Effect of Cell Cycle on Cell Surface Expression of Voltage-Gated Sodium Channels and Na^+,K^+-ATPase

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Abstract

Voltage-gated sodium channels (VGSCs) are the target for many therapies. Variation in membrane potential occurs throughout the cell cycle, yet little attention has been devoted to VGSCs and Na^+,K^+-ATPase in the cell cycle. We hypothesized that in addition to doubling DNA and cell membrane in anticipation of cell division, there should be a doubling of VGSCs and Na+,K+-ATPase compared to non-dividing cells. We tested this hypothesis in eight immortalized cell lines by correlating immunocytofluorescent labeling of VGSCs or Na^+,K^+-ATPase, with propidium iodide or DAPI fluorescence using flow cytometry. Cell surface expression of VGSCs during phases S through M was double that seen during phases G0 - G1. By contrast, Na^+,K^+-ATPase expression increased only 1.5-fold. The increases were independent of baseline expression of channels or pumps. The variation in VGSC and Na^+,K^+-ATPase expression has implications for both our understanding of sodium's role in controlling the cell cycle and variability of treatments targeted at these components of the Na^+ handling system.

Introduction

The modulation of voltage-gated sodium channels (VGSCs) and/or Na^+,K^+-ATPase (sodium pumps) plays a key role in the expression and treatment of several disease states including neuropathic pain Gould [1], cancer [2-4], hypertension [5], and epilepsy [6] For example, mutations in the SCN-9A gene that encodes the $Na_V1.7$ VGSC are responsible for erythromelalgia, [7] paroxysmal extreme pain disorder, and congenital insensitivity to pain [8-10]. Over-expression of VGSCs and Na^+,K^+-ATPase in aggressive carcinomas enhances their ability to invade normal tissues and metastasize, but also makes them suitable targets for cancer therapy [11-13]. In addition, dysregulation of Na^+,K^+-ATPase contributes to disease states such as epilepsy, hypoglycemia or ischemia [14]. Several technologies designed to selectively target the over- or under-expression of sodium channels and sodium pumps to better manage these debilitating diseases have been proposed. Because VGSC and Na^+,K^+-ATPase expression varies based on the status of the organism, cellular environment, and on the stage of disease evolution, an understanding of the dynamics of underlying function and change is essential for developing effective therapeutics and determining the optimum timing for their delivery.

We have known for decades that ion channels and shifts in membrane potential play important roles in the cell cycle that are responsible for preparing the cell for the sequence of events that must take place to ensure maintenance, replication and survival, through control of constantly changing intra- and extracellular ion concentrations [15]. For example, during the G1/S transition, the membrane potential is hyperpolarized due to influx of sodium and efflux of potassium [15]. Depolarization occurs during the G2/M transition caused by efflux of chloride ions [16]. During the G2/M transition, the activity of potassium channels is decreased, leading to a decrease in intracellular potassium, resulting in hyperpolarization [15, 17]. In addition, the increase of intracellular calcium has been noted for its involvement with nuclear envelope breakdown, metaphase-anaphase transition, and cytokinesis [17]. Interactive dynamic changes associated with the role of potassium, calcium and chloride in the cell cycle

have been studied extensively, but in light of the importance of VGSCs and Na^+,K^+-ATPase in membrane excitation and the maintenance of the electrochemical gradient in excitable cells, it is surprising that less is known about the role of VGSCs and Na^+,K^+-ATPase in modulating membrane potential and coordinating function during the cell cycle.

What is known is that shifts in the cell membrane potential are essential for cell cycle progression and that VGSCs and Na^+,K^+-ATPase play an important role in this process [15]. The opening of VGSCs that allows for the entry of sodium into the cell and depolarization of the cell membrane is required for transition from G0 to G1 [15] The sodium pump returns sodium ions to the extracellular space and restores the resting membrane potential and the intracellular sodium concentration. Reestablishing the electrochemical gradient across the cell membrane is necessary for the cell to progress through S phase [15].

In spinal cord astrocytes, the influx of sodium achieves a more than four-fold increase of intracellular sodium in S phase-arrested cells compared to non-arrested controls [18]. It is not clear whether the excess Na^+ concentration is due to an upregulation in the number of cell surface VGSCs, an increase in the frequency of channel opening or an inhibition of sodium pump function [18-20]. Treating spinal astrocytes with tetrodotoxin, a VGSC blocker, has no effect on cell proliferation, but treating with low doses of ouabain, a Na^+,K^+-ATPase inhibitor, increases the proportion of quiescent cells in S phase [18]. This information taken together is evidence that an increase or decrease of extracellular potassium, an increase of intracellular sodium, or membrane depolarization are important contributors to astrocyte proliferation.

Electrophysiologic recording revealed that in many forms of cancer, depolarization initiates robust sodium currents [20]. Additionally, Coombs et al. (1992) discovered that there is an initial increase in sodium currents immediately after depolarization. Sodium currents remain elevated through meiosis and the first cleavage. The increase in sodium currents is evidence for an increase in VGSC activity in S through M phases.

Aggressive carcinomas over-express VGSCs compared to their non-cancerous or stromal counterparts [4, 21], but even in highly malignant cancers, there is relative heterogeneity in VGSC expression in cells within a given solid tumor that fall into 2 groups. The cells with the highest level of expression comprise 30-45% of the total. We hypothesized that the most highly expressing cells are those in the S-M phase of the cell cycle, whereas the cells that express relatively fewer VGSCs are in the G0-G1 phase. Accordingly, we assessed the relative levels of VGSCs and Na^+,K^+-ATPase protein expression in eight immortalized cancer cell lines at phases of the cell cycle in which there are single (G0-G1) or double (S-M) copies of DNA using a flow cytometry paradigm. To determine whether variation of cell-surface VGSC and Na^+,K^+-ATPase expression during the cell cycle is dependent upon the baseline expression of cells, we used both noncancerous and cancerous cell lines.

A representative graph of VGSC cell surface expression from each cell line is displayed in Figure 1. For each replicate, MFI gated for VGSCs in cells with single copy DNA (G0-G1 phases) and double copy DNA (S-M phases) was determined. For all cell lines, the MFI in S-M phase cells was significantly greater than VGSC protein expression in G0-G1 phase cells ($p<.01$), with the mean MFI for cells in S-M phases was between 1.86- and 2.38-fold greater than that of cells in G0-G1 phases ($\bar{X}$ = 2.11 ± 0.065) across all 8 cell lines (Fig. 3A). A one-way ANOVA showed no main effect of relative expression across the eight cell lines.

Likewise, representative graphs of Na^+,K^+-ATPase cell surface expression from each cell line are depicted in Figure 2. For all cell lines, MFI for Na^+,K^+-ATPase protein expression in S-M phase cells was significantly greater than for Na^+,K^+-ATPase protein expression in G0-G1 phase cells with the MFI for Na^+,K^+-ATPase in cells in S-M phases was between 1.47 and 1.86 times greater than for that of cells in G0-G1 phases (Fig. 3B), with an average across all 8 cell lines of 1.64 ± 0.04. The relationship between VGSC and Na^+,K^+-ATPase expression and the phase groupings of the cell cycle were not significantly different whether the cell line was from a human or murine source or were expressed in cancerous or non-cancerous cells.

Discussion

With this series of experiments, we demonstrated that the expression of cell-surface VGSCs doubles during cell division compared to G0/G1 phases of the cell cycle. Similarly, cell-surface expression of Na^+,K^+-ATPase increases by 1.5-fold during the stages of cell division when there is a doubling of DNA. These increases occurred irrespective of the baseline expression of these sodium handling proteins. In addition, the upregulation occurred in cancerous and noncancerous cells lines derived from three different source organs.

It is tempting to simplify these results as VGSC expression doubling because DNA doubles. However, there are far too many modulators of both RNA and protein expression for a direct one-to-one interpretation. Moreover, the DNA for Na^+,K^+-ATPase also doubles, yet the protein increases only by 1.5-fold. Many proteins critical to the cell cycle up- or down-regulate during specific cycle phases. Thus, we propose that our findings of a 2-fold increase for VGSCs and a 1.5-fold increase for Na^+,K^+-ATPase may be important for our understanding of the progression through cell division.

In addition, these results are likely to be important for our understanding of the mechanism or variability in efficacy of current and proposed treatments of diseases that involve modulation of sodium channels and/or sodium pump. Modulation of VGSC subtype distribution and expression has been noted in many genetic and iatrogenic disease states. For example, changes in VGSC expression and distribution have been correlated with alterations in pain perception associated with peripheral nerve injury and inflammation [1] and more recently with inherited channelopathies, such as inherited primary familial erythromelalgia, paroxysmal extreme pain disorder and congenital insensitivity to pain [8-10, 22-24]. VGSC dysregulation has also been implicated in cases of essential hypertension [5] and epilepsy [6]. In

addition, alterations in Na^+,K^+ -ATPase expression and function has been associated with hypertension, inflammation [25-26] and the control of insulin and the development of painful diabetic polyneuropathy [1, 27]. Most recently, VGSCs have been shown to be directly correlated with tissue invasion, malignancy and metastasis in many forms of cancer [2-4, 11-13, 21, 28-30] and Na^+,K^+-ATPase has been shown to play a role in oncogenesis, tumor growth and development and metastasis [31].

The observed changes in VGSC and Na^+,K^+-ATPase expression seen in the present study, may provide the basis for a more thorough understanding of the sodium handling system in cell replication and disease, a focus for seeking targeted treatments and a guide for optimizing the timing of therapeutic intervention.

Materials And Methods

Cell culture

We compared VGSC and Na^+,K^+-ATPase expression between different forms of cancer using human A549 lung and MDA-MB-231 breast cancer and H28 mesothelioma cells compared with the VGSC and Na^+,K^+-ATPase expression in their non-cancerous counterparts, MRC5 non-cancerous lung cells, MCF10a non-cancerous breast cells and MeT-5a pleural effusion cells respectively. Across-species comparisons for VGSC and Na^+,K^+-ATPase expression in malignant cells were also made using murine AB1 mesothelioma and 4T1 triple-negative breast cancer cells. All cell lines were purchased from American Type Culture Collection (www.atcc.org), except AB1 cells, which were purchased from Millipore Sigma (www.sigmaaldrich.com). A549 cells were cultured with F-12K medium (www.fishersci.com) supplemented with 10% Fetal Bovine Serum (FBS) (www.thermofisher.com) and 1% penicillin/streptomycin (pen/strep; www.thermofisher.com). MRC5 cells were cultured with Dulbecco's Modified Eagle s Medium (DMEM; www.thermofisher.com) supplemented with 10% FBS and 1% pen/strep. H28, MDA MB 231, 4T1 and AB1 cells were cultured with Roswell Park Memorial Institute (RPMI) medium (www.atcc.org) with 10% FBS, 1% pen/strep and 25mM N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES; www.thermofisher.com). MCF10a cells were cultured with mammary epithelial growth medium (MEGM) mediPBSa supplemented with 10% FBS, 1% pen/strep and MEGM kit (https://bioscience.lonza.com). MeT-5a cells were cultured with M199 media (www.sigmaaldrich.com) supplemented with 10% FBS, 1% pen/strep, 3.3 nM epidermal growth factor (EGF; www.sigmaaldrich.com), 400 nM hydrocortisone (www.sigmaaldrich.com), 870 nM bovine insulin (www.sigmaaldrich.com), 20 nM HEPES and 0.1 mL trace elements (ecatalog.corning.com).

Antibody conjugation

The panspecific antibodies for VGSCs (www.alomone.com) and Na^+,K^+-ATPase (www.abcam.com) were conjugated to allophycocyanin (APC; Ex/Em: 594/633; biotium.com) or to R-phycoerythrin (RPE; Ex/Em: 488-561/578; biotium.com) using the Biotium Mix-N-Stain protocol.

Flow cytometry

Cells were cultured in T75 flasks to 80-90% confluence and harvested using 2-3 mL Corning CellStripper (ecatalog.corning.com), fixed with 0.5% paraformaldehyde for 10 minutes, blocked with 5% goat serum and 3% bovine serum albumin solution for 1 hour, and washed with 1X phosphate buffered saline (PBS). Cells were then incubated in APC-conjugated antibody at 1:200 in PBS and overnight at 4ºC protected from light. The cells were then washed once in PBS and then were resuspended in propidium iodide (PI; www.bdbiosciences.com) or 1ug/mL 4',6-diamidino-2-phenylindole (DAPI; www.thermofisher.com) Relative fluorescence of DNA label vs. VGSC or Na^+,K^+-ATPase label in samples were determined using BD Fortessa or BD Canto flow cytometers. The cytometer configuration is depicted in Supplementary Table 1. Samples consisting of approximately 250,000 cells were gated into G0-G1 phase (low DNA fluorescence) and S-M phase (high DNA fluorescence) groups, and the degree of APC or RPE fluorescence was measured and plotted as a histogram. There were three replicates of each experiment.

Statistical analysis

For each cell line, the median APC fluorescence (VGSCs) was determined for G0-G1 and S-M phases. To determine relative cell surface expression in these phases, the mean of the three determinations of median fluorescence intensity (MFI) for S-M cells was divided by the mean of the three determinations of MFI for the G0-G1 phases. The relative cell surface expression of Na^+,K^+-ATPase was determined for each cell line in the same manner, using the mean of three MFI and difference among the MFIs analyzed using one-way ANOVAs.

Abbreviations

APC: allophycocyanin

MEGM: mammary epithelial growth medium

MFI: median fluorescence intensity

Na^+,K^+-ATPase: sodium, potassium adenosine trisphosphate

pen/strep: penicilamine / streptomycin

PI: propidium iodide

RPE: R-phycoerythrin

RPMI: Roswell Park Memorial Institute

Declarations

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Author contributions

SIE conducted all experiments. SIE and DP were responsible for the data analysis. All authors contributed to the experimental design, and the writing and editing of the manuscript.

Competing interests

HJG and DP are on the Board of Directors and hold equity stakes in OMT. SIE has no competing interests.

Data availability

The datasets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request.

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Figures

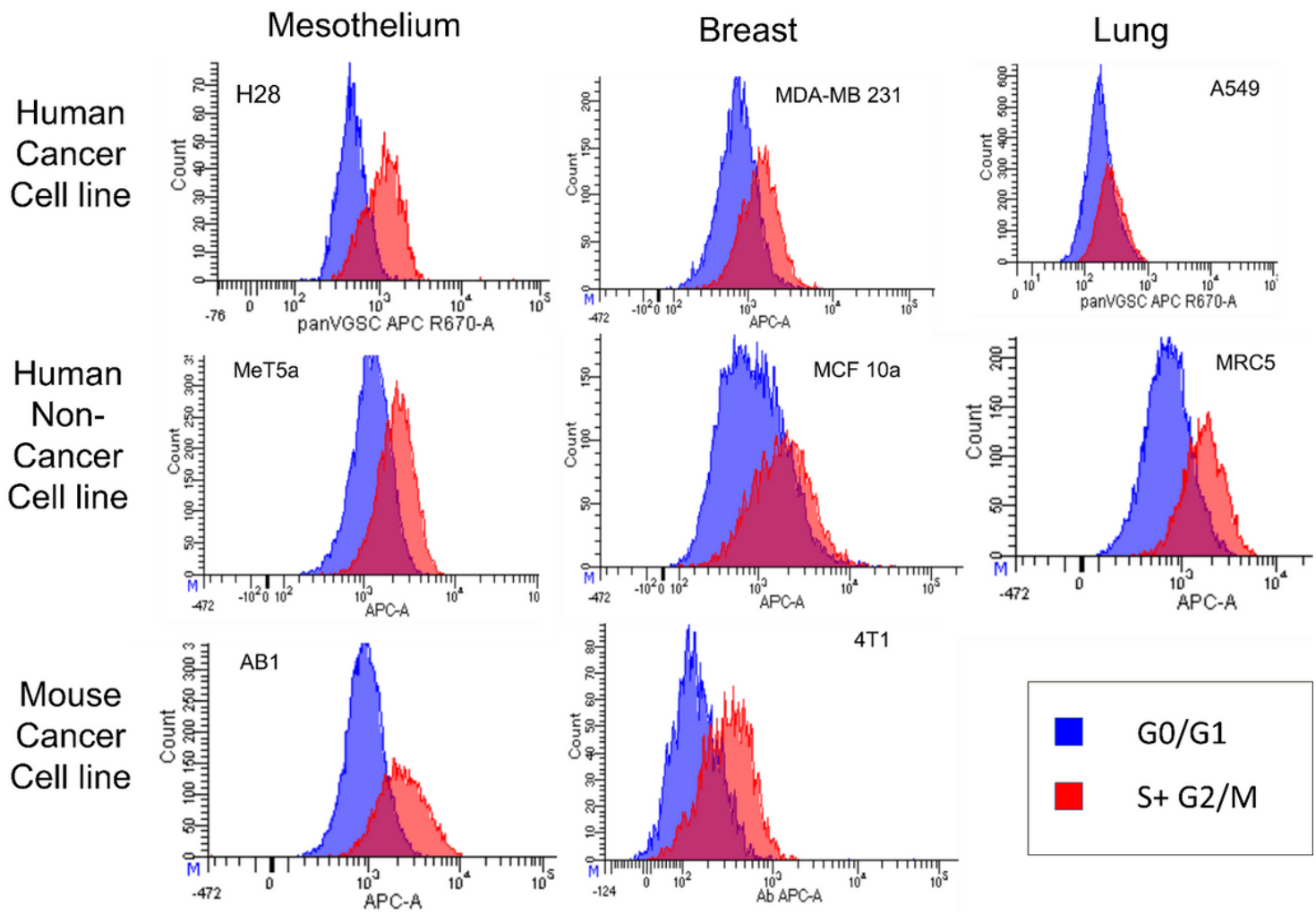

Figure 1

VGSC protein expression of cells in G0/G1 vs. S-M phases. Cells that were labeled with PI and a pan-specific VGSC antibody conjugated to APC were analyzed using flow cytometry. The number of APC fluorescence events per cell were compared in cells with low or high PI fluorescence. Cells with 2 copies of DNA (high PI fluorescence) cells (red) have significantly greater median fluorescent intensity than cells with 1 copy of DNA (low PI fluorescence) cells (blue). Graphs shown are representative of triplicate experiments.

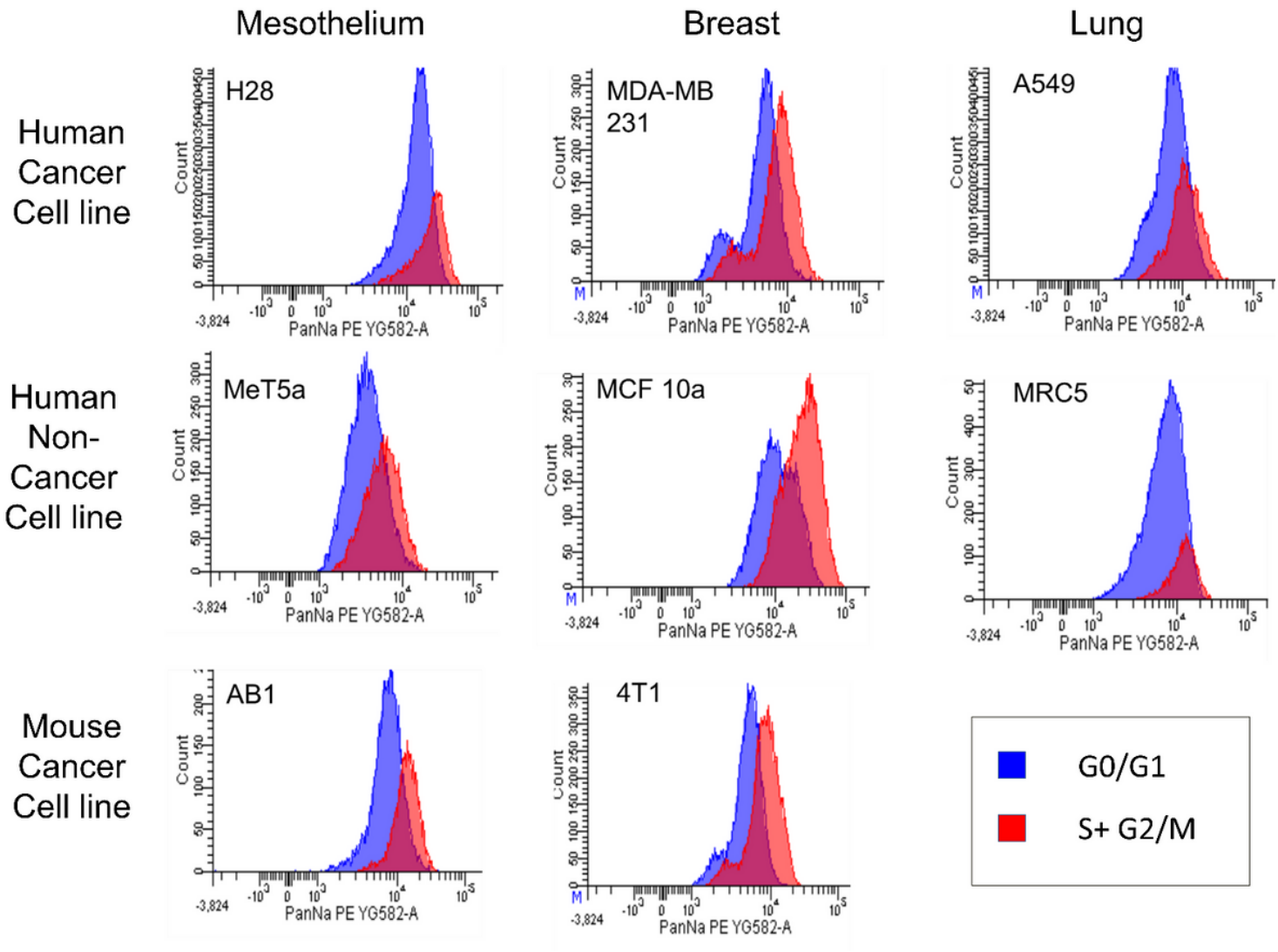

Figure 2

Na+,K+-ATPase protein expression of cells in G0/G1 vs. S-M phases: Cells that were labeled with pan-specific Na+,K+-ATPase antibody conjugated to RPE and DAPI were analyzed using flow cytometry. The number of RPE fluorescence events per cell were compared in cells with low or high DAPI fluorescence. Cells with two copies of DNA (high DAPI fluorescence; red) have significantly greater median fluorescent intensity than cells with 1 copy of DNA (low DAPI fluorescence; blue). Graphs shown are representative graphs of triplicate experiment.

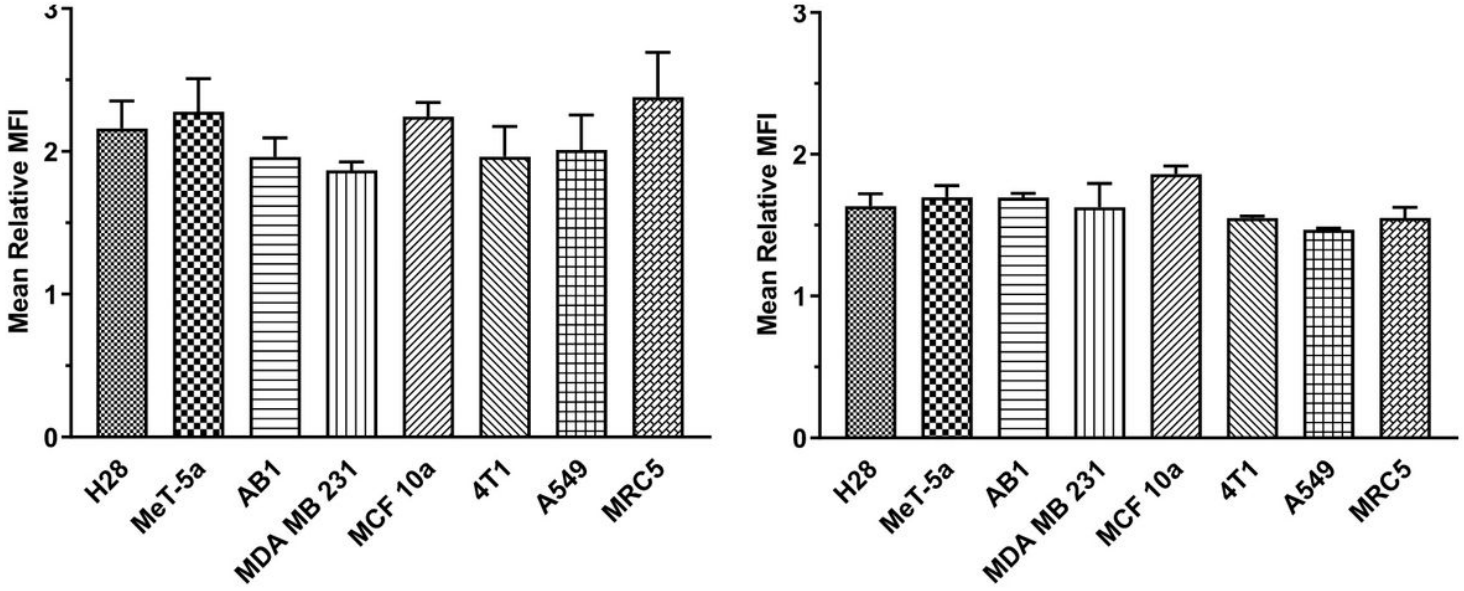

Figure 3

Mean shift in median VGSC (A) and Na+,K+-ATPase (B) expression in the eight cell lines. Bars represent the mean fold difference between median fluorescent intensities of cells with 2 copies of DNA and median fluorescent intensities of cells with one copy of DNA. One-way ANOVAs revealed that there was no significant difference among the cell lines for either VGSCs or Na+,K+-ATPase. Error bars are representative of the standard deviation of separate flow cytometry experiments; N=3.

Supplementary Files

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