# The Intrinsic Mitochondrial Membrane Potential of Colonic Carcinoma Cells Is Linked to the Probability of Tumor Progression

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## Abstract

**We subcloned cell lines from SW620 cells establishing that, despite the dynamic nature of the mitochondrial membrane potential ($\Delta\psi_m$), there are significant and stable differences in the intrinsic $\Delta\psi_m$ among cells within an *in vitro* population of human colonic carcinoma cells. Whereas more dramatic differences in $\Delta\psi_m$ would likely perturb essential mitochondrial functions, the differences in $\Delta\psi_m$ of the subclones did not affect steady-state reactive oxygen species levels, electron transport activity, or cellular viability and growth rates. However, the differences in intrinsic $\Delta\psi_m$ had a significant effect on the tumorigenic behavior of the cells. Subcloned cell lines with higher $\Delta\psi_m$ were more likely to exhibit elevated steady-state levels of vascular endothelial growth factor and matrix metalloproteinase 7, and increased invasive behavior (properties associated with tumor progression), than cells with lower intrinsic $\Delta\psi_m$, whereas cells with lower $\Delta\psi_m$ were more likely to respond to the chemopreventive activities of butyrate, including $\Delta\psi_m$ dissipation, growth arrest, and apoptosis, than cells with higher $\Delta\psi_m$. Therefore, these data establish that the probability for tumor development and progression is linked to stable differences in the intrinsic $\Delta\psi_m$ of colonic epithelial cells.** (Cancer Res 2005; 65(21): 9861-7)

## Introduction

In mammalian cells, the mitochondrial membrane potential ($\Delta\psi_m$) is the predominant component of an electrochemical gradient that is generated across the inner mitochondrial membrane. This gradient originates as a consequence of the transfer of electrons through mitochondrial enzyme complexes I, III, and IV, coupled to the pumping of protons from the mitochondrial matrix to the intermembranous space (1). Numerous studies have established a critical role for the $\Delta\psi_m$ in apoptosis, linking successful initiation of apoptotic cascades to $\Delta\psi_m$ dissipation (2, 3). The $\Delta\psi_m$ is also involved in the translation and stability of mitochondrial encoded and synthesized proteins (4, 5), the translocation and activation of nuclear encoded proteins across mitochondrial membranes (6), and the production of reactive oxygen species and energy (7).

We have shown the necessity for an intact $\Delta\psi_m$ in the initiation of apoptosis and cycle arrest of colonic carcinoma cells induced by the short-chain fatty acid butyrate (NaB), a chemopreventive agent which is found at high concentrations in the colonic lumen (3, 8). Using novel colonic carcinoma cell lines engineered to exhibit differences in their intrinsic $\Delta\psi_m$, we recently showed the significant effect that variations in the $\Delta\psi_m$ have on the extent to which cells enter NaB-mediated growth arrest and apoptotic cascades (8), and on the ability of the cell to adapt to hypoxia, invade the basement membrane, grow under anchorage-independent conditions, and assume shape changes that have been linked to metastatic potential.[1] Based on such studies, we have proposed that the mitochondria, especially the $\Delta\psi_m$, plays a critical role in influencing the probability of colonic tumor development and progression (3, 8, 9).

Solid tumors are composed of populations of cells that are continually evolving, responding, and adapting to fluctuations within their microenvironment. Similarly, the $\Delta\psi_m$ is dynamic, changing in response not only to apoptotic signals (2, 3, 10, 11) but also to changes in the microenvironment, the location of an individual cell within an outgrowth, and the localization of particular mitochondria within a cell (12, 13). To further investigate the role of the $\Delta\psi_m$ in tumor development and progression, we asked whether, in spite of its plasticity, stable differences in the intrinsic $\Delta\psi_m$ of cells exist within a population of colonic carcinoma cells and, if so, how such differences affect cell function.

To address these questions, we have generated single-cell subclones from the SW620 human colonic carcinoma cell line. Using these subcloned lines, we report that, despite the dynamic nature of the $\Delta\psi_m$, there are subtle, significant, and stable differences in the intrinsic $\Delta\psi_m$ among cells within an *in vitro* population of colonic carcinoma cells. Whereas dramatic alterations in the $\Delta\psi_m$ would likely disrupt essential mitochondrial functions, the consequence of which would be loss of viability, neither mitochondrial electron transport activity, reactive oxygen species levels, viability, nor growth was affected by the differences in $\Delta\psi_m$ exhibited by the subcloned lines. However, differences in $\Delta\psi_m$ had a significant effect on the steady-state vascular endothelial growth factor (VEGF) and matrix metalloproteinase (MMP)-7 levels, the invasive capacity of the cell, and the magnitude of NaB-mediated $\Delta\psi_m$ dissipation, growth arrest, and apoptosis. Therefore, combined with our previous work, these data establish that the probability for tumor development and the propensity for tumor progression are influenced by subtle, stable differences in the intrinsic $\Delta\psi_m$.

## Materials and Methods

**Determination of intrinsic $\Delta\psi_m$.** Mitochondrial membrane potential was determined by flow cytometry using the $\Delta\psi_m$-dependent fluorescent dye JC-1 (5,5′ 6,6′ -tetrachloro-1,1′ ,3,3′ tetraethylbenzimidazol

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carbocyanineiodide; Molecular Probes, Eugene, OR), analyzed in fluorescence detection channel 2 (FL-2), as we have previously described (2, 3, 8, 11).

**Quantitation of reactive oxygen species.** Steady-state $O_2^{\bullet-}$ and $H_2O_2$ levels were quantified using dihydroethidium and 2′,7′-dichlorofluorescin diacetate (Molecular Probes), analyzed by flow cytometry in FL-2 and FL-1, respectively, as we have described (2).

**Quantitation of superoxide dismutase activity.** Cells were seeded into replicate 96-well plates. One plate was used to determine the number of viable cells by spectrophotometric quantitation of the reduction of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT; Sigma-Aldrich, St. Louis, MO) to formazan (14) using a standard curve generated by the serial dilution of SW620 cells. Cells in replicate plates were lysed in 100 μL of ice-cold 50 mmol/L D-mannitol, 2 mmol/L Tris-HCl (pH 7.4), 0.1% Triton X-100. Superoxide dismutase (SOD) activity was quantified in lysates using a SOD assay Kit-WST (Dojindo Molecular Technologies, Inc., Gaithersburg, MD) following the protocol of the manufacturer, and expressed as units per viable cell.

**Quantitation of cytochrome *c* oxidase activity.** Mitochondria were isolated and enzymatic activity of cytochrome *c* oxidase was determined as we have previously described (15).

**Quantitation of cell growth.** Cells were seeded into 96-well plates and the number of viable cells was determined at 2-day intervals using the reduction of MTT to formazan (14) and a standard curve was generated by the serial dilution of SW620 cells.

**Quantitation of vascular endothelial growth factor.** Cells were seeded into 96-well plates, tissue culture medium was replaced 3 days later, and the cells were allowed to grow for an additional 3 days. Medium was again replaced and harvested 24 hours later for quantitation of VEGF protein by ELISA (R&D Systems, Inc., Minneapolis, MN) according to the protocol of the manufacturer.

**Quantitation of matrix metalloproteinase 7 (matrilysin).** Cells were seeded into 96-well plates and allowed to grow as described above. Tissue culture medium was then replaced with fetal bovine serum–free medium, which was harvested 24 hours later. MMP7 protein levels were quantified by ELISA (R&D Systems) according to the protocol of the manufacturer.

**Quantitation of cell invasion.** Cells were seeded into chambers consisting of a reconstituted basement membrane supported by an underlying polycarbonate membrane (Chemicon, Temecula, CA). Migration through the basement membrane was determined by staining and subsequent quantification by absorbance of cells adhering to the polycarbonate membrane, according to the protocol of the manufacturer.

**Quantitation of butyrate mediated responses.** Cells were exposed to 5 mmol/L NaB (Sigma, St. Louis, MO) for 16 to 48 hours. Dissipation of the $\Delta\psi_m$ was determined by JC-1 staining as we have described (2, 3, 8, 11). Cell cycle variables were analyzed by propidium iodide staining and flow cytometry, as we have described (2, 3, 10). Active caspase-3 was quantified using phycoerythrin-conjugated anti–active caspase-3 (PharMingen, San Diego, CA) followed by flow cytometry, as we have described (2). Cell viability was determined by the reduction of MTT to formazan (14).

**Statistical analyses.** Data from at least three independent determinations were analyzed using Dunnett's multiple comparison tests. Mean data were also evaluated as a function of the intrinsic $\Delta\psi_m$, determined by mean emission of JC-1 in FL-2, using linear regression analyses.

## Results

**Cells within a population exhibit stable differences in intrinsic $\Delta\psi_m$.** JC-1 is a fluorescent dye that is taken up by the mitochondria where it forms $\Delta\psi_m$-dependent complexes (16). The emission intensity of these complexes at 590 nm, analyzed by flow cytometry in FL-2, is a sensitive index of $\Delta\psi_m$ of the cell (2, 3). In the course of our previous work (2, 3, 8), we noted that JC-1–stained SW620 cells generated a distribution of emission intensity, which we reasoned either reflected the dynamic nature of the $\Delta\psi_m$ or, instead, the presence of cells within the population that had stable differences in their intrinsic $\Delta\psi_m$. To address the latter possibility, a suspension of SW620 cells was diluted such that there was a high probability that a single cell was placed into each of 301 standard tissue culture wells. From these wells, 162 subcloned cell lines were generated and expanded.

To determine whether there were differences in the intrinsic $\Delta\psi_m$ among the 162 subclones, each subclone line, as well as the original population of SW620 cells, was stained with JC-1, analyzed by flow cytometry, and the mean emission of the dye plotted as a frequency distribution. As shown in Fig. 1*A* (*solid line*), emission in the 162 subcloned cell lines ranged in intensity from ~110 to 610. Importantly, the median emission of the 162 subclones was 202.62, comparable to the mean emission of the original SW620 cell population (207.72). Therefore, among the subclone lines there is a normal distribution of $\Delta\psi_m$, the midpoint of which is equivalent to the $\Delta\psi_m$ of the original population of cells.

Six of the 162 subcloned lines, which exhibited JC-1 emission ranging from the lower to the upper 95% confidence interval of the mean, were then selected from frozen stocks for further study.

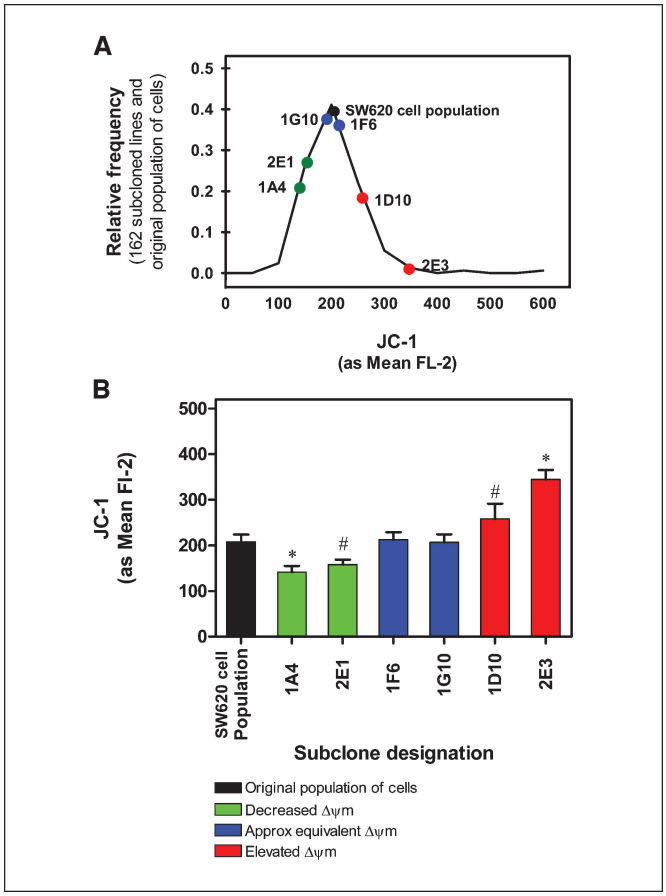


**Figure 1.** Cells within a population of human colonic carcinoma cells exhibit significant and stable differences in intrinsic $\Delta\psi_m$. *A,* the original population of SW620 and the 162 subcloned cell lines were stained with JC-1 and analyzed by flow cytometry in FL-2. Mean dye emission of three individual analyses of each cell line is plotted as a frequency distribution using a bin width of 50. Symbols correspond to the mean emission of specific cell lines: *black symbols,* original SW620 cell population; *green, red,* and *blue symbols,* selected subcloned lines (1A4, 2E1, 1F6, 1G10, 1D10, and 2E3, based on their well and plate position during subcloning) exhibiting mean emission lower, higher, and comparable to that of the original population, respectively. *B,* mean and SE of seven individual determinations of the mean emission of JC-1 in FL-2 of the original population and six selected subcloned cell lines made over an 8-month period. Mean emission of the subclones compared with the original population by Dunnett's test; # and *, $P < 0.05$ and $P < 0.01$, respectively.

Compared with JC-1 staining of the original population, the subcloned cell lines designated 1A4 and 2E1 (based on their well and plate position during subcloning) had lower emission intensity, corresponding to decreases in intrinsic $\Delta\psi_m$ of ~32% and 24%, respectively; subclones 1D10 and 2E3 had higher emission intensity, corresponding to increases in intrinsic $\Delta\psi_m$ of ~24% and 66%, respectively; and the emission intensity of the subclones 1G10 and 1F6 was similar to that of the cell population, indicative of $\Delta\psi_m$ comparable to that of the original population (Fig. 1*A*).

To investigate whether these differences in intrinsic $\Delta\psi_m$ were stable, multiple determinations were made over an 8-month period. As shown in Fig. 1*B*, the standard errors (SEs) in JC-1 staining of the subcloned lines were comparable to that of the original SW620 cell population, establishing the stability of their intrinsic $\Delta\psi_m$. The $\Delta\psi_m$ of subclones 1A4 and 2E1 are significantly lower, whereas the $\Delta\psi_m$ of D10 and 2E3 are significantly higher than that of the original population. Moreover, the $\Delta\psi_m$ of subclones 1D10 and 2E3 are approximately double that of 1A4 and 2E1. Therefore, despite the dynamic nature of the $\Delta\psi_m$ (12, 13), significant and stable differences in the intrinsic $\Delta\psi_m$ of cells exist within the population of SW620 colonic carcinoma cells.

**Differences in the intrinsic $\Delta\psi_m$ of subclones do not perturb mitochondrial functions that would compromise viability and growth.** It is important to appreciate that although the differences in the intrinsic $\Delta\psi_m$ of the subcloned lines are subtle, they are significant and stable. Furthermore, it is critical to recognize that greater variations in the $\Delta\psi_m$ would likely lead to disruptions in mitochondrial functions that would result in the loss of viability. Mitochondrial electron transport/oxidative phosphorylation is not only the major source of energy production of the cell but also the major source of endogenous reactive oxygen species (17). Approximately 2% to 5% of the electrons that pass through the electron transport chain escape, reacting with molecular oxygen to generate superoxide ($O_2^{\cdot P-}$). $O_2^{\cdot P-}$ is then converted to $H_2O_2$ by SOD. Because alterations in the $\Delta\psi_m$ can affect reactive oxygen species production (7), leading to growth arrest and apoptosis (18, 19), reactive oxygen species and SOD levels were quantified and plotted as a function of the $\Delta\psi_m$ of each cell line. As shown in Fig. 2*A*, differences in the intrinsic $\Delta\psi_m$ did not affect the steady-state levels of either $O_2^{\cdot P-}$ or $H_2O_2$ [$P$ = 0.42 and 0.57, respectively (linear regression analysis)] and, consistent with the absence of an affect on $H_2O_2$, did not effect steady-state SOD activity (Fig. 2*B*; $P$ = 0.56, linear regression analysis).

Each of the mitochondrial enzyme complexes directly involved in electron transport/oxidative phosphorylation is constructed from subunits encoded by mitochondrial and nuclear genes. For example, complex IV, cytochrome *c* oxidase, is assembled from 13 subunit peptides; 3 are encoded by mitochondrial DNA and synthesized in the mitochondria, 10 by nuclear genes and synthesized in the cytoplasm and transported into the mitochondria. A functional enzyme can be assembled only when stoichiometric amounts of each of these subunits are available within the mitochondria (20, 21). Because of the critical roles that the $\Delta\psi_m$ has in the translation and stability of mitochondrial encoded and synthesized proteins (4, 5) and in the transport of nuclear-encoded proteins across the mitochondrial membrane (6), cytochrome *c* oxidase activity was quantified (15) and plotted as a function of the $\Delta\psi_m$ of each cell line. As shown in Fig. 2*C*, the differences in the intrinsic $\Delta\psi_m$ of the subclones did not affect steady-state cytochrome *c* oxidase activity ($P$ = 0.68, linear regression analysis).

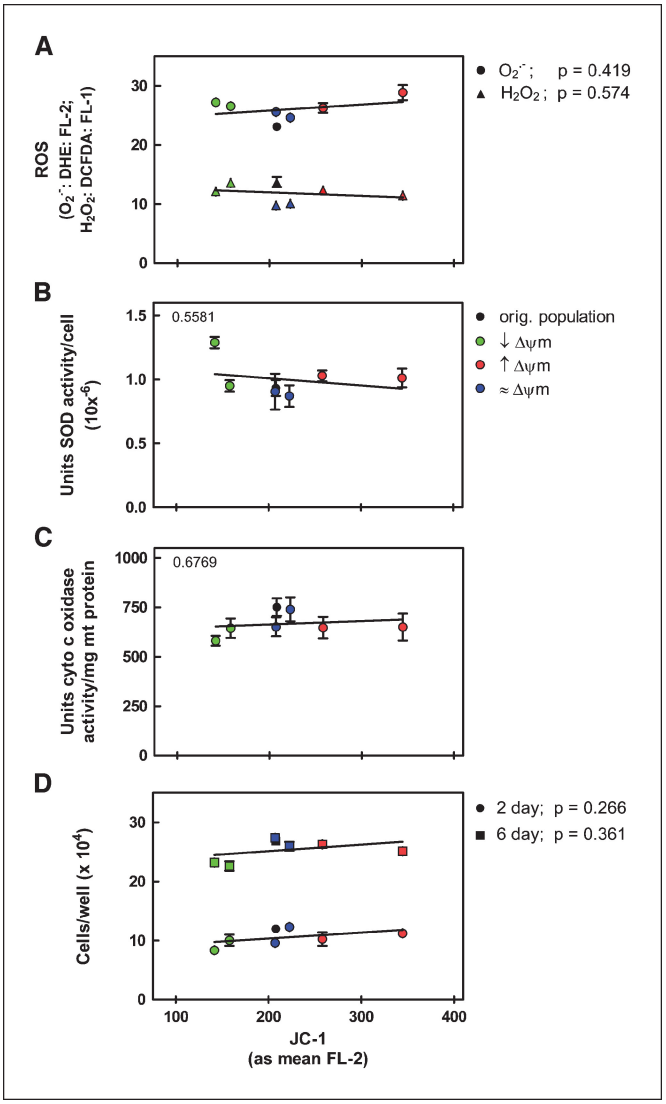


**Figure 2.** Stable differences in the intrinsic $\Delta\psi_m$ of subclones do not perturb essential mitochondrial functions or compromise cell viability and growth. *Black symbols,* original SW620 cell population; *green, red,* and *blue symbols,* subclones with intrinsic $\Delta\psi_m$ that are decreased, increased, or comparable to that of the original population, respectively. Mean and SE of steady-state levels of $O_2^{\cdot P-}$ and $H_2O_2$ (*A*), SOD enzymatic activity (*B*), cytochrome *c* oxidase enzymatic activity (*C*), and the number of viable cells determined 2 and 6 days following seeding into standard tissue culture wells (*D*). Data are plotted as a function of intrinsic $\Delta\psi_m$ of each cell line, determined as emission of JC-1 in FL-2, and analyzed by linear regression; $O_2^{\cdot P-}$ and $H_2O_2$, $P$ = 0.41 and 0.57, respectively; SOD enzymatic activity, $P$ = 0.56; cytochrome *c* oxidase enzymatic activity, $P$ = 0.68; steady-state viability and growth, $P$ = 0.25 at 2 days and $P$ = 0.36 at 6 days.

Consistent with the absence of affects on steady-state cytochrome *c* oxidase and SOD activities and $O_2^{\cdot P-}$ and $H_2O_2$ levels, the differences in $\Delta\psi_m$ of the subcloned lines did not affect cell viability or growth rates 2 and 6 days following seeding [Fig. 2*D*; $P$ = 0.26 and 0.36, respectively (linear regression analysis)]. Therefore, because the $\Delta\psi_m$ of the subcloned cell lines do not affect mitochondrial activities that compromise cell viability and growth, they provide a novel system with which to dissect the effect of stable differences in intrinsic $\Delta\psi_m$ on the tumorigenic behavior of colonic carcinoma cells.

**Differences in the intrinsic $\Delta\psi_m$ of subclones are linked to cellular properties associated with tumor progression.** Among

the key factors in colonic tumor progression and expansion are angiogenesis and the ability of malignant cells to invade normal tissue and generate metastatic lesions. VEGF promotes new endothelial and lymphatic vessel formation and has been linked to liver metastasis and poor prognosis in colon cancer (22). To determine whether steady-state VEGF production was influenced by the intrinsic $\Delta\psi_m$, VEGF protein levels were quantitated in conditioned medium and plotted as a function of the $\Delta\psi_m$ of each line. As shown in Fig. 3*A*, the intrinsic $\Delta\psi_m$ was linked to steady-state VEGF secretion levels ($P = 0.04$, linear regression analysis) and the mean levels produced by the subcloned lines with decreased $\Delta\psi_m$ were significantly lower than that of the original population of cells.

For successful invasion and metastasis, tumor cells must attach to the basement membrane and then degrade and penetrate the extracellular matrix. The key enzymes responsible for degradation of extracellular matrix proteins are a family of MMPs. Whereas most MMPs are produced by stromal cells, MMP7 (matrilysin) is synthesized by tumor cells and its expression is a marker of tumor progression (23, 24). To determine whether steady-state MMP7 production was linked to differences in the intrinsic $\Delta\psi_m$, MMP7 protein levels were quantified in conditioned medium and plotted as a function of the $\Delta\psi_m$ of each cell line. As shown in Fig. 3*B*, MMP7 levels were associated with the intrinsic $\Delta\psi_m$ ($P = 0.03$, linear regression analysis). Moreover, the levels of MMP7 in conditioned medium from subclones with decreased intrinsic $\Delta\psi_m$ were more than 10-fold lower than the level in the original population of SW620 cells.

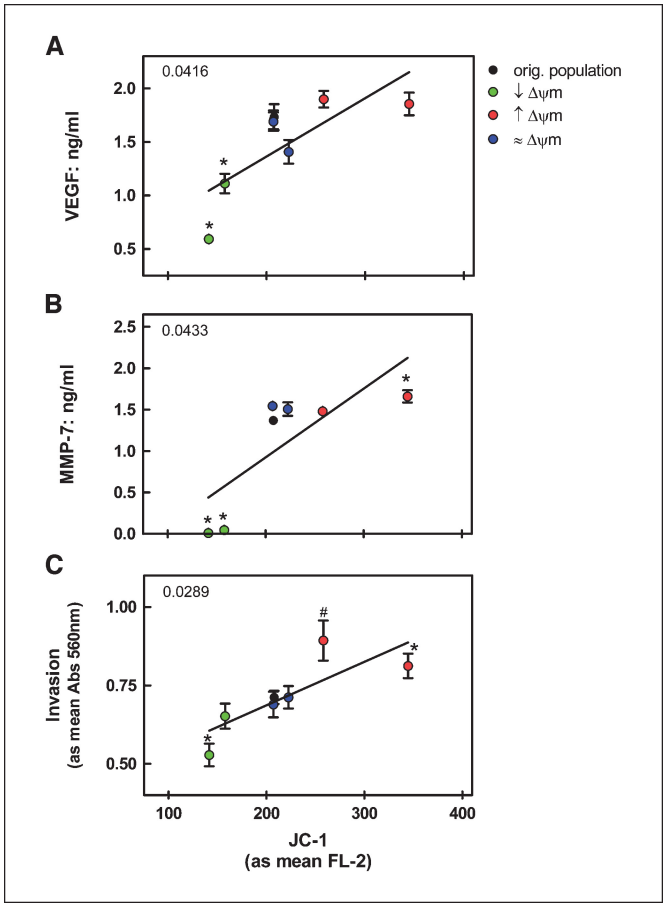


**Figure 3.** Stable differences in the intrinsic $\Delta\psi_m$ of subclones are linked to cellular properties that are associated with tumor progression. Mean and SE of steady-state VEGF secretion (*A*), steady-state MMP7 secretion (*B*), and cellular migration (*C*) through a reconstituted basement membrane, quantified by absorbance of stained invasive cells (described in Materials and Methods). Data are plotted as a function of intrinsic $\Delta\psi_m$ of each cell line, determined as emission of JC-1 in FL-2, and analyzed by linear regression (*top left corner*; $P < 0.05$). In addition, the levels of VEGF or MMP7 secretion and the invasiveness of the subclones were compared with the original population by Dunnett's test; # and *; $P < 0.05$ and $P < 0.01$, respectively.

In colonic tumors, MMP7 expression is highest at the invasive edge of the tumor (24) and in metastatic lesions (23, 25, 26). Suppression of MMP7 expression inhibits the ability of cells to penetrate the basement membrane and metastasize (26, 27). The extremely low levels of MMP7 secretion in the subcloned cells with decreased $\Delta\psi_m$, combined with evidence that the $\Delta\psi_m$ of migrating cells (28, 29) and cells at the leading edge of a colony outgrowth (12, 13) is elevated, led us to reason that differences in the intrinsic $\Delta\psi_m$ were likely to affect the ability of cells to cross the basement membrane. Therefore, cells were seeded onto a reconstituted basement membrane and those that migrated through and attached to a supporting polycarbonate membrane were stained and quantified by absorbance. Mean absorbance data were then plotted as a function of the intrinsic $\Delta\psi_m$ of each cell line. As shown in Fig. 3*C*, the intrinsic $\Delta\psi_m$ was significantly associated with the ability of cells to cross the basement membrane ($P = 0.03$, linear regression analysis). The subcloned lines with elevated $\Delta\psi_m$ were significantly more invasive, whereas the subcloned cells with lower $\Delta\psi_m$ were less invasive than the original population of cells.

Therefore, the significant effect that the intrinsic $\Delta\psi_m$ has on steady-state secretion of VEGF and MMP7 and the ability of cells to invade the basement membrane show that, within a population of colonic carcinoma cells, the cells with higher intrinsic $\Delta\psi_m$ are more likely to participate in tumor expansion and progression than the cells with lower $\Delta\psi_m$.

**Differences in the intrinsic $\Delta\psi_m$ of subclones are linked to their response to sodium butyrate.** The unbranched short-chain fatty acid butyrate (NaB) is a natural constituent of the colonic contents, generated during fiber fermentation (30). Rapidly taken up by cells, NaB enters the mitochondria where it undergoes β-oxidation (31). In addition to functioning as the primary energy source for colonic epithelial cells (32), NaB mediates maturation pathways *in vitro* and *in vivo* (33–35) and has been linked to chemoprevention (36–38).

Our previous work has shown that the $\Delta\psi_m$ plays a critical role in NaB-initiated cell cycle arrest and apoptotic pathways in SW260 cells, and that the extent to which cells enter these pathways is linked to dissipation of the $\Delta\psi_m$ (3, 8). To determine whether the differences in the $\Delta\psi_m$ of the subclones affected their responses to NaB, cells were exposed to 5 mmol/L NaB for 16 to 48 hours and analyzed for $\Delta\psi_m$ dissipation, alterations in cell cycle, and the initiation and completion of an apoptotic cascade. The time course of each of these NaB-mediated responses, expressed as a percentage of untreated cells, is shown in Fig. 4*A* to *D*. Data from critical time points for each response are then plotted as a function of the intrinsic $\Delta\psi_m$ of each cell line (Fig. 4-*H*).

As shown in Fig. 4*A* and *E*, the intrinsic $\Delta\psi_m$ had a dramatic affect on the kinetics and magnitude of NaB-induced dissipation of the $\Delta\psi_m$. During the first 16 hours of NaB treatment, there was little affect on the $\Delta\psi_m$ of the original population of cells or the subcloned lines with $\Delta\psi_m$ approximately equivalent to that of the original population; from 24 to 48 hours, there was a comparable dissipation of the $\Delta\psi_m$ among these cell lines. In contrast, NaB

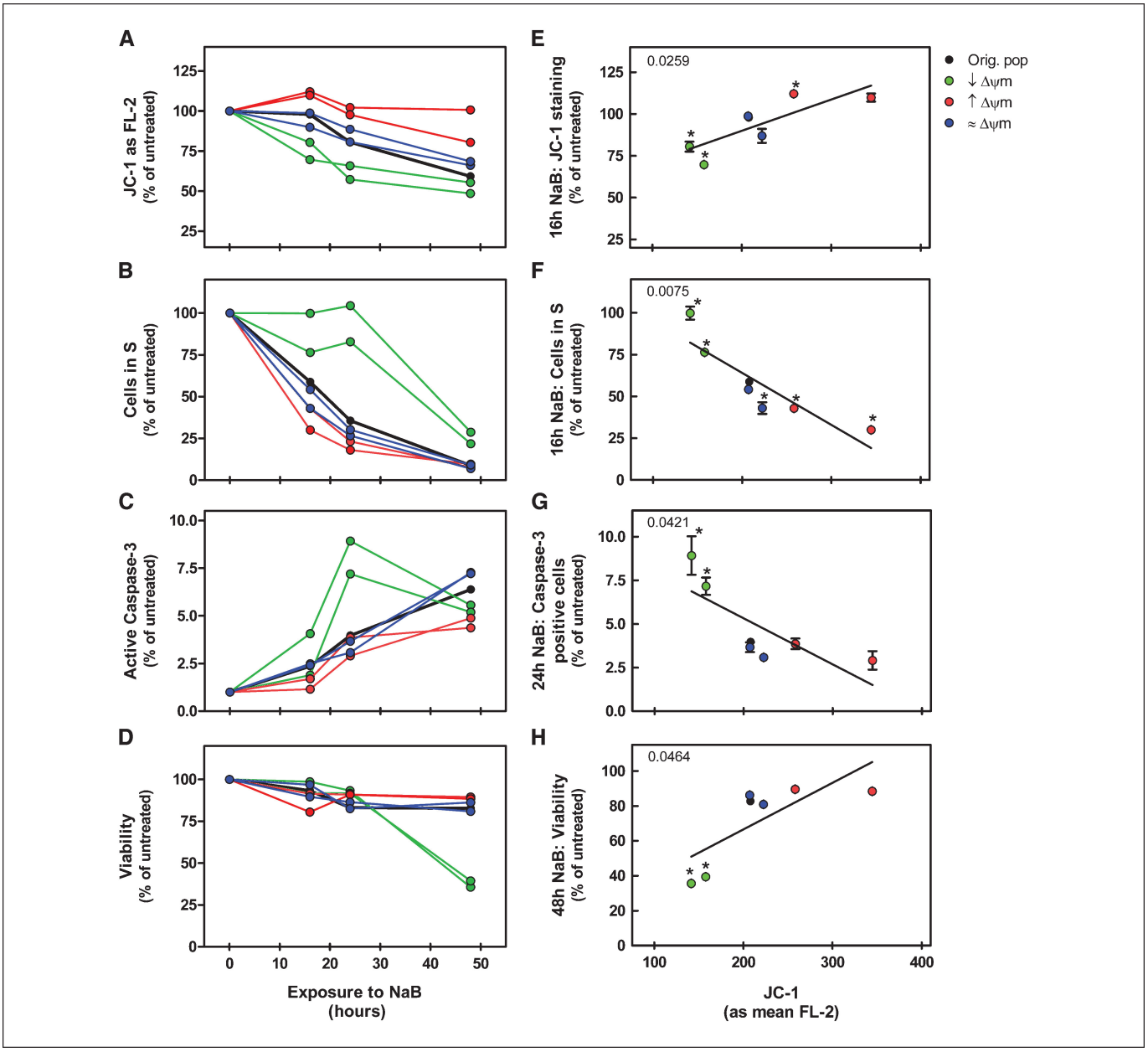


**Figure 4.** Stable differences in the intrinsic $\Delta\psi_m$ of subclones are linked to the response to NaB. *A* to *D,* the original population and subcloned cell lines were exposed to 5 mmol/L NaB for 16 to 48 hours, stained with JC-1 and evaluated by flow cytometry for alterations in $\Delta\psi_m$ (*A*); stained with propidium iodide and evaluated by flow cytometry for alterations in cell cycle (*B*); stained with phycoerythrin-conjugated anti–active caspase-3 and analyzed by flow cytometry for induction of an apoptotic cascade (*C*); or incubated with MTT to quantitate cellular viability (*D*). The mean of these cellular responses to NaB is expressed as percent of untreated cells and plotted as a function of time of exposure to NaB. *E* to *H,* mean and SE of these cellular responses to NaB are plotted at critical time points as a function of the intrinsic $\Delta\psi_m$ of each cell line, determined as emission of JC-1 in FL-2, and analyzed by linear regression (*top left corner*; $P$ <0.05 to <0.01). In addition, the responses of the subcloned lines were compared with those of the original population by Dunnett's test; *, $P < 0.01$.

induced a more rapid $\Delta\psi_m$ dissipation in the subcloned lines with decreased intrinsic $\Delta\psi_m$, detected as early as 16 hours. Moreover, the magnitude of dissipation at subsequent time points was greater in these cells than in cells with higher $\Delta\psi_m$. On the contrary, in the cells with elevated intrinsic $\Delta\psi_m$, NaB-mediated dissipation of the $\Delta\psi_m$ was not detected in one of the lines until 48 hours and, even then, the extent of dissipation was less than that induced in cells with lower intrinsic $\Delta\psi_m$. Furthermore, in the other subcloned line with an elevated intrinsic $\Delta\psi_m$, NaB failed to induce $\Delta\psi_m$ dissipation—even after 48 hours, the $\Delta\psi_m$ of NaB-treated cells was comparable to that of untreated cells.

The intrinsic $\Delta\psi_m$ also had a striking affect on NaB-induced cell cycle arrest (Fig. 4*B* and *F*). After 16 hours, ~40% to 70% of the original population of cells, the subclones with $\Delta\psi_m$ comparable to that of the original population, and the subclones with elevated $\Delta\psi_m$ were lost from S phase; by 48 hours, this increased to ~90%. In marked contrast, the subcloned lines with decreased intrinsic $\Delta\psi_m$ were relatively resistant to this early NaB-mediated decrease in cycling cells. Compared with untreated cells, after 16 hours, fewer than 25% of NaB-treated cells in one of these lines were lost from S phase, and in the second line, there was, in fact, no loss of cycling cells.

In addition, the time course and extent of NaB-induced caspase-3 activation and subsequent cell death were affected by the intrinsic $\Delta\psi_m$ (Fig. 4*C* and *G,* and *D* and *H*, respectively). Activation of caspase-3 in the original population, the subclones with $\Delta\psi_m$ comparable to the original population, and the subcloned lines with elevated $\Delta\psi_m$ progressively increased with exposure to NaB (Fig. 4*C*), and after 24 hours, active caspase-3 was detected in 3% to 4% of these cells. In marked contrast, after 24 hours, active caspase-3 was detected in 7% to 9% of the cells with decreased intrinsic $\Delta\psi_m$. After this peak at 24 hours, the percentage of cells with active caspase-3 declined, consistent with their loss by apoptosis. In fact, as shown in Fig. 4*D*, <40% of cells with decreased $\Delta\psi_m$ were viable after 48 hours of NaB treatment. On the contrary, despite caspase-3 activation levels that approached the peak attained by the subclones with decreased intrinsic $\Delta\psi_m$, almost 90% of the cells with higher $\Delta\psi_m$ were still viable 48 hours after treatment with NaB.

Taken together, the kinetics of these responses to NaB shows that, in the cells with decreased intrinsic $\Delta\psi_m$, the spike in caspase-3 activation coincides with dissipation of the $\Delta\psi_m$ by ~60% (Fig. 4*A*) and, similar to cell death, is followed by the loss of cells from S phase (Fig. 4*B*). Thus, these data implicate the magnitude of $\Delta\psi_m$ dissipation as a key in the regulation of the cellular response to NaB: Cells with a lower intrinsic $\Delta\psi_m$ are more likely than cells with higher $\Delta\psi_m$ to achieve a critical threshold of $\Delta\psi_m$ dissipation, which is required for the initiation and completion of an apoptotic cascade that targets cells in S phase.

Furthermore, plotting the responses to NaB sequentially as a function of the intrinsic $\Delta\psi_m$ of each of the cell lines and analyzing the data by Dunnett's multiple comparison tests and by linear regression emphasize the significant effect of the intrinsic $\Delta\psi_m$ on the extent to which cells undergo NaB-initiated dissipation of the $\Delta\psi_m$, cell cycle arrest, caspase-3 activation, and death (Fig. 4*E-H*, respectively; $P$ <0.05 to <0.01, linear regression analysis). Thus, within a population of colonic carcinoma cells, the cells with lower intrinsic $\Delta\psi_m$ respond to the chemopreventive activities of NaB more quickly and to a greater extent than cells with higher $\Delta\psi_m$.

## Discussion

As a consequence of the high proliferative rate of colonic epithelial stem cells, over a life span of 6 to 7 decades, more than $10^{12}$ cell divisions will have taken place in the colonic mucosa (39). Because the initiation of tumorigenesis for most colon cancer patients is linked to only one of these cell divisions, subtle changes in cell composition and/or function have the potential of dramatically affecting the probability of tumor formation and progression.

Regardless of the etiology, the malignant transformation of colonic epithelial cells includes alterations in the mitochondrial genome (40), defects in mitochondrial activity (41), and elevations in the $\Delta\psi_m$ (1, 12, 42). The fact that the $\Delta\psi_m$ affects electron transport/oxidative phosphorylation (4–6, 17), generation and accumulation of reactive oxygen species (7, 18, 19), growth arrest and apoptosis (2, 3), and cellular migration (12, 13, 28, 29) implies that variations in the $\Delta\psi_m$ can influence tumor development and progression.

We generated single-cell subcloned lines from SW620 cells establishing that cells with stable differences in their intrinsic $\Delta\psi_m$ exist within a population of colonic carcinoma cells. Although these differences did not perturb essential mitochondrial functions or compromise cell viability and growth, they had a significant effect on characteristics associated with tumor progression and on the cellular response to the chemopreventive agent butyrate.

Although the mechanisms involved in generating and maintaining these differences in $\Delta\psi_m$ are unclear, they may be reflected in, or paralleled by, modulations in the composition of mitochondrial membranes. Alterations in mitochondrial membrane phospholipids influence the $\Delta\psi_m$ (43) and, similar to the effect of differences in the intrinsic $\Delta\psi_m$, affect dissipation of the mitochondrial membrane and apoptosis (44, 45), as well as the metastatic potential (46, 47) and butyrate sensitivity of colonic carcinoma cells *in vivo* (48) and *in vitro* (49). Thus, stable differences in the intrinsic $\Delta\psi_m$ may be the result of alterations in molecular and/or biochemical processes that are regulated by the nuclear genome and/or environmental factors, and affect the generation, regulation, and maintenance of mitochondrial membrane phospholipids (50, 51).

The differences in the intrinsic $\Delta\psi_m$ may also reflect an enrichment of a particular mitochondrial population within cells. For example, heteroplasmy refers to the differences in genotype among the hundreds of mitochondria within each colonic epithelial cell. During the division of these cells, mitochondria with different genotypes are randomly distributed into the daughter cells. Therefore, over many generations, the mitochondria of a particular cell, and hence the majority of its progeny, can drift toward a predominant subtype, a state known as homoplasmy (17).

In conclusion, the data show that subtle, stable differences in the intrinsic $\Delta\psi_m$, likely exhibited by cells within a colon tumor, are linked to changes in cell function that play fundamental, critical roles in defining the probability of tumor development and the propensity for tumor expansion and progression. How and when these differences arise are important questions for future investigation.

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