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The plasma membrane transporter SLC5A8 suppresses tumor progression through depletion of survivin without involving its transport function

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Summary

SLC5A8 is a sodium-coupled transporter for monocarboxylates. Among its substrates are the HDAC inhibitors butyrate, propionate, and pyruvate. Expression of *SLC5A8* is silenced in cancers via DNA methylation, and ectopic expression of SLC5A8 in cancer cells induces apoptosis in the presence of its substrates that are HDAC inhibitors. Here we show that ectopic expression of SLC5A8 in cancer cells translocates the anti-apoptotic protein survivin to plasma membrane through protein-protein interaction resulting in depletion of nuclear survivin and also decreases cellular levels of survivin through inhibition of transcription. These SLC5A8-induced changes in the location and levels of survivin result in cell cycle arrest, disruption of the chromosome passenger complex involved in mitosis, induction of apoptosis, and enhancement in chemosensitivity. These effects are seen independent of the transport function of SLC5A8 and histone acetylation status of the cell; in the presence of pyruvate, a SLC5A8 substrate and also an HDAC inhibitor, these effects are amplified. Ectopic expression of SLC5A8 in the breast cancer cell line MB231 inhibits the ability of the cell to form colonies in vitro and to form tumors in mouse xenografts in vivo. The suppression of survivin transcription occurs independent of HDAC inhibition, and the underlying mechanism is associated with decreased phosphorylation of STAT3. The observed effects are specific for survivin with no apparent changes in expression of other inhibitor-of-apoptosis proteins. These studies unravel a novel, hitherto unrecognized, mechanism for the tumor-suppressive role of a plasma membrane transporter independent of its transport function.

Keywords

SLC5A8; survivin; histone deacetylases; tumor suppressor; STAT3

INTRODUCTION

SLC5A8, also known as SMCT1 (sodium-coupled monocarboxylate trasporter 1), is a plasma membrane transporter for monocarboxylates such as lactate, pyruvate, acetate, propionate, butyrate, nicotinate, and β-hydroxybutyrate [1–4]. Its expression is silenced in a wide variety of cancers including cancers of brain, colon, thyroid, pancreas, prostate, lung and breast [1–4]. This transporter was first identified as a putative tumor suppressor in colon even before the identity of its transport function was established [5]. Soon after this discovery, two independent studies have identified its biological function as a Na^+-coupled

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transporter for monocarboxylates [6, 7]. Butyrate, one of the initial substrates identified for the transporter, is an inhibitor of histone deacetylases [8]. This monocarboxylate is generated at high concentrations in colonic lumen by bacterial fermentation of dietary fiber, and the transporter is expressed on the luminal surface of colonic epithelial cells [9], suggesting that transport of this bacterial metabolite into colonic epithelial cells is likely to be one of the physiological functions of the transporter. Butyrate inhibits specifically HDAC1 and HDAC3, and this process is related to the tumor-suppressive function of this transporter in colon [10, 11]. Interestingly, the expression of this transporter is suppressed not only in colon cancer but also in cancers of various systemic organs (e.g., mammary gland, stomach, prostate, pancreas, thyroid) [1–4], which are not exposed to any significant concentrations of butyrate (butyrate concentration in colonic lumen, ~10 mM; butyrate concentration in plasma, ~50 μM). Why would the transporter be silenced in cancers of tissues where there is no possibility of butyrate-induced HDAC inhibition? This conundrum led to the discovery that pyruvate, another transportable substrate of SLC5A8, can also act as an HDAC inhibitor, thus providing a plausible molecular basis of the tumor-suppressive function of SLC5A8 in non-colonic tissues [12]. Ectopic expression of SLC5A8 in cancer cells leads to apoptosis in the presence of butyrate or pyruvate [11–13]. One of the changes observed in this apoptotic process is depletion of the anti-apoptotic protein survivin [11, 12]. Interestingly, a recent report demonstrated that SLC5A8 interacts with survivin [14].

Survivin is a member of the inhibitor of apoptosis (IAP) family, and the proteins belonging to this family play an important role in inhibiting apoptosis. The IAP family proteins were first identified in baculovirus and all of them contain variable number of baculoviral IAP repeat (BIR) domains [15]. Several human and *Drosophila* IAPs inhibit apoptosis by directly binding and inhibiting caspases [15]. Hence, it was suggested that survivin, which has one BIR domain, inhibits caspases. Though the evidence for survivin directly inhibiting caspases remains controversial, knockdown of survivin does increase cell death in cancer cells [16]. In normal cells, the expression of survivin is tightly regulated in a cell cycledependent manner with a marked increase in G2/M phase [17]. Increased amount of survivin present during G2/M regulates mitosis through its role as a component of the chromosomal passenger complex (CPC) [18]. Survivin is known to function both at centrosomes and microtubules, ensuring accurate separation of sister chromatids during mitosis. Survivin also localizes to the kinetochores, the mid region of chromosomes, and here it is associated with other regulators of cytokinesis such as Aurora B kinase, the inner centromere protein (INCENP) and Borealin/Dasra [19]. Malignant cells overexpress survivin, which contributes to resistance of these cells to apoptosis and anticancer drugs. The present study was undertaken to investigate whether SLC5A8 has any role in the biology and function of survivin and if the transport function of SLC5A8 and associated HDAC inhibition have any relevance to this process.

MATERIALS AND METHODS

Cell culture

The cancer cell lines MCF7 (a estrogen receptor-positive breast cancer cell line) and MB231 (a estrogen receptor-negative breast cancer cell line) were maintained in DMEM and RPMI respectively with 10% FBS and antibiotics. Two different techniques were used for ectopic expression of SLC5A8 in cancer cell lines: an inducible expression system and a constitutive expression system. MCF7-SLC5A8-TetOn and MB231-SLC5A8-TetOn cells were generated, representing the expression system that is inducible by doxycycline. For constitutive expression, SLC5A8 cDNA was subcloned into pCDH-CMV-MCS-EF1-puro vector. Recombinant lentivirus was produced by co-transfection of pCDH vector or SLC5A8-pCDH with helper plasmids (ViraPower Lentiviral Expression System, Invitrogen) into 293FT cells using Lipofectamine 2000 transfection reagent. MCF10A-IV (a mammary

epithelial cell line transformed with oncogenic $HRAS^{G12D}$), MCF7 and MB231 cells were infected with the virus for 24 h and selected with puromycin. SLC5A8 expression was confirmed by RT-PCR and Na^+-coupled [^{14}C]-nicotinate uptake. MCF10A series (M-I, M-II, M-III, M-IV) cell lines were obtained from Dr. L. M. Wakefield (National Cancer Institute, Bethesda, MD, U.S.A.). MCF10A-I represents immortalized but non-malignant mammary epithelial cell line. Transfection of MCF10A-I with constitutively active $HRAS^{G12D}$ and selection by xenografting gave rise to MCF10A-II cell line, which predominantly forms premalignant lesions. MCF10A-III and MCF10A-IV were derived from occasional carcinomas arising from xenografts of MCF10A-II. MCF10A-III predominantly forms well-differentiated carcinomas in xenografts, while MCF10A-IV forms relatively undifferentiated carcinomas and metastasizes to the lung upon injection into the tail vein.

We also used a human pancreatic cancer cell line (Miacapa-2) to confirm the changes that occurred in human breast cancer cell lines in response to overexpression of SLC5A8. We selected Miacapa-2 cell line because it has been demonstrated that *SLC5A8* is silenced in this pancreatic cancer cell line via DNA methylation (20) as is the case with the breast cancer cell lines MCF7 and MB231.

Reverse-transcriptase PCR

The expression of various genes was analyzed by RT-PCR. The expression of SLC5A8 was induced with doxycycline in MCF7-SLC5A8-TetOn and MB231-SLC5A8-TetOn cells for 72 h. Pyruvate (1 mM) was added after 48 h of doxycycline treatment as indicated. At 72 h, RNA was isolated and used for RT-PCR. Primer sequences are given in Supplementary Materials. The reactivation of SLC5A8 expression by 5'-azacytidine was performed as described previously [12].

Immunoblotting and immunoprecipitation

Cell lysates were prepared in RIPA buffer with a protease inhibitor cocktail (Sigma-Aldrich). Protein samples were fractionated on SDS-PAGE and transferred to Immobilon FL PVDF membrane (Millipore, Billerica, MA, U.S.A.). The membranes were incubated with primary antibody at 4 °C overnight followed by incubation with IR secondary antibodies and imaged using Odyssey infrared imaging system (LICOR). For immunoprecipitation, cells were lysed in Tris-HCl buffer containing 1% Nonidet P-40. The samples were incubated with appropriate antibodies overnight at 4 °C followed by incubation with Sepharose protein-A/G (Roche) for 3 h at 4 °C. Samples were washed three times in lysis buffer and eluted in sample buffer. To analyze the interaction between survivin and aurora B, the cells were treated with 100 μg/ml nocodazole and then shifted to fresh media for 30 min; these cells were then used for immunoprecipitation with survivin antibody.

Immunofluorescence

For colocalization studies, cells were grown in chamber slides, fixed with 4% paraformaldehyde, and stained with anti-survivin and SLC5A8 antibody. Cells were then incubated with Alexa Fluor 488- and Alexa Fluor 568-conjugated secondary antibodies (Invitrogen) for 1 h. Unbound secondary antibody was washed, Hoechst 33342 was used to stain the nucleus, and the slides were mounted using ProLong Gold Antifade reagent (Invitrogen). For binucleated cell analysis, cell mask (Invitrogen) was used to visualize plasma membrane. For survivin and Aurora B localization analysis, the cells were treated with nocodazole and immunofluorescence analysis was carried out using antibodies specific for survivin, tubulin and Aurora B. All images were captured using a Carl Zeiss LSM510 Meta upright confocal microscope.

Chemosensitivity

Control cells and SLC5A8-expressing cells were exposed to increasing concentrations of chemotherapeutic agents for 72 h. Following the treatment, cells were collected, and apoptotic and necrotic cells were analyzed by annexin V-FITC and propidium iodide staining.

Colony formation assay

MB231-pCDHpuro (vector) and MB231-SLC5A8 cells were seeded in 6-well plates (10,000 cells/well), and allowed to form colonies for 2 weeks, changing the medium every 3 days. The cells were then fixed in 100% methanol for 30 min and stained with KaryoMax Giemsa stain (Invitrogen) for 1 h. The stain was eluted in 1% SDS in 0.2 N NaOH for 5 min and the absorbance was measured at 630 nm.

Cell cycle analysis

Cells were synchronized at G1-S phase by overnight treatment with thymidine (2.5 mM) for two consecutive nights with normal growth media between the two treatments. Distribution of cells at different stages of cell cycle was analyzed by propidium iodide staining using flow cytometry.

Caspase 3 activity

Caspase 3 activity was measured using a kit according to the manufacturer's instructions (R&D systems, Minneapolis, MN, U.S.A.).

TUNEL assay

Survivin was overexpressed in vector-only MB231 cells and in MB231-SLC5A8 cells by transfection with a plasmid containing survivin cDNA. Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) was used to detect apoptosis according to manufacturer's instructions (Clontech, Mountain view, CA, U. S. A.). The labeled cells were counted in six random fields and the percentage of apoptotic cells were analyzed using Image J software (National Institutes of Health, Bethesda, MD, U. S. A.).

Survivin promoter activity

Cells were co-transfected with the luciferase reporter plasmid pTL-Luc containing 843 bp of *survivin* promoter (Cat. No: LR1016, Panomics, Santa Clara, CA, U.S.A.) and β-actin in renilla luciferase expression plasmid. Luciferase activity was measured after 48 h using the dual luciferase assay system (Promega, Madison, WI, U.S.A.), and relative luciferase units (RLU) were calculated following normalization with Renilla luciferase activity.

Mouse xenograft

Female athymic nude mice (6- to 8-week old) were purchased from Jackson Laboratories (Bar Harbor, ME, U.S.A.) and housed in standard conditions. MB231-pCDHpuro and MB231-SLC5A8 cells were injected subcutaneously into the mammary fat pad (1×10^7 cells in 100 μl serum-free medium) as described previously [21, 22]. Tumor size was measured periodically by caliper, and tumor volume was calculated using the formula ($\text{width}^2 \times \text{length}$)/2. These experiments were approved by the Institutional Animal Care and Use Committee.

Statistical analysis

Data presented are means ± SE. Statistical significance was determined by Student's *t* test; $p<0.05$ was considered significant.

RESULTS

Downregulation of survivin expression by SLC5A8

MCF10A-I is a transformed mammary epithelial cell line that is used widely as a model for normal mammary epithelial cells. This cell line expresses SLC5A8 [ref. 12]. But, expression of survivin is low in this cell line (Figure 1A). However, on oncogenic transformation with ectopic expression of constitutively active $HRAS^{G12D}$, these cells (MCF10A-II) show a dramatic decrease in SLC5A8 expression accompanied with an increase in survivin expression (Figure 1A). The tumorigenic MCF10A-III and the metastatic MCF10A-IV cells also show decreased expression of SLC5A8 and increased expression of survivin (Figure 1A). It is already known that oncogenic transformation is associated with silencing of *SLC5A8* and upregulation of survivin expression [1–4, 23]. Hence, to find out whether there is any direct correlation between SLC5A8 expression and downregulation of survivin, we ectopically expressed SLC5A8 in MCF10A-IV cells and monitored the expression of survivin. MCF10A-IV cells, a high-grade metastatic carcinoma cell line, do not express SLC5A8 but express survivin (Figure 1B). Ectopic expression of SLC5A8 in these cells caused a significant decrease in survivin expression (Figure 1B), suggesting a possible direct link between SLC5A8 and survivin. To investigate this relationship further, we developed two different breast cancer cell lines, one estrogen receptor-positive (MCF7) and the other estrogen receptor-negative (MB231), with doxycycline-inducible SLC5A8: MCF7-SLC5A8-TetOn and MB231-SLC5A8-TetOn. Expression of SLC5A8 upon doxycycline treatment was verified by RT-PCR, western blot, and transport activity (Figure S1). As observed in MCF10A-IV, induction of SLC5A8 expression in these cells was associated with a decrease in survivin mRNA and protein (Figure 1C, D). These experiments were first carried out in the absence of pyruvate, a transportable substrate of SLC5A8 and also an inhibitor of HDACs. HDAC inhibitors are known to decrease survivin expression [24]. The SLC5A8-dependent decrease in survivin expression was seen in the absence of pyruvate. When pyruvate was present in the medium, there was a detectable increase in the magnitude of the effect on survivin expression, but only at mRNA level and that too only in MCF7 cells. There was no change in survivin protein levels in both cell lines as a result of pyruvate exposure. These data show that SLC5A8 decreases the expression of survivin regardless of the presence or absence of pyruvate, showing that SLC5A8-dependent suppression of survivin is independent of the transporter's ability to transport HDAC inhibitors into cancer cells.

SLC5A8 interacts with survivin

SLC5A8 is silenced in cancer by promoter methylation, and inhibition of DNA methylation induces its expression in cancer cells [5, 11, 12]. We wanted to determine if induction of endogenous *SLC5A8* with the DNA methylation inhibitor 5'-azacytidine would also decrease survivin expression. Treatment of MB231 cells with 5'-azacytidine resulted in an increase in SLC5A8 expression and a decrease in survivin expression (Figure 2A). In MB231-SLC5A8-TetOn cells, downregulation of survivin was completely dependent on SLC5A8 expression as removal of doxycycline from the medium with a resultant decrease in SLC5A8 expression increased survivin expression (Figure 2B). It has recently been demonstrated that SLC5A8 complexes with survivin by protein-protein interaction [14]. Therefore, we examined the interaction between these two proteins by co-immunoprecipitation (Figure 2C). These studies showed that SLC5A8 does indeed interact with survivin in MB231 cells, suggesting that expression of SLC5A8 in breast cancer cells not only decreases the expression of survivin but also may alter the subcellular localization of survivin as a result of this interaction because the former is a plasma membrane protein whereas the latter is an intracellular protein. This possibility was examined by immunofluorescence analysis in MCF7 cells (Figure 2D). Exposure of vector-only cells to

doxycycline with or without pyruvate did not alter the nuclear location of survivin whereas exposure of MCF7-SLC5A8-TetOn cells to doxycycline shifted the location of survivin from nucleus to plasma membrane even in the absence of pyruvate. Survivin was not detected in non-malignant MCF10A-I cells, which expressed SLC5A8; in contrast, survivin was present and localized to nucleus in MCF10A-IV cells, which did not express SLC5A8 (Figure S2). The data obtained with MCF7-SLC5A8-TetOn cells were also reproducible with MB231-SLC5A8-TetOn cells (Figure S2).

We confirmed the influence of ectopic expression of SLC5A8 on the localization of survivin using a human pancreatic cancer cell line (Miacapa-2). This cell line does not express SLC5A8 due to promoter hypermethylation [20]. We generated a Miacapa-2 cell line that overexpresses SLC5A8 as we did for the breast cancer cell lines, and used the vector-transfected cells as the control. As observed in breast cancer cell lines, we found abundant survivin expression in the nucleus of vector-only cells. However, when SLC5A8 was expressed ectopically, there was translocation of survivin from nucleus to plasma membrane (Figure S3).

SLC5A8 impairs survivin role in chromosomal passenger complex (CPC)

Survivin is a very mobile protein; it shuttles in and out of the nucleus and the localization of survivin is tightly regulated during cell cycle [25]. Since SLC5A8 expression led not only to a decrease in total survivin levels but also to a displacement of survivin from nucleus to plasma membrane, we wanted to examine whether SLC5A8 would impair the ability of survivin to act as a component of CPC. For these studies, MB231 cells constitutively expressing SLC5A8 (MB231-SLC5A8) were used. First, the cells were synchronized in G1 phase and the cell cycle progression was analyzed. At 8 h following release from synchronization, 13% of cells remained in the G1 phase in vector-only cells whereas a significantly higher percent of SLC5A8-expressing cells were in G1 phase (Figure 3A). At 12 h following release, vector-only cells showed a distribution with 60% in G1 and 29% in G2/M phase; in contrast, SLC5A8-expressing cells had a distribution of 42% of cells in G1 phase and 38% in G2/M phase. These results show that cell cycle is delayed in cancer cells by SLC5A8 expression. Increased accumulation of cells in G2/M phase with a decrease in survivin expression has already been reported [26]. These findings support an essential role of survivin as CPC protein during mitosis. We then wanted to know whether SLC5A8 expression disrupts the formation of a functional CPC. Reports show that survivin localizes Aurora B to its substrates and increases its kinase activity [27]. Survivin is sequentially colocalized with Aurora B and the inner centromere protein INCENP to the centromeres, the spindle midzone, and then to the cleavage furrow during mitosis, a typical behavior of chromosomal passenger proteins. In the absence of survivin, Aurora B cannot localize to the spindle midzone during anaphase [27]. To examine the formation of functional CPC, vector-only MB231 cells and SLC5A8-expressing MB231 cells were blocked with nocodazole in mitotic phase, and then survivin was immunoprecipitated to analyze the presence of Aurora B in the immunoprecipitate. In vector-only cells, survivin co-immunoprecipitated with Aurora B; there was a significant reduction in the binding of Aurora B to survivin in SLC5A8-expressing cells (Figure 3B). These data provide evidence for impaired CPC formation. Immunofluorescence analysis also showed decreased colocalization of survivin and Aurora B during mitosis in SLC5A8-expressing cells (Figure 3C). Impaired formation of functional CPC and abnormal mitotic structures observed in SLC5A8-expressing cells prompted us to check for cells with abnormal nuclei. Vector-only MB231 cells and SLC5A8-expressing MB231 cells were stained with a stain specific for plasma membrane (Cellmask™) for analysis of binucleated cells as a read-out for defective mitosis. As expected, SLC5A8-expressing cells had an increase in the number of binucleated cells

(Figure 3D, E). Similar results were obtained with SLC5A8-expressing MCF7 cells (Figure 3D, E).

SLC5A8 decreases the anti-apoptotic activity of survivin

Survivin is also an inhibitor of caspases. Therefore, we compared caspase activity, PARP cleavage, and apoptosis between vector-only MB231 cells and SLC5A8-expressing MB231 cells (Figure 4A–C). There was a 2-fold increase in caspase activity in MB231 cells as a result of SLC5A8 expression. The *N*-terminal extension of the BIR2 domain of IAPs inhibits caspase 3 by blocking the substrate-binding pocket [28]. Studies by Tamm *et al* [29] have shown that survivin inhibits the activities of caspase 3 and 9 whereas studies by Liu *et al* [30] suggest that the anti-apoptotic function of survivin is related to its ability to inhibit mitochondrial and apoptosis-inducing factor-dependent pathways. Thus, a decrease in survivin causes apoptosis in caspase-dependent and independent pathways. Our studies show that SLC5A8-induced depletion of survivin results not only in increased caspase activity but also in increased PARP cleavage and in increased apoptotic cells. In addition, the tumor-forming ability of MB231 cells in mouse xenografts was markedly reduced when SLC5A8 was expressed in these cells (Figure 4D). Similarly, there was also a decrease in colony formation capability in SLC5A8-expressing MB231 cells (Figure 4E, F). The increase in apoptosis and the decrease in colony formation observed in SLC5A8-expressing MB231 cells were seen even in the absence of pyruvate in the culture medium; however, the presence of pyruvate enhanced these effects to some extent.

If SLC5A8-induced depletion of survivin is responsible for activation of the apoptotic process, forced expression of survivin in SLC5A8-expressing cancer cells should prevent apoptosis. To test this, we overexpressed survivin in vector-only MB231 cells and in SLC5A8-expressing MB231 cells, and then monitored apoptosis using the TUNEL assay. As expected, there were more TUNEL-positive cells in SLC5A8-MB231 cells than in vector-only MB231 cells (Figure S4). Overexpression of survivin suppressed apoptosis not only in vector-only MB231 cells but also in SLC5A8-MB231 cells (Figure S4A). More importantly, the SLC5A8-induced apoptosis was mitigated to a marked extent by overexpression of survivin (Figure S4B).

SLC5A8 expression increases chemosensitivity in breast cancer cells

Survivin is considered as an important protein determining the sensitivity of cancer cells to chemotherapeutic drugs or radiation. It has been shown that increased expression of survivin during chemotherapy is associated with increased chemoresistance and poor prognosis [31]. Therefore, we wanted to investigate whether the SLC5A8-induced decrease in survivin expression would increase the sensitivity of breast cancer cells to chemotherapeutic drugs. MCF7-SLC5A8 and MB231-SLC5A8 cells along with corresponding vector-only cells were exposed to varying concentrations of cisplatin and 5-fluorouracil, and the extent of apoptosis induced was monitored (Figure 5). Ectopic expression of SLC5A8 in both cancer cell lines enhanced their sensitivity to these two chemotherapeutic agents.

SLC5A8 expression decreases survivin levels independent of histone acetylation status

Ectopic expression of SLC5A8 in breast cancer cells was able to decrease survivin expression and thereby disrupt CPC formation, and enhance apoptosis and chemosensitivity. These effects are specific to survivin because expression of other IAPs was not altered as a result of SLC5A8 expression (Figure S5). The tumor-suppressive potential of SLC5A8 is attributed to the transport of HDAC inhibitors into cancer cells. Our studies have provided evidence for the tumor-suppressive role of this transporter even in the absence of any substrate of the transporter that functions as an HDAC inhibitor. To rule out the possibility of HDAC inhibition in our experiments by some, hitherto unrecognized, HDAC inhibitors

present in the medium, we examined the acetylation status H4K16 under the same conditions that were employed in previous experiments. There was no change in the level of H4K16 acetylation in MCF7 and MB231 cells as a result of SLC5A8 expression (Figure 6A), showing that the observed effects of SLC5A8 on survivin expression is independent of HDAC inhibition. Even though there was no involvement of HDACs, the decrease in total survivin content of cancer cells as a result of SLC5A8 expression was due to inhibition of survivin expression at transcriptional level as evidenced from the *survivin*-promoter activity using a reporter assay (Figure 6B). There are many transcriptional regulators of survivin, of which p53, NF-κB and STAT3 are of major importance [32]. MCF7 cells have a functional wild type p53 whereas MB231 cells have a mutant p53; but the ability of SLC5A8 to decrease survivin expression was seen in both cell lines, thus ruling out involvement of p53 in the observed effects. Similarly, immunoblot analysis of phosphorylated NF-κB did not show any significant difference between control and SLC5A8-expressing cells (Figure S6). But, there was a significant decrease in STAT3 phosphorylation (Tyr705) in SLC5A8-expressing cells compared to vector-only controls (Figure 6C). Immunofluorescence analysis confirmed the western blot data (Figure 6D).

DISCUSSION

The present studies demonstrate a novel molecular mechanism for the tumor-suppressive function of the plasma membrane transporter SLC5A8 that is independent of its transport function. SLC5A8 is the first transporter with a demonstrated role in tumor suppression. Until now, the tumor-suppressive role of this transporter was focused on the ability of the transporter to mediate the entry of the HDAC inhibitors pyruvate and butyrate into cells. Butyrate is physiologically relevant to the tumor-suppressive role of SLC5A8 in colon whereas pyruvate is relevant in other systemic organs. The present studies show that the transporter is capable of tumor suppression not only through HDAC inhibition but also through another, hitherto unrecognized, mechanism involving survivin depletion. The latter process involves at least two distinct mechanisms. First, the transporter binds to survivin and facilitates the sequestration of the latter to the plasma membrane. This causes a decrease in the levels of survivin in nucleus. Second, the transporter inhibits survivin expression at the transcriptional level, and this process occurs through suppression of STAT3 phosphorylation. STAT3 conveys signals from the cell surface to the nucleus upon activation by cytokines and growth factors. Binding of ligands to respective cell-surface receptors induces tyrosine phosphorylation of STAT3 by Janus kinase, or growth factor receptor tyrosine kinases. The phospho-STAT3 forms a dimer, which translocates to nucleus and regulates expression of target genes. Survivin is a target of STAT3; therefore, the observed SLC5A8-induced decrease in pSTAT3 might explain the inhibition of survivin expression at the transcriptional level. The identity of molecular targets upstream of STAT3 that are affected by SLC5A8 remains to be determined. The change in the subcellular localization and in the expression of survivin resulting from ectopic expression of SLC5A8 in cancer cells diminishes the anti-apoptotic function of survivin, causing cell death in cancer cells subsequent to forced expression of the transporter. Based on what is known on the role of survivin in cell cycle and cell division, ectopic expression of SLC5A8 in cancer cells also leads to blockade of cell cycle and cell division. Collectively, these data show that SLC5A8 is indeed a tumor suppressor and that this function does not depend solely on the ability of the transporter to alter histone acetylation through butyrate or pyruvate.

The significance of the present studies lies not only in the identification of a novel molecular mechanism for the tumor-suppressive function of SLC5A8 but also in the demonstration of such a role in vivo. Several studies have speculated a tumor-suppressive role for SLC5A8 solely based on the observations that the transporter is silenced in a wide variety of cancers [1–4]. Until a few years ago, among the substrates of SLC5A8, only butyrate was known to

be an HDAC inhibitor and hence considered to be relevant to the ability of the transporter to induce apoptosis in colon cancer cells. Indeed, we were able to demonstrate that colon cancer cells do not express this transporter, but when made to express the transporter ectopically, these cells underwent apoptosis in the presence of butyrate [11]. Our recent discovery that pyruvate is also an HDAC inhibitor and an excellent substrate for SLC5A8 provided a logical explanation as to why the transporter expression is silenced in cancers of non-colonic tissues [12]. Pyruvate is present in circulation at ~100 μM, a concentration relevant to the Michaelis constant of the transporter (~50 μM). Therefore, silencing of the transporter expression in non-colonic tissues upon tumor formation is likely to prevent the entry of pyruvate from blood into cancer cells. The present studies reporting a new mechanism for the tumor-suppressive function of the transporter are relevant to all tissues (colonic as well as non-colonic) because this function is independent of the transport function of SLC5A8 and hence is not related to whether or not the physiological substrate for the transporter is butyrate or pyruvate.

These studies demonstrate the existence of a reciprocal relationship between the expression levels of SLC5A8 and survivin. In non-malignant cells, SLC5A8 expression is high and survivin expression is low; in contrast, in malignant cells, the situation is reversed with low expression of SLC5A8 and high expression of survivin. The high level of survivin expression is obviously advantageous to cancer cells for promotion of mitosis and suppression of apoptosis. Interestingly, even in the presence of decreased levels of survivin, normal cells do not undergo apoptosis; they also perform mitotic function, though at a lower rate than malignant cells. This is necessarily not a paradox because normal cells and cancer cells do not differ solely in the relative levels of SLC5A8 and survivin expression. We have shown previously that normal colon and mammary epithelial cells express high levels of SLC5A8 and take up the HDAC inhibitors butyrate and pyruvate, but still do not undergo apoptosis [11, 12]. In contrast, colon cancer cells and breast cancer cells do not express SLC5A8 and thus do not take up butyrate and pyruvate; however, when these cells are forced to express SLC5A8 and to take up the HDAC inhibitors, the cells undergo apoptosis [11, 12]. This suggests that SLC5A8-mediated entry of HDAC inhibitors is not detrimental to normal cells but it is to cancer cells, thus highlighting the relevance of other biological differences between normal and cancer cells to this phenomenon.

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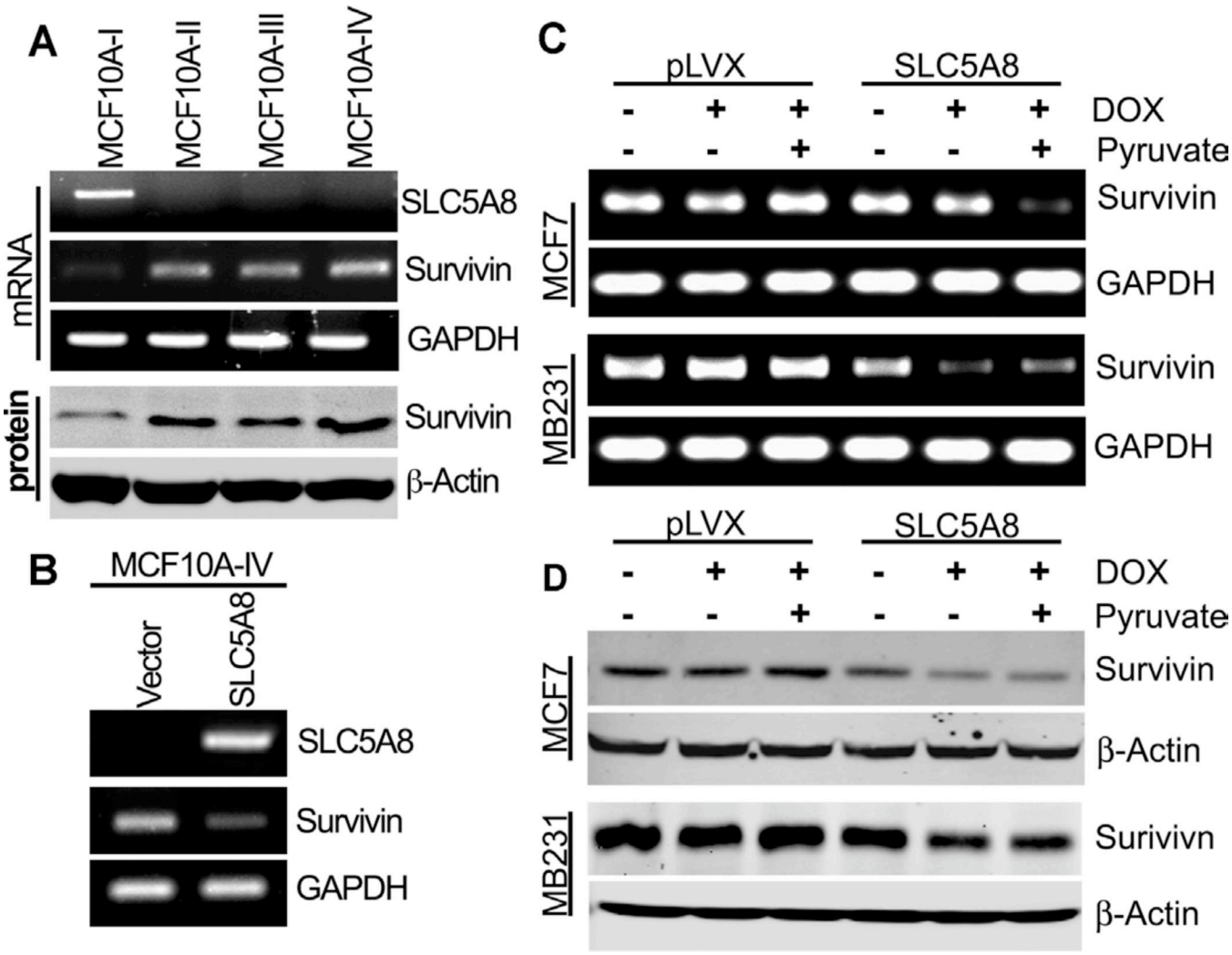

Figure 1.

SLC5A8 decreases survivin mRNA and protein. A, Semi-quantitative RT-PCR of relative mRNA levels of survivin and SLC5A8 in MCF10A series cell lines: MCF10A-I (normal epithelium), MCF10A-II (oncogenically initiated premalignant epithelium), MCF10A-III (low grade carcinoma), and MCF10A-IV (high-grade metastatic carcinoma). GAPDH was used as internal control. B, MCF10A-IV cells were made to stably express SLC5A8 through lentiviral transfection. RNA was isolated and expression of survivin was analyzed by RT-PCR. C and D, The expression of SLC5A8 was induced with doxycycline in MCF7-SLC5A8-TetOn and MB231-SLC5A8-TetOn for 72 h. Pyruvate (1 mM) was added to the medium after 48 h of doxycycline treatment as indicated. At 72 h, expression of survivin was analyzed by RT-PCR (C) and immunoblotting (D). β-Actin was used as an internal control for Western blot.

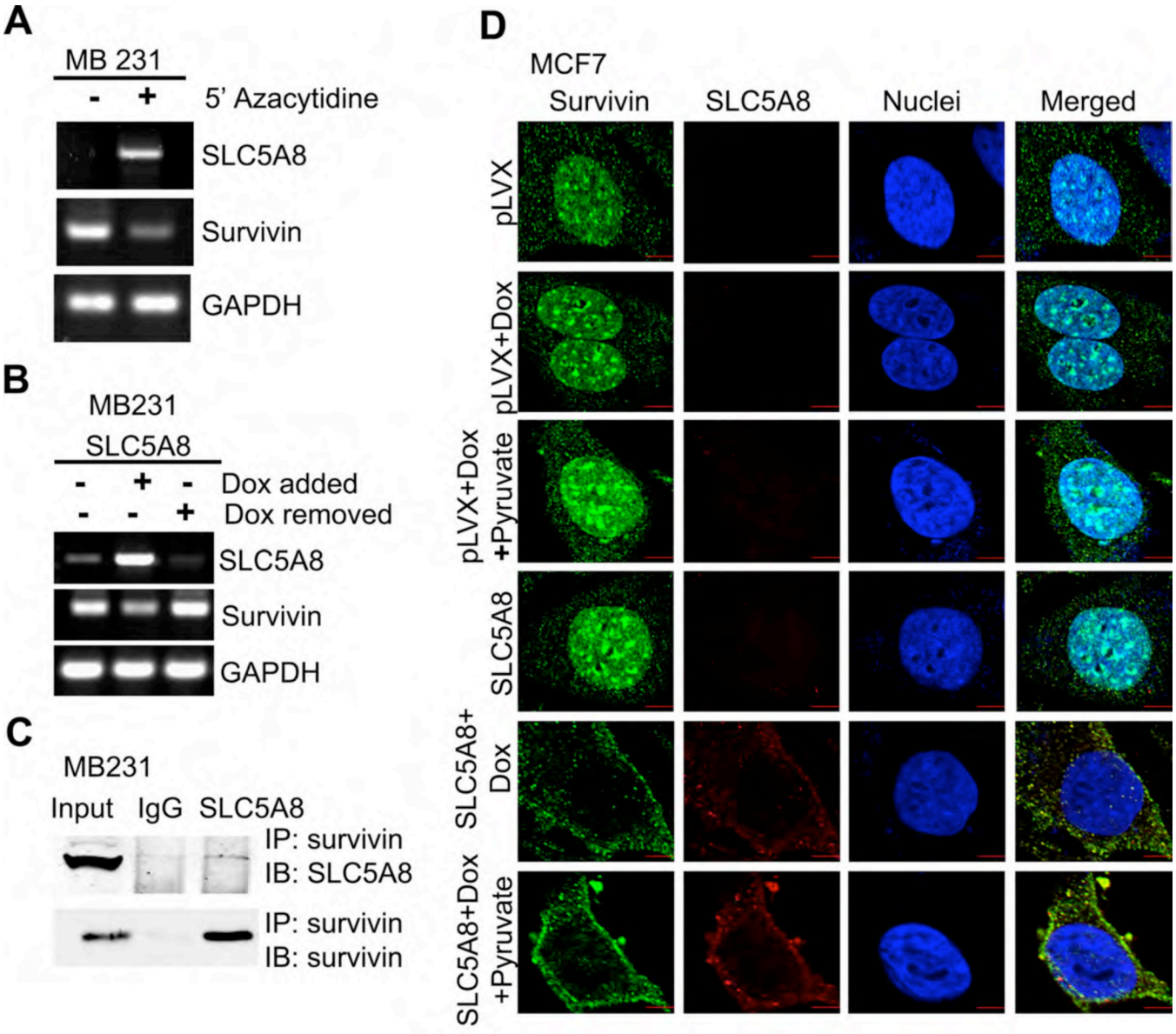

Figure 2.

SLC5A8 decreases survivin levels in nucleus through sequestration to plasma membrane. A, Cells were treated with 3 μg/ml of 5'-azacytidine and expression of SLC5A8 and survivin was analyzed by semi-quantitative RT-PCR. B, The expression of SLC5A8 was induced with doxycycline in MB231-SLC5A8-TetOn for 3 days. After 3 days, doxycycline was removed and the cells were cultured for an additional 3 days. RNA was isolated at day 3 and 6, and survivin and SLC5A8 expression was analyzed by semi-quantitative RT-PCR. C, Survivin was immunoprecipitated from MB231-SLC5A8 cells using anti-survivin antibody and immunoblotted with anti-SLC5A8 antibody. D, The expression of SLC5A8 was induced with doxycycline in MCF7-SLC5A8-TetOn cells for 72 h. Corresponding vector-only MCF7 cells were also used in parallel. Pyruvate (1 mM) was added to the indicated cells after 48 h of doxycycline treatment. Cells were fixed at 72 h and immunofluorescence analysis was carried out with anti-survivin and anti-SLC5A8 antibodies and visualized with

Alexafluor 488- and Alexafluor 568-conjugated secondary antibodies respectively. Hoechst was used to locate the nucleus. Scale bar represents 10 μm.

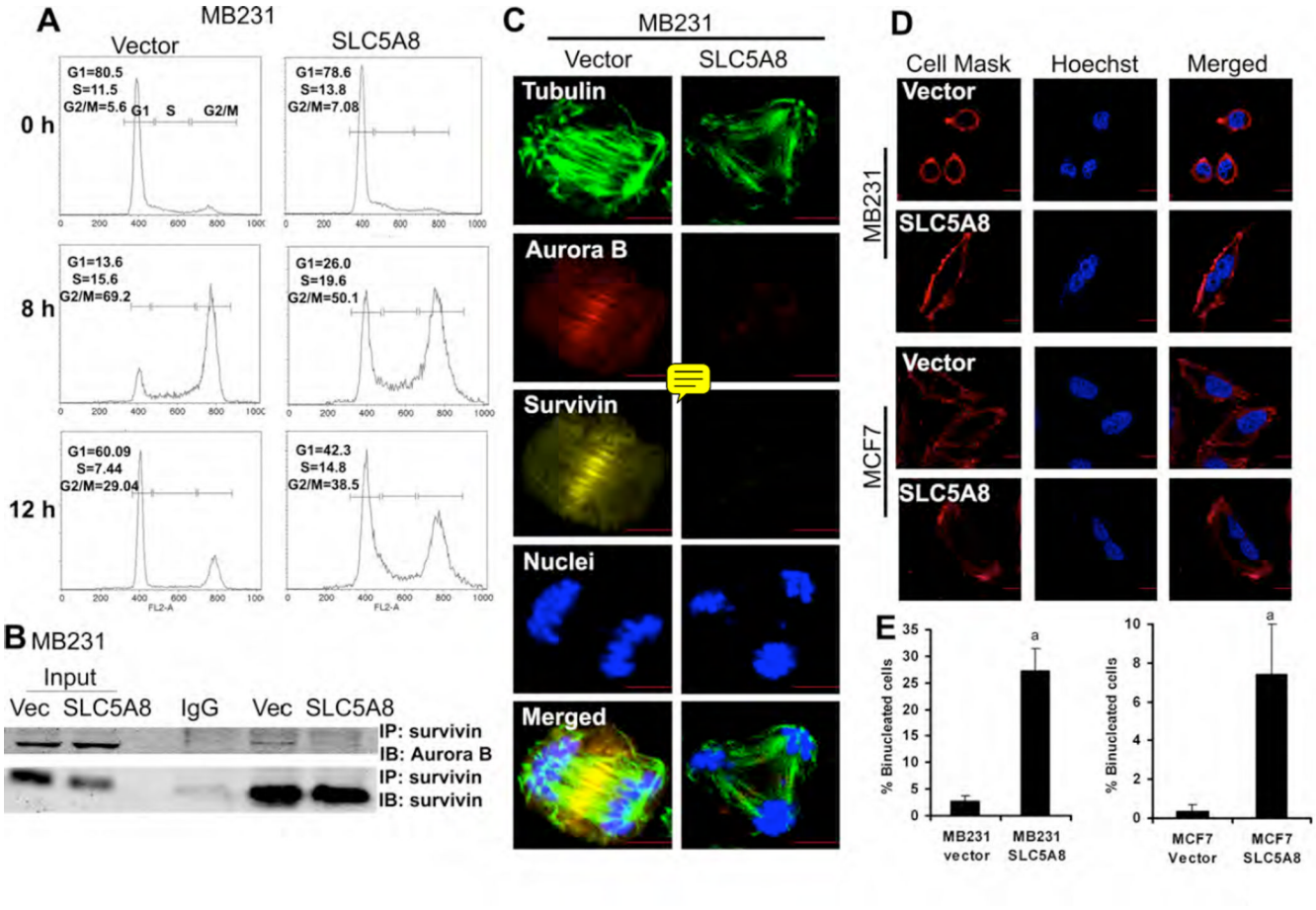

Figure 3.

Depletion of survivin by SLC5A8 impairs CPC formation. A, MB231-SLC5A8 cells were synchronized in G1 phase with double thymidine block and released in fresh media with 10% FBS. The progression through cell cycle was followed at indicated time points using propidium iodide staining. Values presented are means of three independent experiments. B, MB231-SLC5A8 cells were synchronized in mitotic phase with nocodazole, and survivin was immunoprecipitated using anti-survivin antibody and immunoblotted with anti-Aurora B antibody. C, MB231-SLC5A8 cells were synchronized in mitotic phase with nocodazole, and immunofluorescence analysis was carried out with specified antibodies. Hoechst was used to locate the nucleus. Scale bar represents 5μm. D, Binucleated cells were analyzed using Cell mask stain in MB231-SLC5A8 and MCF7-SLC5A8 and corresponding vector cells. Scale bar represents 10μm. Binucleated cells were counted in six independent fields and are depicted in (E). Data are presented as means ± SE. a, $p<0.05$.

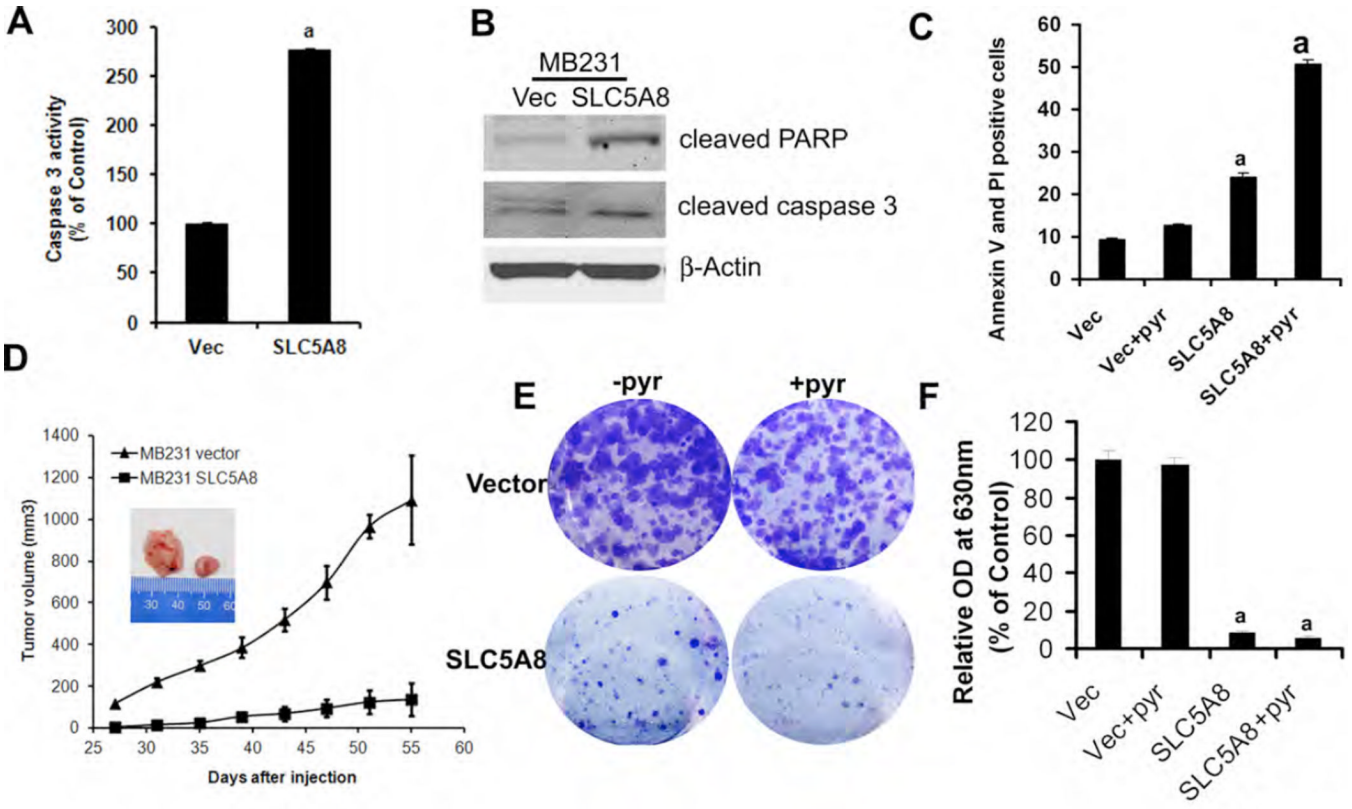

Figure 4.

SLC5A8 increases cell death in breast cancer cells and decreases tumor formation in mouse xenografts. A, Caspase 3 activity was increased in MB231-SLC5A8 cells compared to control vector-only cells. Values (means ± SE) are from three independent experiments. a, $p<0.05$. B, Immunoblot analysis of cleaved PARP and active caspase 3 in MB231-SLC5A8 and vector-only cells. C, Flowcytometric analysis of Annexin V-propidium iodide positive cells in MB231-SLC5A8. Values (means ± SE) are from three independent experiments. a, $p<0.05$. D, MB231-SLC5A8 and vector-only cells (1×10^7 cells) were injected into mammary pads of athymic nude mice, and the growth of tumor was monitored using a caliper. Inset shows the size of the tumors at the end of experimental period (left, vector-only MB231 cells; right, MB231-SLC5A8 cells). E, Colony formation assay was performed with vector-only MB231 cells and MB231-SLC5A8 cells in the presence and absence of pyruvate. The colonies were stained with Giemsa and quantified (F). a, $p<0.05$.

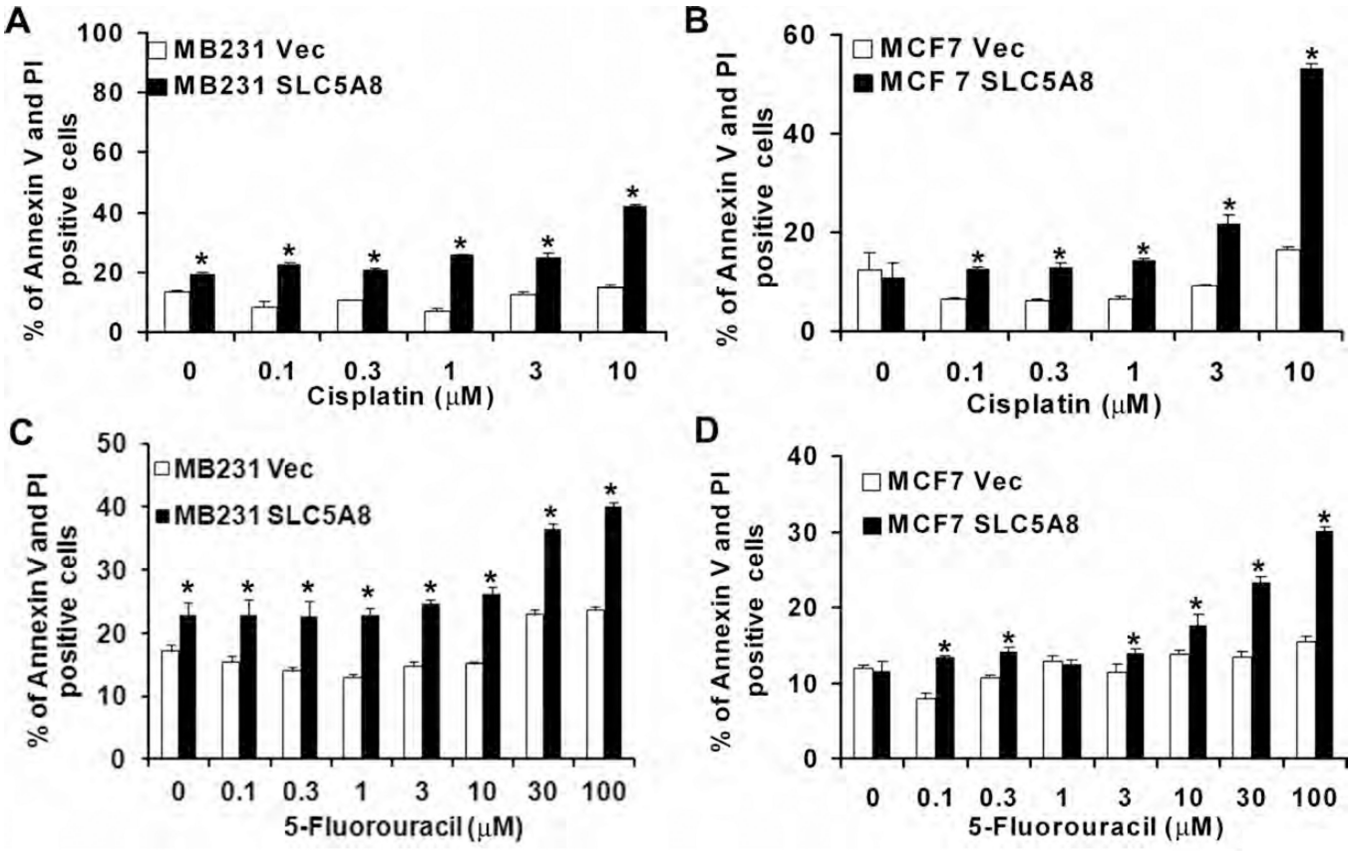

Figure 5.

SLC5A8 increases chemosensitivity in breast cancer cells. MCF7-SLC5A8 and MB231-SLC5A8 cells and corresponding vector-only cells were treated with indicated concentrations of 5-fluorouracil or cisplatin for 72 h. The apoptotic and necrotic cells were quantified by Annexin Vpropidium iodide staining. Values (means ± SE) are from three independent experiments. *, $p<0.05$.

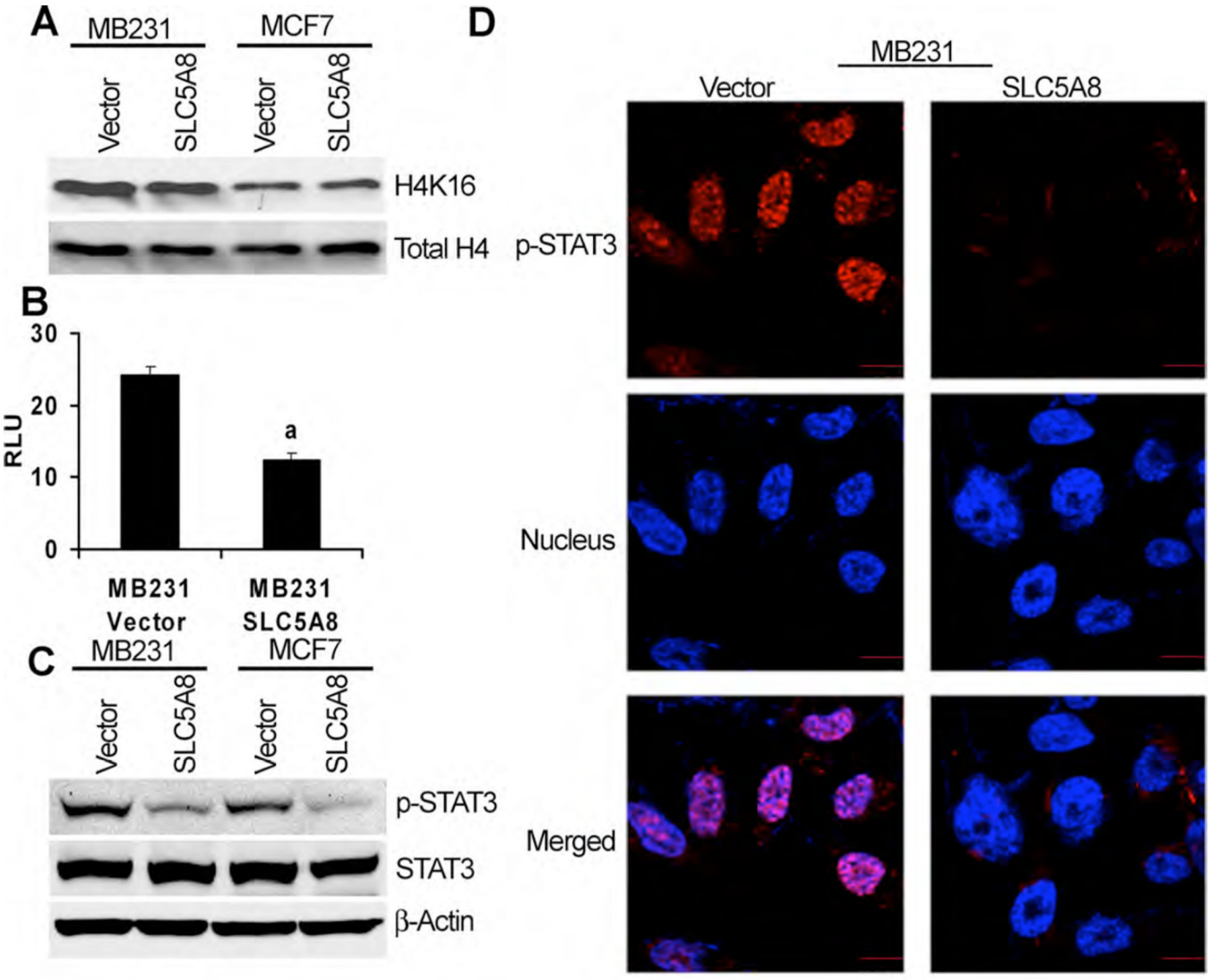

Figure 6.

SLC5A8 expression has no effect on histone acetylation but decreases STAT3 phosphorylation in breast cancer cells. A, Immunoblot analysis of H4K16 and total H4 in MCF7-SLC5A8 and MB231-SLC5A8 cells and corresponding vector-only cells B, MB231-SLC5A8 cells and corresponding vectoronly cells were transfected with *survivin*-promoter and β-actin renilla luciferase constructs. After 48 h, the reporter activity was measured using the dual luciferase assay; data represent values after normalization with renilla luciferase. a, $p<0.05$. C, Immunoblot analysis of phosphorylated and total STAT3 in MCF7-SLC5A8 and MB231-SLC5A8 cells and corresponding vector-only cells. D, Immunofluorescence analysis of phosphorylated STAT3 in MB231-SLC5A8 cells and corresponding vector-only cells. Scale bar represents 10 μm.