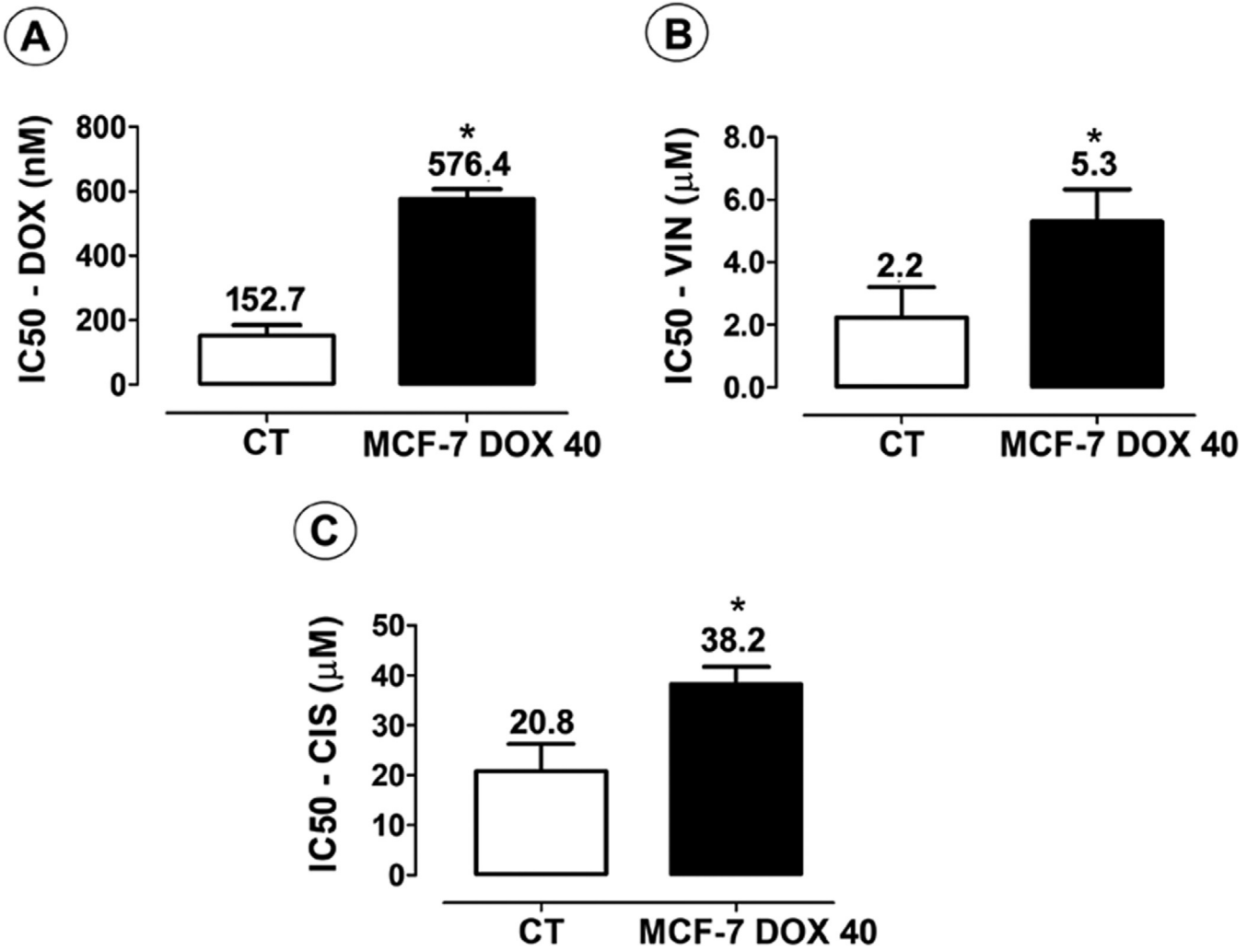

Fig. 2. IC50 values for doxorubicin, cisplatin and vincristine in MDR MCF-7 cells. After 6 months of treatment with DOX, the emergence of the MDR phenotype was monitored by investigating the effect of DOX (A), VIN (B) and CIS (C) on the viability of chronically treated cells. MTT assays were performed to monitor cell viability, and determination of IC50 for each tested drug. Results are shown as $\pm$ SEM. $n = 3$; * $p \leq 0.05$ vs. CT (MCF-7 parental cells).

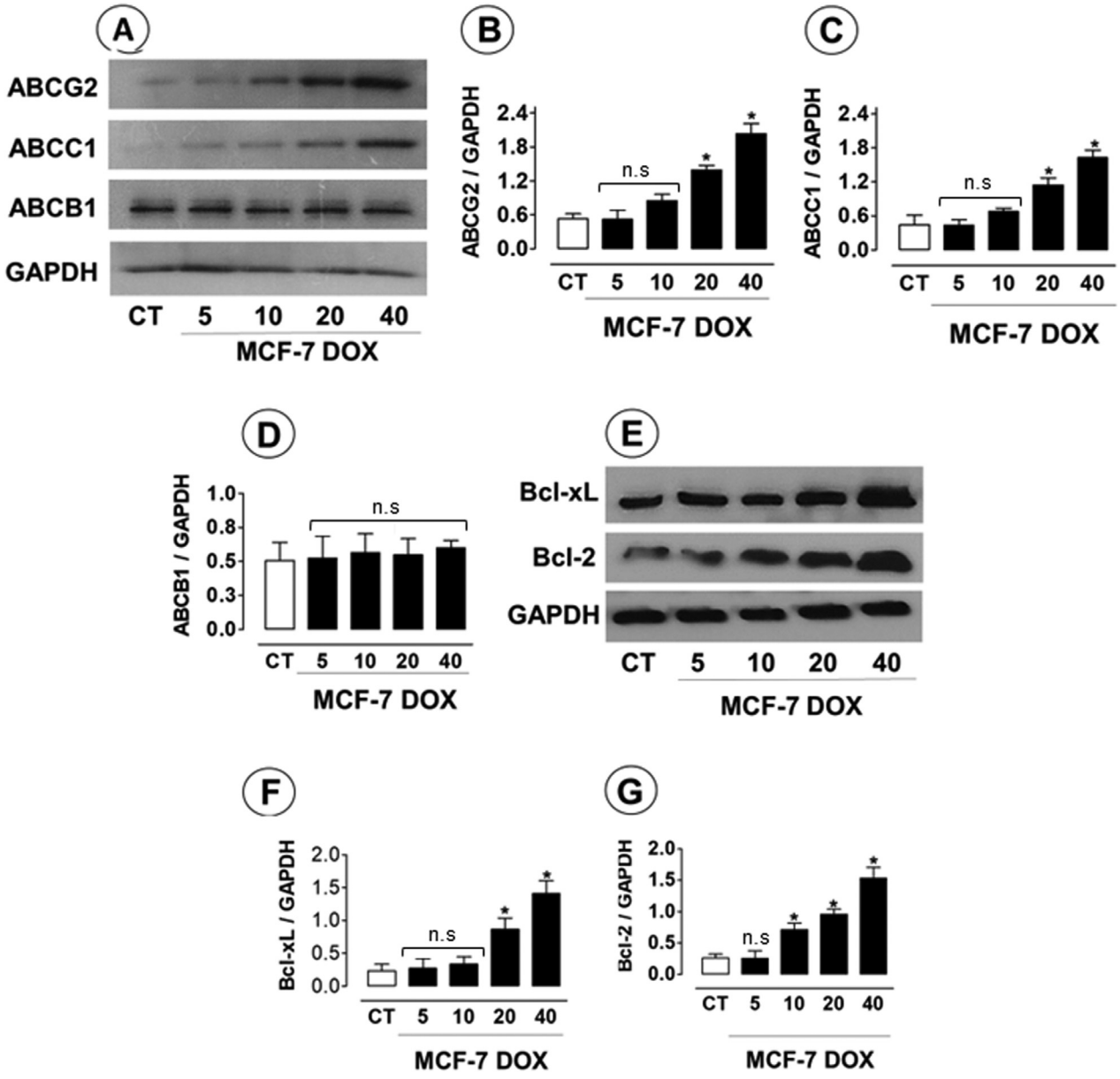

Fig. 3. Analysis of the multidrug resistance phenotype. In order to validate the acquisition of the MDR phenotype, parental MCF-7 cells, as well as cells chronically treated with DOX (MCF-7 DOX 5, 10, 20 and 40), were cultured in six-well plates, and after 48 h, the monolayers were lysed as described in the Materials and Methods, and analyzed by WB. The expression of ABC superfamily proteins (A-D), as well as anti-apoptotic proteins (E-G) were evaluated. Images are representative of three independent experiments ± SD. *$p \leq 0.05$ vs. CT (MCF-7 parental cells). n.s. not significant.

mRNA levels being observed for both total FN (Fig. 5D) and IIICS-FN (Fig. 5E), which carry the hexapeptide VTHPGY, which can be *O*-glycosylated on its threonine residue by a specific GalNAc-T, creating the oncofetal epitope required for the FDC-6 binding [28]. In addition, the acquisition of chemoresistance was accompanied by increased mRNA levels for pp-GalNAc-T6 when compared to control cells (Fig. 5F), which was confirmed by WB analysis (Fig. 5G–H). Conversely, MCF-7 cells chronically treated with DOX, showed reduced mRNA (Fig. 5I) and protein expression (Fig. 5J, K) for pp-GalNAc-T3, which was previously proposed as a possible candidate for onf-FN biosynthesis [31]. To give more

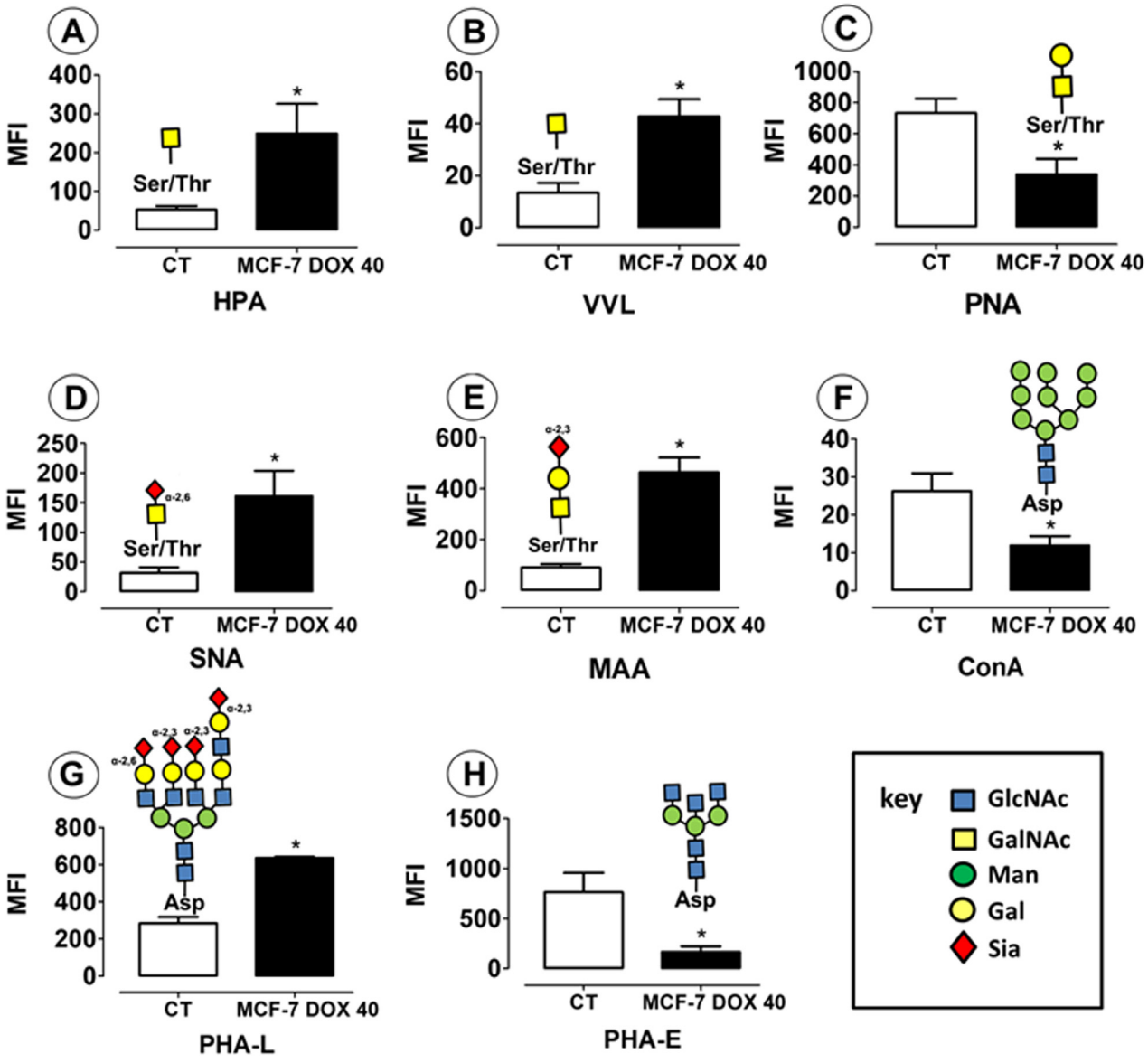

Fig. 4. Evaluation of the cellular glycophenotype in both parental and chronically treated MCF-7 cells. To monitor possible changes in cellular glycophenotype, MDR and parental MCF-7 cells were incubated with fluorescein-5-isothiocyanate (FITC)-conjugated lectins with distinct saccharide specificities, and analyzed by flow cytometry as described in Materials and Methods. Results are shown as average ± SEM. $n = 3$; * $p \leq 0.05$ vs. CT (MCF-7 parental cells).

value to our hypothesis, we also used both HUH-7 and HUH-7 T6 cells lines. HUH-7 T6 was generated as previously described [28,29] and provided by Dr. Hakomori. HUH-7 T6 is a hepatoma cell line that overexpresses pp-GalNAc-T6, and as consequence, secretes high amounts of *O*-glycosylated onf-FN (Supp. Fig. 3D) [29]. The HUH-7 T6 presented higher resistance to the chemotherapeutics tested when compared to the parental cell line (Supp. Fig. 3A−C). These, corroborated the data obtained with the MCF7-DOX 40 cell line, which also showed increased expression for pp-GalNAc-T6 (Fig 5F−H).

Impact of pp-GalNAc-T6 and pp-GalNAc-T3 silencing on MDR phenotype and FNs expression

In order to evaluate the importance of both pp-GalNAc-T6 and pp-GalNAc-T3 in the MDR

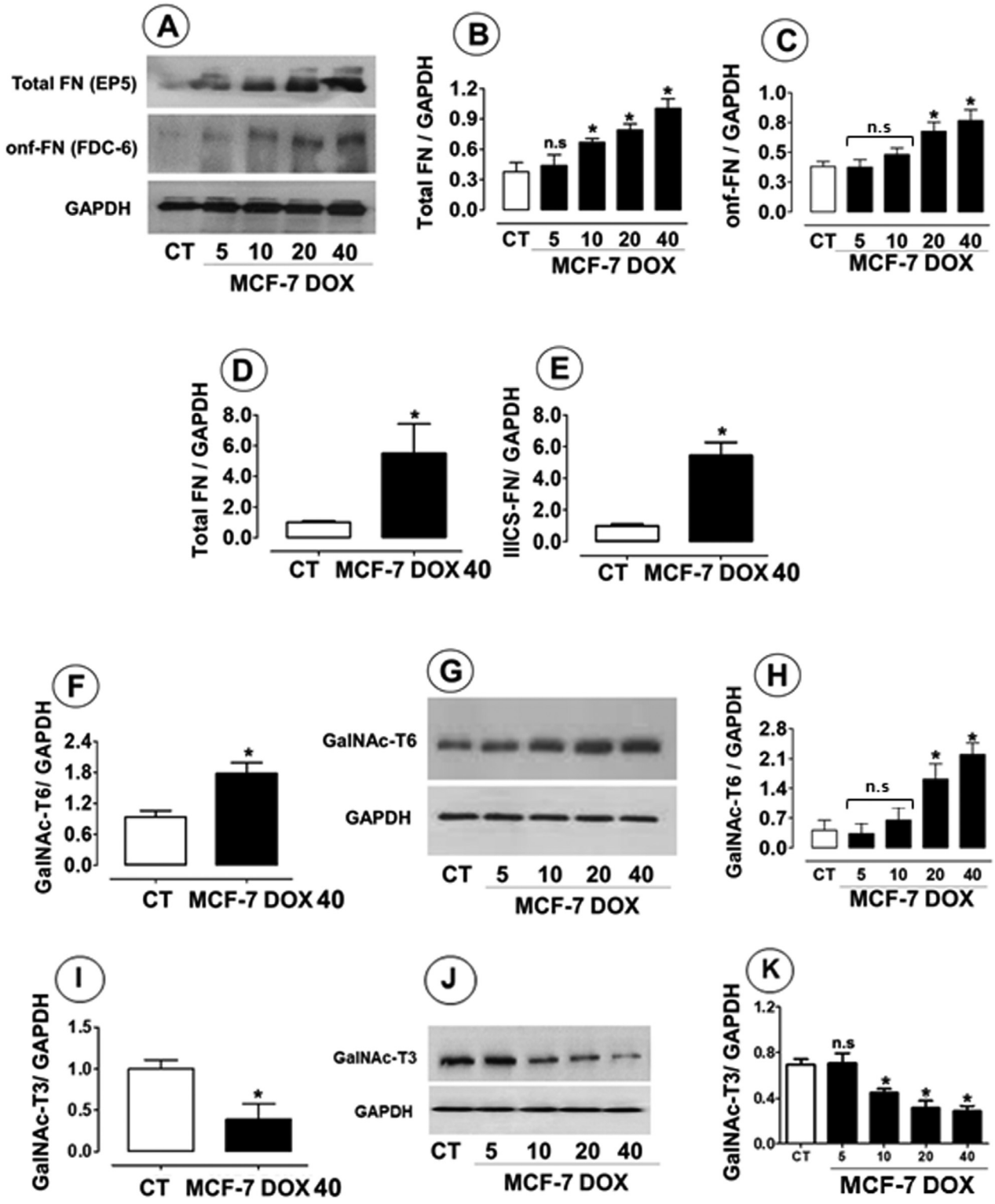

Fig. 5. Monitoring the expression of fibronectin isoforms, pp-GalNAc-T3 and pp-GalNAc-T6 in parental and MDR MCF-7 cells. Parental and resistant MCF-7 cells were cultured in 6-well plates for 48 h prior to WB assay to

phenotype, we used siRNAs to silence the glycosyltransferases in both parental and MCF-7 DOX 40 cell lines. Their expressions were subsequently monitored by both RT-qPCR and WB analysis. Since the expression of pp-GalNAc-T3 was downregulated in MDR cell line variants (Fig. 5I–K), we investigated its role through knockdown of the parental cell line (Supp. Fig. 4). The results demonstrated that silencing was efficient, since it significantly reduced mRNA (Supp. Fig. 4A) and protein expression (Supp. Fig. 4B, C) for pp-GalNAc-T3 when compared to cells treated with the siRNA negative control. In addition, the knockdown did not influence the expression of other analyzed glycosyltransferases (Supp. Fig. 4D, E). Regarding FNs expression, parental knockdown cells presented no reduction in mRNA levels for both IIICS-FN (Supp. Fig. 4F) and total FN (Supp. Fig. 4G), nor were any significant changes observed on protein levels (Supp. Fig. 4H–J). Besides not interfering in the expression of FNs, the silencing of pp-GalNAc-T3 did not modulate the mRNA (Supp. Fig. 5A–C) and proteins levels (Supp. Fig. 5D–G) of ABC transporters (ABCG2, ABCC1 and ABCB1) or the mRNA (Supp. Fig. 6A, B) or proteins levels (Supp. Fig. 6C–E) for both Bcl-2 and Bcl-xL. pp-GalNAc-T3 silencing also did not influence parental MCF-7 cell line sensitivity to DOX, CIS and VIN (Supp. Fig. 6F–H). On the other hand, as the expression of pp-GalNAc-T6 was increased in MCF-7 cells chronically treated with DOX (Fig. 5F–H), we focused on analyzing its possible involvement in the MDR phenotype. As demonstrated, the silencing of pp-GalNAc-T6 was efficient, since the expression of the enzyme was significantly reduced when compared to cells treated with the siRNA negative control (Fig. 6A–C). Furthermore, the knockdown for pp-GalNAc-T6 proved to be specific for the glycosyltransferase, since assays performed by RT-qPCR by using specific primers for pp-GalNAc-T2 (Supp. Fig. 7A) and pp-GalNAc-T10 (Supp. Fig. 7B) did not show differences between the experimental points. Additional RT-qPCR analysis demonstrated that the silencing of pp-GalNAc-T6 did not negatively modulate the mRNA levels for both IIICS-FN domain (Supp. Fig. 7C) and total FN (Supp. Fig. 7D), as previously documented [28]. After silencing pp-GalNAc-T6, the next step was to investigate the expression of the FN isoforms. For this purpose, in addition to the EP5 and FDC-6 mAbs, we also used the YKH-1 mAb, which reacts with the naked (non-*O*-glyosylated) hexapeptide VTHPGY, expressed by FN isoforms found in healthy tissues [29]. The results showed that after silencing the glycosyltransferase in MCF-7 DOX 40 cell line, it was possible to observe a significant reduction in FDC-6 labeling (Fig. 7A, C). No significant changes were detected using the EP5 mAb (Fig. 7A, B), corroborating previous results [28]. However, after pp-GalNac-T6 knockdown, a significant increase in the labeling for YKH-1 mAb was observed (Fig. 7A, D). This data can be explained by the presence of an *O*-linked glycan, which before silencing, masked the binding site for YKH-1. Regarding parental cells, the knockdown of either pp-GalNAc-T6 or pp-GalNAc-T3, did not significantly reduce onf-FN expression (Fig. 6B, C), as previously documented [28]. Knowing the acquisition of the MDR phenotype is associated with an increased expression of proteins belonging to the ABC superfamily and anti-apoptotic proteins, the expression of the efflux pumps ABCB1, ABCC1 and ABCG2, as well as Bcl-2 and Bcl-xL were investigated (Fig. 8). The results showed that silencing pp-GalNAc-T6 had no effect on the expression of the investigated ABC transporters (Fig. 8A–D). Regarding the anti-apoptotic proteins Bcl-2 and Bcl-xL, the silencing of pp-GalNAc-T6 significantly reduced the expression of both (Fig. 8E–G), suggesting that glycoproteins decorated with *O*-linked glycans, such as onf-FN, may directly influence cell death in MDR cancer cells. Assays performed by real time RT-qPCR corroborated the data obtained by WB related to ATP-binding cassette proteins (Supp. Fig. 8A-C), as well as the expression of Bcl-2 and Bcl-xL (Supp. Fig. 8D-E). Taken together, these data sets strengthens previous results [27,29,31], indicating that pp-GalNAc-T6 plays an important role in the expression of onf-FN. Furthermore, it demonstrates for the first time that pp-GalNAc-T6, but not pp-GalNAc-T3 has a direct role in the emergence and/or maintenance of the MDR phenotype in cancer cells.

Effect of pp-GalNAc-T6 knockdown on the modulation of apoptosis and MDR phenotype

The results on the expression of bcl-2 proteins point to the conclusion that the MCF-7 DOX 40 cell line exhibited greater apoptosis resistance when compared to the parental cell line. In order to confirm if MCF-7 DOX 40 is more resistant to apoptosis than the parental MCF-7, an annexin V (AV)-propidium iodide (PI) binding assay was performed to tag cells undergoing cell death. Fig. 9A shows that when treated with DOX 150 nM, the MCF-7 parental cells

investigate the expression of total fibronectin (A, B), oncofetal fibronectin (A, C), pp-GalNAc-T6 (G, H) and pp-GalNAc-T3 (J, K). In parallel, mRNA levels for total FN (D), IIICS-FN (E), pp-GalNAc-T6 (F) and pp-GalNAc-T3 (I) were investigated by RT-qPCR. Images are representative of three independent experiments. Graphs B, C, H and K are displayed as average ± SD, and graphs D, E, F and I are displayed as average ± SEM. $n = 3$; * $p \leq 0.05$ vs. CT (MCF-7 parental cells). n.s. not significant.

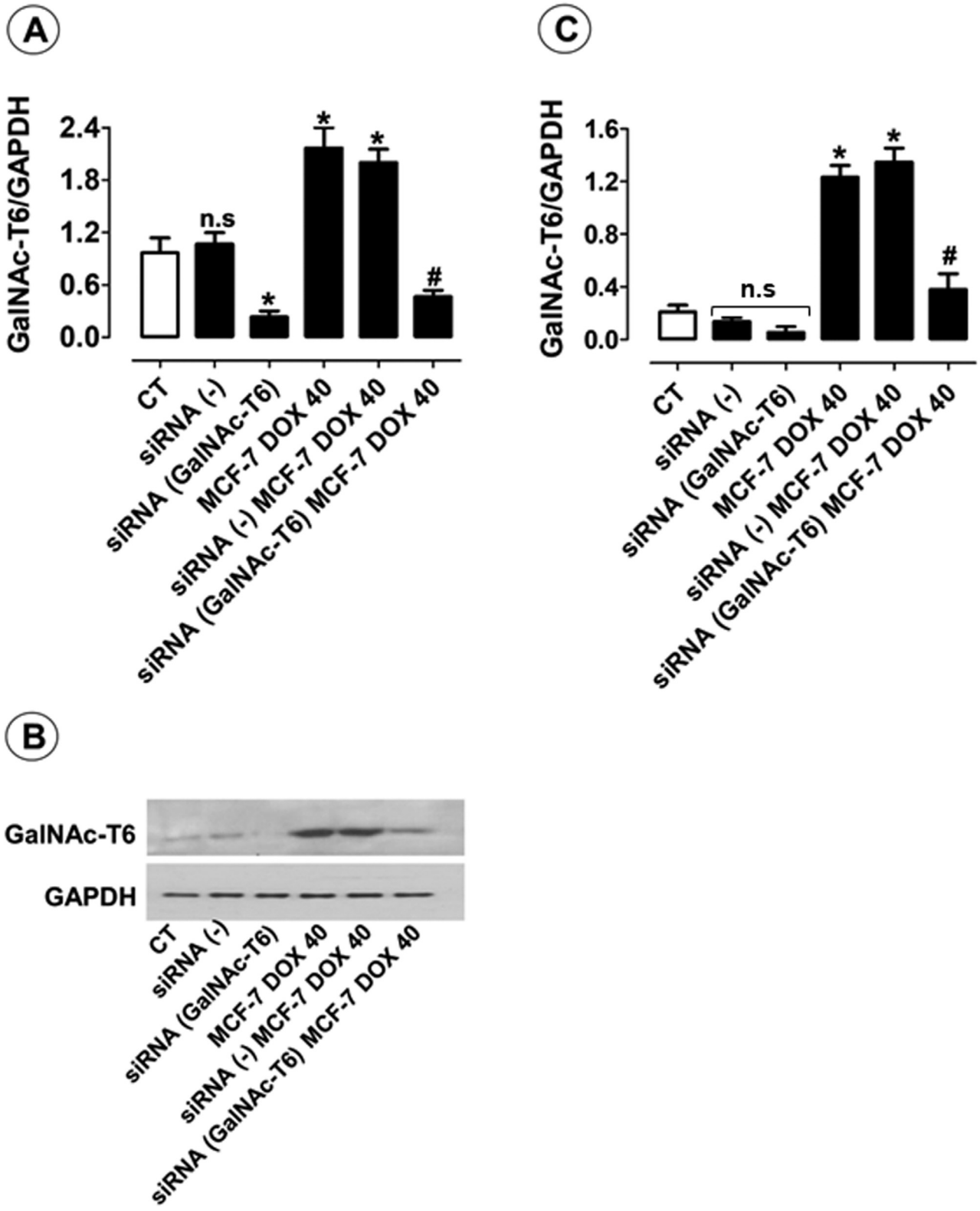

Fig. 6. Analysis of pp-GalNAc-T6 expression after glycosyltransferase knockdown by the siRNA technique. Parental or MDR MCF-7 cells were transfected with specific siRNA for pp-GalNAc-T6 or control siRNA as described in Materials and

(Fig. 9A$_i$) exhibited significant viability reduction (Fig. 9A$_{iii}$), with most cells becoming positive for AV-PI. It also was noticeable that the MCF-7 DOX 40 cell line (Fig. 9A$_{iv}$) displayed similar AV-PI binding levels to the untreated MCF-7 cells, both in the presence or absence of DOX, as expected for the resistant cell line (Fig. 9A$_{ii,}$ 9A$_{iv,}$ 9A$_{vi}$ and 9A$_{viii}$). Interestingly, after silencing pp-GalNAc-T6 (Fig. 9A$_{v,}$ 9A$_{vi,}$ 9A$_{vii}$ and 9A$_{viii}$), MCF-7 DOX 40 exhibited AV-PI binding levels similar to what can be seen for the parental cell line when challenged with DOX (Fig. 9A$_{viii}$), suggesting an abrogation of the resistance phenotype. Figure 9B shows the average of three individual experiments performed by flow cytometry, where AV-PI staining was monitored in both parental and MCF-7 DOX 40 cell line silenced or not for pp-GalNAc-T6. Since the silencing of pp-GalNAc-T6 promoted a significant reduction of onf-FN, and compromised the expression of anti-apoptotic proteins, we investigated how MDR MCF-7 cells would respond to cytotoxic insults induced by different classes of chemotherapeutic agents, such as DOX (Fig. 10A), CIS (Fig. 10B) and VIN (Fig. 10C). The results revealed that after silencing pp-GalNAc-T6, the MDR cells became more sensitive to the drugs, further confirming the partial reversion of the MDR phenotype.

Discussion

The development of resistant cell lines through chronic exposure to chemotherapeutic agents, represents one of the main protocols used to understand the molecular events related to MDR phenotype, which is a multifactorial phenomenon [2]. Most studies in this area seek to understand the role played by proteins belonging to the ABC superfamily, as well as proteins that modulate apoptotic pathways [47]. It is also important to point out that a large part of the protocols described to generate MDR cancer cells are characterized by the continuous exposure to high doses of chemotherapeutic agents, which leads to the development of variants that are phenotypically very different from what is observed in primary cancer cells isolated from cancer patients [62]. For this reason, in the present work, we employed a continuous protocol previously described by our group [51], where parental MCF-7 cells were exposed for a long period in culture medium supplemented with non-lethal concentrations of DOX (5, 10, 20 and 40 nM), which were initially selected by MTT assay. Interestingly, the characteristics adopted by MDR MCF-7 cells developed in this study showed glycophenotypic similarities to other resistant cell lines established by our and other research groups, even using different chemotherapeutic drugs [51,63,64]. Some of these changes included increased sialylation, which has been extensively associated with tumor growth and spreading, and resistance to apoptosis [65]. This highlights the importance of analyzing the MDR-associated glycan changes to better understand the role of glycoconjugates in cancer biology, and their impact in clinical outcomes. In an interesting work developed by Saw and colleagues (2017), the authors demonstrated that DOX-resistant MCF-7 cells expressed an unusual FN isoform, also called onf-FN [66]. However, in this work, the alterations observed in the ECM component were not related to the glycosylation profile of the molecule, but rather to the differential expression of extra-domain B (EDB) [66], a spliced domain detected during embryogenesis and neoangiogenesis, a process common to many cancers [67]. It is also important to point out, that many studies related to MDR phenotype in transformed cells, reported changes in glycans structures of several cancer models [17,50,51]. Most of these studies revealed the expression of membrane proteins carrying structurally atypical *O*- and *N*-linked glycans [68]. To the best of our knowledge, there are no studies demonstrating how glycophenotypic changes in ECM glycoproteins may impact tumor cell behavior, especially the acquisition of the MDR phenotype, a serious obstacle faced by oncologists in the fight against cancer. Although it is still a neglected research area, studies related to glycosylation changes are growing exponentially in number [69,70]. In the initial studies related to the glycobiology of cancer, several unusual glycoconjugates were observed. However, such glycomolecules were used only as glycobiomarkers [71], ignoring their roles in cancer cell biology. Currently, thanks to biotechnological advances, many research groups have already demonstrated how atypical glycoconjugates are able to modulate the functionality of cancer cells [72−74]. Currently, alteration in protein glycosylation is considered a hot topic in cancer biology, but there is no information on how structurally unusual glycan structures carried by ECM components may influence for example, the establishment and/or maintenance of the MDR phenotype. Recently, De Giorgi and colleagues (2021) demonstrated that collagen, an important ECM component, undergoes glycosylation through the addition of glucose and/or galactose units to specific hydroxylysine residues in alpha chains of procollagen [75]. In this way, the authors confirmed that both

Methods. After 48 h, the levels of mRNA (A) and proteins (B, C) were monitored by real-time qPCR and WB, respectively. Image is representative of three independent experiments. Data is shown as average ± SD. $n = 3$; $^*p \leq 0.05$ vs. CT (MCF-7 parental cells) and # $p \leq 0.05$ vs. MCF-7 DOX 40 cells. n.s. not significant.

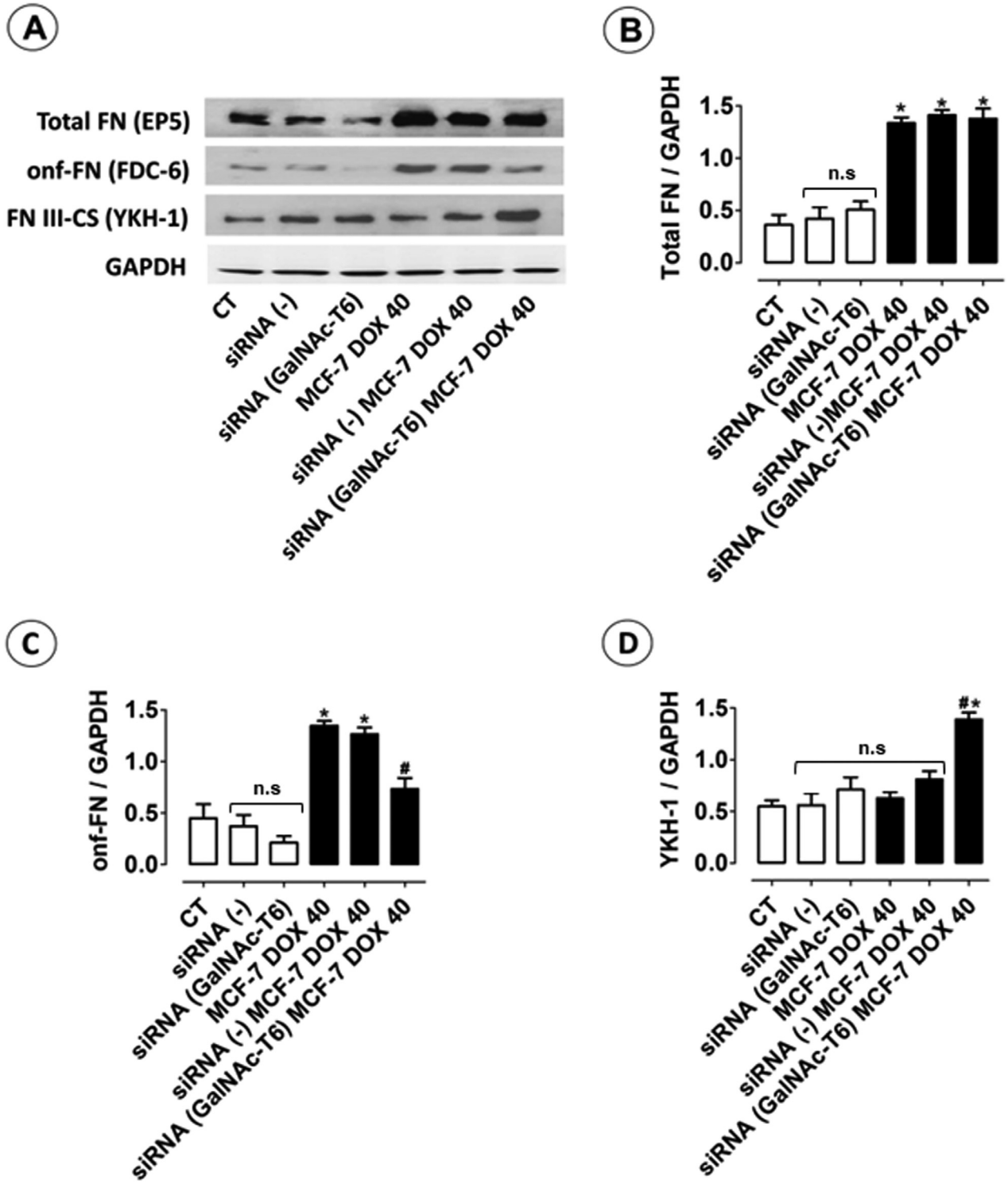

Fig. 7. Evaluation of the expression of different fibronectin isoforms in knockdown cells for pp-GalNAc-T6. Parental or MDR MCF-7 cells were transfected with specific siRNA for pp-GalNAc-T6 or control siRNA as described in Materials and Methods section. After 48 h, the protein expression for total fibronectin (A, B), *O*-glycosylated oncofetal fibronectin (A, C) and non-*O*-glycosylated fibronectin (A, D) were monitored by WB. Image is representative of three independent experiments. Data is shown as average ± SD. $n = 3$; * $p \leq 0.05$ vs. CT (MCF-7 parental cells), and # $p \leq 0.05$ vs. MCF-7- DOX 40 cells. n.s. not significant.

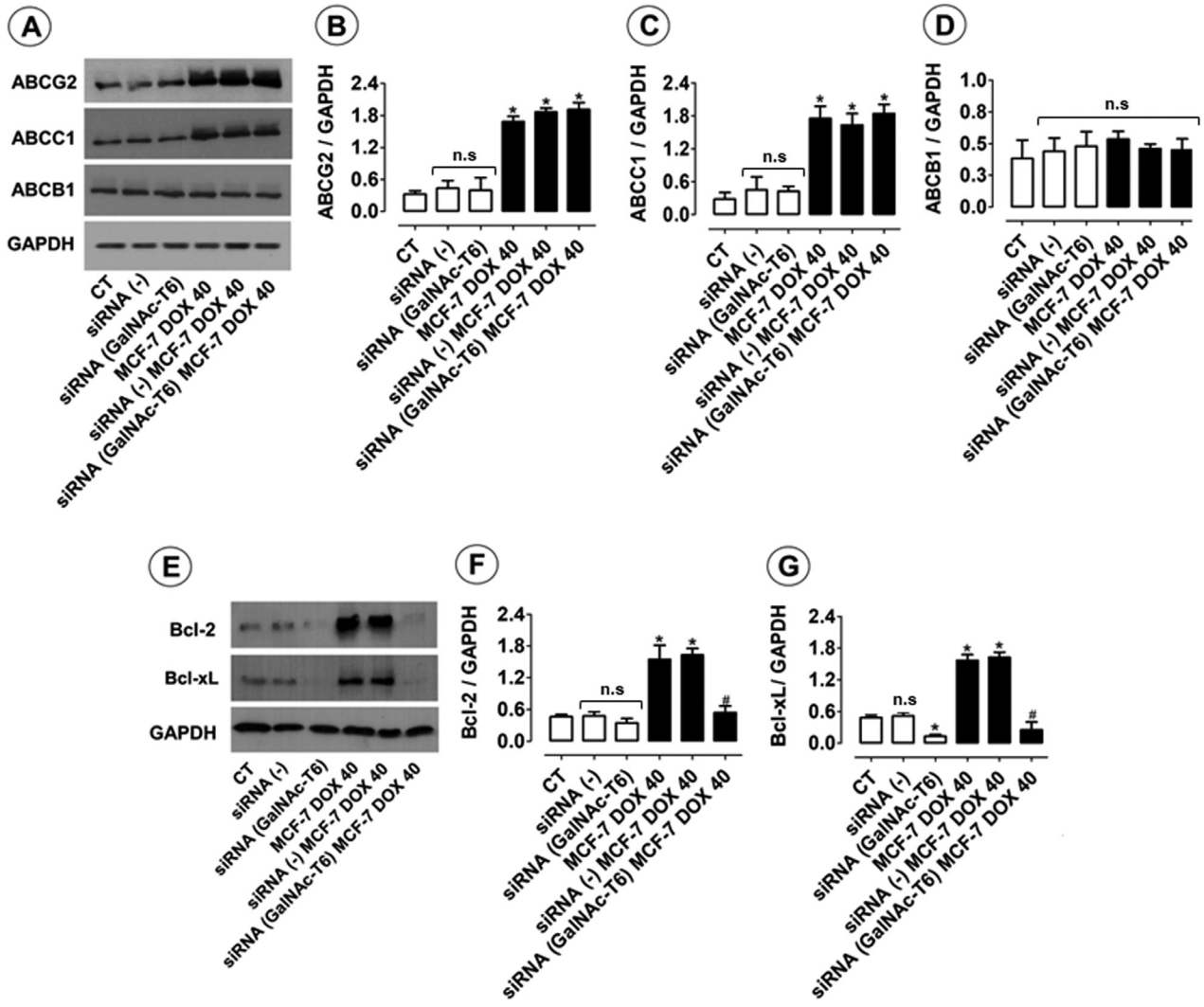

Fig. 8. Monitoring the expression of proteins belonging to the ABC superfamily (ABCB1, ABCG2 and ABCC1) and anti-apoptotic proteins (Bcl-2 and Bcl-xL) in parental and MDR knockdown cells for pp-GalNAc-T6. MCF-7 or MCF-7 DOX 40 cells were plated into 6-well plates and transfected with specific siRNA for pp-GalNAc-T6 or control siRNA as described in Materials and Methods section. After 48 h, the monolayers were lysed, and the samples subjected to the WB technique to monitor the expression of the efflux pumps ABCG2 (A, B), ABCC1 (A, C) and ABCB1 (A, D), as well as anti-apoptotic proteins Bcl-2 (E, F) and Bcl-xL (E, G). Images are representative of three independent experiments. Data is shown as average ± SD. *$p \leq 0.05$ vs. CT (MCF-7 parental cells), and # $p \leq 0.05$ vs. MCF-7- DOX 40 cells. n.s. not significant.

galactosyl-hydroxylysine (Gal-Hyl) and glucosyl-galactosyl-hydroxylysine (Glc-Gal-Hyl) are essential for collagen and ECM homeostasis [75]. In addition, it has been also demonstrated that Glc-collagen, but not Gal-collagen, has the ability to induce the expansion of cancer stem cells (CSC), defined by the phenotype CD133+ CXCR4+, which have elevated metastatic potential and therefore a high ability to initiate tumors [76]. This work clearly demonstrates that the biological role played by this ECM glycoprotein is determined by its glycophenotype. onf-FN was initially described in the 1980s, and remained in use as a glycobiomarker for over 25 years [32,77-80]. Only in 2011, we demonstrate for the first time, the role of the oncofetal glycoprotein in cancer cells undergoing EMT process [28]. Since then, we have already detected its expression in both alternatively activated macrophages and cancer cells kept in high-glucose culture conditions [26,61], supporting the hypothesis that diabetic patients are more susceptible to the development and/or progression of different types of cancer [81]. However, its role in the MDR phenotype has never been addressed. The addition of GalNAc to threonine and serine residues

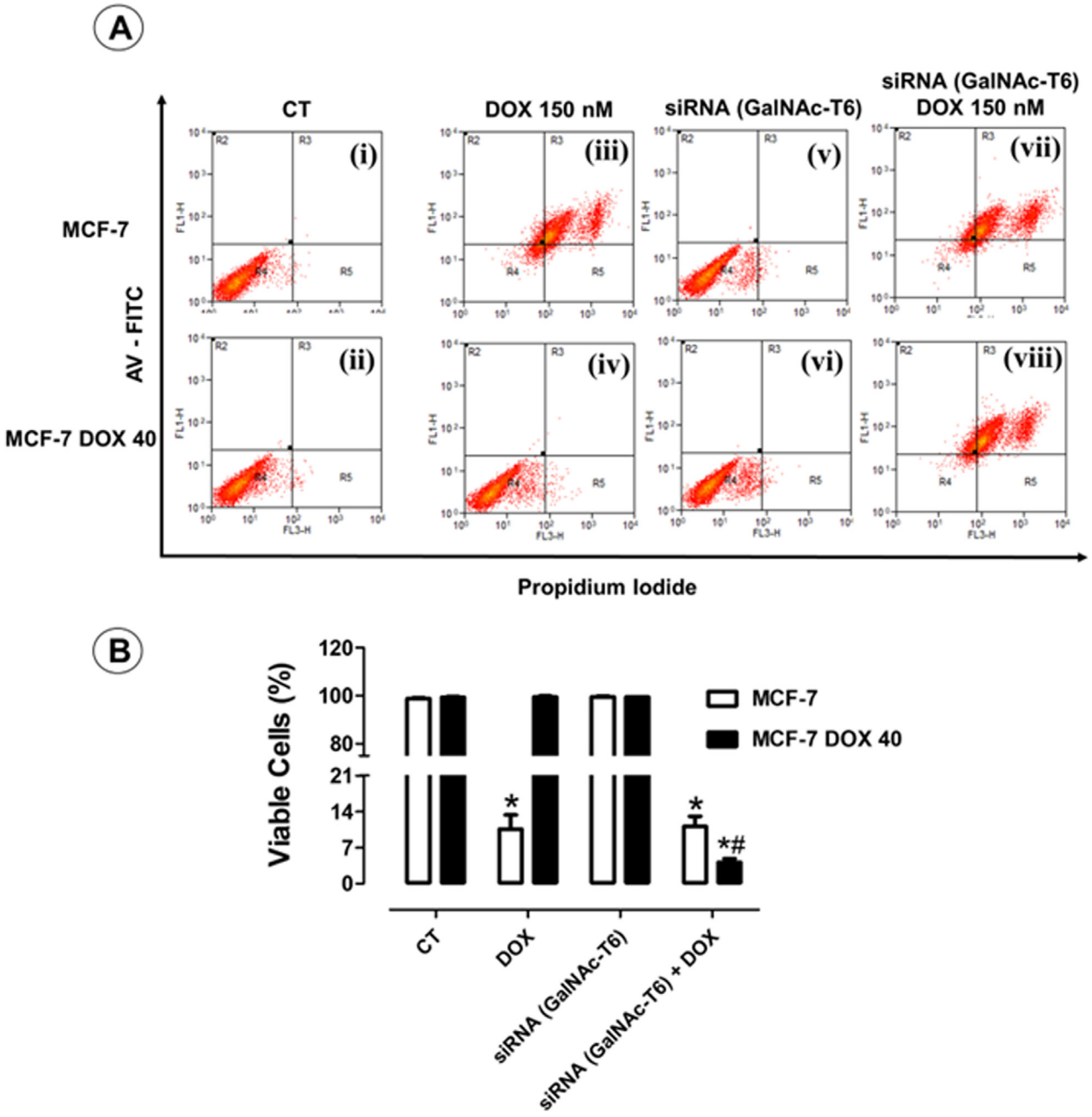

Fig. 9. Analysis of annexin V and propidium Iodide binding in knockdown cells for pp-GalNAc-T6. MCF-7 DOX 40 (A_{ii}, A_{iv}, A_{vi}, A_{viii}) or MCF-7 (A_i, A_{iii}, A_v, A_{vii}), were silenced (A_v, A_{vi}, A_{vii}, A_{viii}) or not (A_i, A_{ii}, A_{iii}, A_{iv}) for pp-GalNAc-T6, as described in the Materials and Methods section. After transfection, cell viability was monitored by flow cytometry to detect cells undergoing apoptosis and / or necrosis. Dot plots (A) represent three independent experiments ± SD. (B) Graphical representation for AV/PI staining of three individual experiments. Results are shown as average ± SD. * $p \leq 0.05$ vs. CT (MCF-7 parental cells), and # $p \leq 0.05$ vs. MCF-7- DOX 40 cells.

is within the purview of the pp-GalNAc-T family, represented by 20 isoforms in humans, which exhibit tissue-specific expression and activity [82]. A couple of years after the first description of the oncofetal glycoprotein [32], Matsuura and colleagues suggested that one or more pp-GalNAc-Ts would be responsible for its biosynthesis [78]. Twelve years later, Clausen's group showed that recombinant

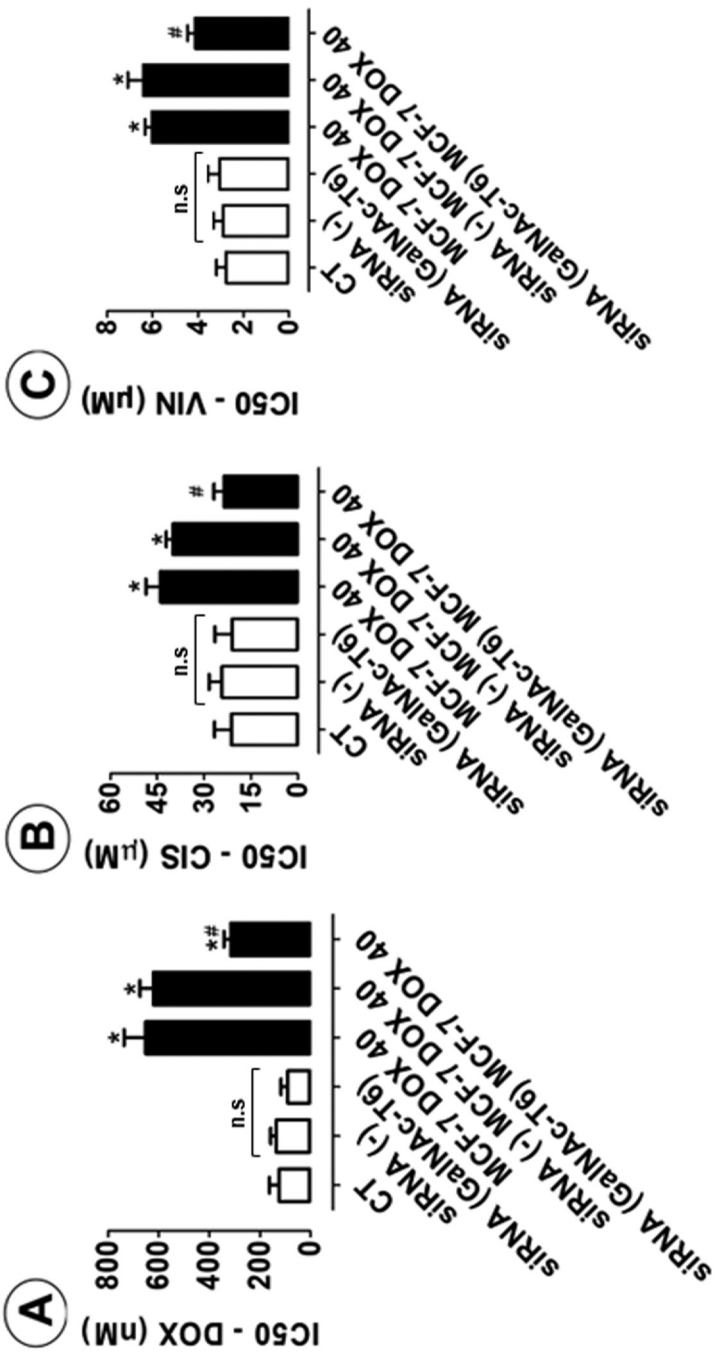

Fig. 10. Evaluation of the maintenance of MDR phenotype in knockdown cells for pp-GalNAc-T6. MCF-7 and MCF-7 DOX 40 cells were or not silenced for pp-GalNAc-T6, and cell viability was investigated by exposure to the chemotherapeutic drugs DOX (A), CIS (B) and VIN (C). After 48 h of treatment, the IC50 for each drug was determined by MTT assay. Results are shown as average $\pm$ SD. $^{*}p \leq 0.05$ vs. CT (MCF-7 parental cells) and $\#\, p \leq 0.05$ vs MCF-7- DOX 40 cells.

GalNAc-T3 was able to glycosylate the VTHPGY sequence [31]. Additional studies developed the same group, evinced that recombinant pp-GalNAc-T6, not only was able to glycosylate the hexapeptide, but also showed superior kinetic parameters when compared to pp-GalNAc-T3 [27]. In addition, it was demonstrated in the same work, that a fetal fibroblast cell line (WI38), which expresses GalNac-T6, but not GalNAc-T3 generates high amounts of onf-FN, suggesting that pp-GalNAc-T6 might be responsible for onf-FN biosynthesis in lieu of pp-GalNAc-T3 [27]. This explains why in more recent works [26,29,61], only the expression of pp-GalNAc-T6 has been investigated when onf-FN is the subject of the studies. Previous results published by Freire-de-Lima and colleagues in 2011 could not conclude whether both enzymes or only pp-GalNAc-T6 would be responsible for generating onf-FN, since in that study, the authors performed a double knockdown for both pp-GalNAc-T3 and pp-GalNAc-T6 [28]. However, further studies developed by the same research group, demonstrated that the overexpression of pp-GalNAc-T6, but not pp-GalNAc-T3 in the HUH-7 cell line, promoted the expression of huge amounts of onf-FN [29], corroborating previously published results by Clausen's group [27]. Further biochemical and molecular studies are needed to identify other potential candidates that can participate in the generation of the oncofetal epitope found in the structure of FN. Despite similarities in structure and substrate specificity, pp-GalNAc-T6 and T3 are differentially expressed [83] and have been pointed as independent prognostic factors in cancer [37,41]. In the present study, we evaluated the role of both pp-GalNAc-T3 and pp-GalNAc-T6 in MDR cancer cells, as so far, they are the only enzymes described as potential candidates for onf-FN biosynthesis. Even though functional redundancy has been shown for pp-GalNAc-T3 and pp-GalNAc-T6 [84], this does not seem to be the case regarding onf-FN expression in MDR cancer cells. Using DNA microarray data from previously published studies, Raghu and colleagues observed low expression of GALNT3 in breast cancer cells standing mesenchymal characteristics [85]. Since EMT-related events have been deeply associated with the acquisition of the MDR phenotype in transformed cells, including breast cancer [86], such results may explain why pp-GalNAc-T3 presented reduced expression in MDR MCF-7 cell lines. However, further studies are needed to better understand this phenomenon. Results obtained by RT-qPCR revealed that our multiresistant cells, especially MCF-7 DOX 40 cell line, in addition to showing increased expression of onf-FN, also expresses high levels of other mesenchymal markers, such as N-cadherin and vimentin (data not shown), confirming that our MDR breast cancer cell line, which was generated by chronic exposure to DOX, presents a mesenchymal phenotype, as previously documented by other research groups [44,45]. The possible roles of these enzymes in cancer progression are complex. Both pp-GalNAc-T3 and pp-GalNAc-T6 can promote or suppress cancer progression, depending on the model [87]. For example, Liu et al. described that linc01296, a subset of Long non-coding RNAs (lncRNAs), acts as a molecular sponge for miR-26a, a negative regulator of pp-GalNAc-T3. In 2016, it has been demonstrated that loss of GALNT3 occurs in poorly differentiated pancreatic ductal adenocarcinoma (PDAC), which was associated with the increased aggressiveness and altered glycosylation of ErbB family proteins [88]. Inhibition of miR-26a leads to increased expression of pp-GalNAc-T3, which is responsible for the *O*-glycosylation of MUC1. Increased *O*-glycosylated MUC1 contributes to colorectal cancer progression via activation of the PI3K/AKT signaling pathway [89]. On the other hand, overexpression of pp-GalNAc-T6 suppressed progression of colorectal cancer through inhibition of AKT expression [90]. Also, the knockdown of GALTN6 in colorectal carcinoma cells has been proved to lead to decreased proliferation and invasiveness, as well as to increased sensitivity to 5-fluouracil, a drug extensively used in the treatment of several types of solid tumors [72]. In addition, it has also been demonstrated that pp-GalNAc-T3 suppresses lung cancer by inhibiting self-renewal and angiogenesis [91], while pp-GalNAc-T6 and its gene, GALNT6, have already been associated with metastasis through the glycosylation of the chaperone GRP78, which enhances the ERK1/2 signaling pathway responsible for activating EMT in lung cancer cell lines [70]. A study developed in 2017 suggested that pp-GalNAc-T3 plays an important role in promoting metastasis in breast cancer cell models, proposing it as a therapeutical target, as its inhibition leads to reduced invasion capacity [92]. There is a lot more information regarding pp-GalNAc-T6 in breast cancer models, and its increased expression has been described as an immunohistochemical marker [25], associated with tumorigenicity [93], promotion of metastasis [93,94] and cell growth [95]. In this study, we demonstrated for the first time, that the increased expression of pp-GalNAc-T6, the main if not the only pp-GalNAc-T responsible for onf-FN biosynthesis in breast cancer cells, is associated with MDR phenotype. Although our results do not demonstrate if the effect on chemoresistance is due directly to onf-FN or to another of pp-GalNAcT6's targets, or even if it depends on a combination of more than one of its products, it is sensible to imagine that the impairment of the onf-FN biosynthesis has a role, direct or not, in this multifactorial phenomenon, since it already was demonstrated that cancer cells treated with chemotherapeutic agents while being exposed to ECM components, including FN, present their viability preserved [96–101]. As

such, further studies by using both *O*-glycosylated and non-*O*-glycosylated FNs purified by immunoaffinity columns as described in our previous work [29] will be essential to better understand the role of the oncofetal glycoprotein in the emergence and/or maintenance of the MDR phenotype in cancer cells, as well as in other oncobiological events, since the altered glycosylation is already considered a hallmark of cancer.

Experimental procedures

Cell line and reagents

The human breast adenocarcinoma cell line MCF-7 was purchased from the American Type Culture Collection (ATCC, Manassas, VA, USA). In all experiments, cells were cultured in Dulbecco's modified Eagle's medium (DMEM) (Sigma-Aldrich, St. Louis, MO, USA cat# D5523) supplemented with 25 mM D (-) glucose (Sigma Chemical CO, USA), 10% fetal bovine serum (FBS), 100 IU/mL penicillin and 100 μg/mL streptomycin. All lectins used in this study to monitor the cell glycophenotype were obtained from Vector Labs (Newark, CA, USA). The antibodies against ABCC1 (sc-18835, dilution 1:1000), ABCB1 (cat# sc-55510, dilution 1:1000), ABCG2 (cat# sc-377176, dilution 1:500), total FN (cat# sc-8422. dilution 1:2000), Bcl-2 (cat# sc-7382, dilution 1:500), Bcl-xL (cat# sc-8392, dilution 1:2000), GalNAc-T6, clone Y5J (cat# sc-100755, dilution 1:200) and Glyceraldehyde 3-phosphate dehydrogenase (GAPDH, cat# sc-47724; dilution 1:5000) were purchased from Santa Cruz Biotechnology (Santa Cruz, Dallas, TX, USA). The antibody directed against GalNAc-T3 cat# MBS8245290, dilution 1:500) was purchased from MyBiosource (San Diego, CA, USA). Secondary antibodies HRP-conjugated anti-mouse IgG (cat# AP308P, dilution 1:5000) and HRP-conjugated anti-rabbit IgG (cat# AP307P, dilution 1:5000) were purchased from Millipore (Burlington, MA, USA). Protease inhibitor (cat# P8340), MTT (cat# M5655), dimethyl sulfoxide (DMSO) (cat# 276855), and the chemotherapeutic drugs: doxorubicin (DOX) (cat# D1515), cisplatin (CIS) (cat# PHR1624) and vincristine (VIN) (cat# V8388) were acquired from Sigma Aldrich (St. Louis, MO, USA). siRNA for GalNac-T6 (cat # D-012366-01) and pp-GalNAc-T3 (cat # D-011866-02) were obtained from Dharmacon Research (Lafayette, CO, USA). Negative control siRNA (cat# SIC001) was purchased from Qiagen (São Paulo, SP, Brazil). BCA Protein Assay Kit (cat# 23225) and lipofectamine (cat# 11668019) were purchased from Thermofisher Scientific (Waltham, MA, USA). The ECL chemiluminescence kit (cat# RPN2108) was purchased from GE healthcare (Little Chalfont, Buckinghamshire, UK). Annexin V/ PI double staining kit (cat# 556547) was obtained from BD Pharmingen (San Diego, CA, USA). The FDC-6 and YKH-1 mAbs, which react with *O*-glycosylated oncofetal FN and non-*O*-glycosylated FN, respectively, were donated by Dr. Sen-Itiroh Hakomori (Seattle, WA, USA). The PCR primers used in this study are listed in Supp. Table 2, and were obtained from Life Technologies (São Paulo, SP, Brazil).

MTT assay

In this study, MCF-7 cells were initially treated with increasing concentrations of the chemotherapeutic agent DOX to select non-lethal doses of the drug used to induce chemoresistance as previously described [50]. The effects of DOX on MCF-7 cells were evaluated through the 3-(4,5-dimethylthizol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay [49,102]. Briefly, the cell lines were seeded onto a 96-well plate at a concentration of 4.0×10^3 cells/well. After 18 h, the medium was renewed, and the cells were treated with increasing concentrations of DOX (0.005 − 1.28 μM) for the next 48 h. At the end of the exposure period, the cells were incubated with 20 μL of MTT solution (5 mg/mL) for 4 h at 37 °C [51]. After the medium was removed, 100 μL of DMSO was added to each well, and the absorbance was measured with a plate reader (Model AD340, Beckman Coulter, Brea, CA, USA) at a wavelength of 570 nm. The cell viability index was calculated using the following formula: experimental optical density value/control OD value.

DOX resistance−inducing process

The MCF-7 cell line was exposed to non-lethal concentrations of DOX (5, 10, 20 and 40 nM), which were selected after MTT assay as described above. Cells in different culture flasks were treated with the non-lethal concentrations of the drug, with at least three passages per week being performed for each experimental condition. Every fifteen days of drug exposure, the acquisition of multidrug resistance was monitored by the MTT assay, using in addition to DOX, the drugs CIS and VIN. The expression of the ATP-binding cassette proteins ABCB1, ABCC1 and ABCG2, as well as anti-apoptotic (Bcl-2 and Bcl-xL) proteins were monitored by WB, in order to validate the phenotypic changes associated with the drug resistance phenotype [50]. After six months of exposure to DOX, both resistance acquisition and phenotypic changes were validated by MTT and WB, respectively, resulting in the four cell variants we used in this article: MCF-7 DOX 5, MCF-7 DOX 10, MCF-7 DOX 20 and MCF-7 DOX 40 cell lines.

RNA extraction and RT-qPCR reaction

RNA extraction and purification from the parental and MCF-7 DOX 40 cell lines were performed 48 h after seeding of 3.0×10^5 cells onto six well microplates, using the RNeasy kit (cat# 74004, QIAGEN, Hilden, Germany) as previously described [102]. cDNA was synthesized using the MMLV RNAse Minus First-Strand cDNA Kit (cat# 13−10,504−005, LGC Biotecnologia, Cotia, Brazil). For the amplification, we used the quantitative polymerase chain reaction (qPCR)-SYBR Green Plus 2x kit (cat# 13−100RTSY, LGC Biotecnologia, Cotia, Brazil) in a LineGene 9600 plus Real-Time PCR device. Data analysis was performed with the LineGene 9600 plus Real-Time PCR for Research software.33. All procedures were performed according to the manufacturer's instructions. The RT-qPCR reactions were performed using the primers described in Supp. Table 2.

Western blotting

Parental MCF-7 and MCF-7 DOX 40 cells were seeded onto six well plates at a density of 3.0×10^5 cell per well. After a 48-h period of incubation, cells were washed with phosphate buffered saline 1 x (PBS), and submitted to lysis with RIPA buffer (10 mM Tris, pH 7.4; 100 mM NaCl; 1 mM EDTA; 1 mM EGTA; 1% Triton X-100; 10% glycerol; 0.1% SDS; 0.5% deoxycholate), and a protease inhibitor cocktail. Lysis was performed over 5 min, after which the lysate was collected and centrifuged for 10 min at 10,000 g and 4 °C. The supernatant was retrieved and transferred to microtubes. Protein concentration was determined using the BCA kit, and 40 μg of total protein were loaded in each well for every assay as previously described [28]. Band intensity was analyzed with the Scion Image software.

Flow cytometry analysis

Flow cytometry analysis was employed by using the FACS Callibur equipment (BD Biosciences, CA, USA). The lectins used to analyze the cell glycophenotype, are listed in the Supp. Table 1. The lectin analysis was performed as described in our previous work [50] In addition, flow cytometry analysis was also employed to monitor cell death by using Annexin V/ PI double staining kit as previously described [50].

Effect of GalNAc-T6 and pp-GalNAc-T3 knockdown on drug-resistance phenotype, MDR phenotype and FNs expression

A total of 1.0 to 1.5×10^5 parental MCF-7 or MCF-7 DOX 40 cells were seeded onto a six-well plate. After 24 h, cells were transfected with 100 nM specific siRNAs for human pp-GalNAc-T6 (CCAUCGACCUUAAUACUUU), pp-GalNAc-T3 (CCAUAGAUCUGAACACGUU) or a negative control siRNA, using Lipofectamine 2000 reagent, following the manufacturer's instructions, as previously described [28]. After 24 h of incubation, the cells in each well were detached or not with trypsin/EDTA, and seeded into 96-well cell culture plate. After 48 h, the effect of knockdown on drug-resistance phenotype was assessed by MTT assay after treatment with DOX, CIS and VIN [51]. The IC50 for each drug was determined by using the GraphPad Prism 5 program by nonlinear regression of the normalized curve [49]. In order to investigate the expression of pp-GalNAc-T6, pp-GalNAc-T3, FN isoforms, as well as the efflux pumps and anti-apoptotic proteins in the knockdown cells, after performing the transfection protocol as described above, the cells grown in six-well plates were lysed as previously described [28], and the expression of proteins monitored by WB.

Statistical analysis

Statistical analyzes were performed with the aid of the GraphPad Prism 5 program. The student *t* test was used to compare two independent samples. The ANOVA test has been used to analyze three or more distinct experimental points. In association with ANOVA, the Bonferroni or Tukey post-tests were employed as a second method of multiple comparisons between the experimental points. Results were considered significant when $p \leq 0.05$.

Authors' contributions

Conceptualization: Santos-dos-Reis, J; da Costa Santos; MAR, Fonseca; LM.

Data curation: Santos-dos-Reis, J; da Costa Santos; MAR.

Formal Analysis: Santos-dos-Reis, J; da Costa KM; Morrot, A.

Funding acquisition: Fonseca, LM; Freire-de-Lima, L; Previato, JO; Mendonça Previato, L.

Investigation: Santos-dos-Reis, J; da Costa Santos; MAR; Freire-de-Lima, CG.

Methodology: Santos-dos-Reis, J; da Costa Santos; MAR.; da Costa KM.

Project administration: Freire-de-Lima, L.

Resources: Fonseca, LM; Freire-de-Lima, L; Morrot, A; Freire-de-Lima, CG; Previato, JO; Mendonça Previato, L.

Supervision: Freire-de-Lima, L.

Validation: Santos-dos-Reis, J; da Costa Santos; MAR.

Visualization: da Costa KM.

Writing - original draft, review & editing: Fonseca, LM; Freire-de-Lima, L; Santos-dos-Reis, J.

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Data availability

Data will be made available on request.

Declaration of Competing Interest

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.matbio.2023.03.002.

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Glycoproteins

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