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Evidence for a potential tumor suppressor role for the Na,K-ATPase $ß_1$-subunit

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Summary. The Na,K-ATPase, consisting of two essential subunits (α, ß), plays a critical role in the regulation of ion homeostasis in mammalian cells. Recent studies indicate that reduced expression of the $ß_1$ isoform (NaK-$ß_1$) is commonly observed in carcinoma and is associated with events involved in cancer progression. In this study, we present evidence that repletion of NaK-$ß_1$ in Moloney sarcoma virus-transformed Madin-Darby canine kidney cells (MSV-MDCK), a highly tumorigenic cell line, inhibits anchorage independent growth and suppresses tumor formation in immunocompromised mice. Additionally, using an *in vitro* cell-cell aggregation assay, we showed that cell aggregates of NaK-$ß_1$ subunit expressing MSV-MDCK cells have reduced extracellular regulated kinase (ERK) 1/2 activity compared with parental MSV-MDCK cells. Finally, using immunohistochemistry and fully quantitative image analysis approaches, we showed that the levels of phosphorylated ERK 1/2 are inversely correlated to the NaK-$ß_1$ levels in the tumors. These findings reveal for the first time that NaK-$ß_1$ has a potential tumor-suppressor function in epithelial cells.

Key words: Tumorigenicity, Cell adhesion, Na,K-ATPase ß-subunit, ERK 1/2

Introduction

The Na,K-ATPase, also known as the sodium pump, is an oligomeric transmembrane protein localized to the basolateral plasma membrane in most epithelial cells. It consists of two essential non-covalently linked α- and ß-subunits. Of the four isoforms of NaK-α and three isoforms of NaK-ß subunits known, only the $α_1$ and $ß_1$ subunits are expressed in a wide variety of tissue types (Sweadner, 1989; Lingrel and Kuntzweiler, 1994; Kaplan, 2002). The α-subunit has the catalytic activity of the enzyme, and the ß-subunit is involved in the translation, stability, and transport of the α-subunit to the plasma membrane (McDonough et al., 1990; Kaplan, 2002; Rajasekaran, et al., 2004). The NaK-$α_1ß_1$ heterodimer is the major isoform of Na,K-ATPase detected in the renal epithelium, including in Madin-Darby canine kidney (MDCK) cells. Recent studies from our laboratory and others indicate that the NaK-$ß_1$ subunit also functions as a cell-cell adhesion molecule (Rajasekaran et al., 2001; Kitamura et al., 2005; Shoshani et al., 2005). We also show that the transmembrane domain of NaK-$ß_1$ has critical amino acid residues that regulate its role in pump activity and cell-cell adhesion (Barwe et al., 2007).

In general, epithelial cells lose key cell adhesion molecules, such as E-cadherin, during cancer progression and gain motility and invasive behavior in a process called epithelial-to-mesenchymal transition (EMT). Several recent findings indicate that reduced NaK-$ß_1$ expression is associated with EMT (Rajasekaran et al., 1999, 2003; Espineda et al., 2003). Well-differentiated epithelial cells (cells with tight junctions and polarity) express high levels of this protein, whereas expression of NaK-$ß_1$ is highly reduced in poorly differentiated carcinoma cells (epithelial cancer cells lacking junctions and polarity) (Espineda et al., 2004). The transcription suppressor Snail is up-regulated in poorly differentiated carcinoma and is a key mediator of EMT (Thiery, 2002). Snail binds to the E-box promoter element of the NaK-$ß_1$ and suppresses its transcription, reducing both protein and mRNA levels (Espineda et al., 2004). Another characteristic of cells undergoing EMT is their increased motility. Reduced expression of NaK-$ß_1$ in poorly differentiated Moloney sarcoma virus-transformed Madin-Darby canine kidney cells (MSV-MDCK) is associated with increased motility of these

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cells; repletion of NaK-ß$_1$ reduces their motility, suggesting that diminished expression of NaK-ß$_1$ is associated with increased motility of carcinoma cells (Rajasekaran et al., 2001; Barwe et al., 2005). Further, repletion of both NaK-ß$_1$ and the cell-cell adhesion molecule E-cadherin in the poorly differentiated MDCK cells reverted these cells back to a well-differentiated morphology (Rajasekaran et al., 2001). Collectively, these studies indicate that a certain level of NaK-ß$_1$ expression in epithelial cells is important to maintain their well-differentiated phenotype, and its reduced levels are associated with events leading to cancer progression.

Anchorage-independent growth (ability of tumor cells to grow in soft agar) and the ability to form tumors in immunocompromised mice (tumorigenicity) are the distinct features of malignant transformation and tumor suppressors inhibit both of these characteristics (Rodrigues et al., 1985; Leone et al., 1991; Weinberg, 1991; Hanahan and Weinberg, 2000). Tumorigenicity of carcinoma cells does not necessarily correlate with invasiveness. For example, the tumor suppressor APC inhibits tumorigenicity, while having a negligible effect on the invasive potential of carcinoma cells (Empereur et al., 1997; Sansom et al., 2006). However, molecules involved in the regulation of cell-cell adhesion, such as E-cadherin, suppress tumorigenicity and invasiveness to a certain extent and thus have tumor-suppressor as well as invasion-suppressor functions (Behrens et al., 1989; Tsukita et al., 1993; Ewing et al., 1995; Bullions et al., 1997; Christofori and Semb, 1999). Since NaK-ß$_1$ has cell-cell adhesion function and is localized to apical junctions in epithelial cells (Rajasekaran et al., 2007), we investigated whether this protein also has tumor-suppressor function. In this study, we have shown that repletion of NaK-ß$_1$ in MSV-MDCK cells, a highly tumorigenic cell line, suppresses both anchorage-independent growth and tumorigenicity in immunocompromised mice, suggesting that the NaK-ß$_1$ has a potential tumor-suppressor role in epithelial cells.

Materials and methods

Cell lines and cell culture

MSV-Vector, MSV-NaK-ß$_1$ clone 1 (MSV-NaK-ß$_1$-cl1), and MSV-NaK-ß$_1$ clone 2 (MSV-NaK-ß$_1$-cl2) cell lines used in this study were grown as previously described (Rajasekaran et al., 2001).

Soft agar assay

25,000 cells (MSV-Vector, MSV-NaK-ß$_1$-cl1, and MSV-NaK-ß$_1$-cl2) were placed into DMEM (20% FBS) containing 0.3% Noble Agar and placed over a layer of DMEM (12% FBS) containing 0.5% Noble Agar. After hardening, an additional layer of DMEM (12% FBS) containing 0.5% Noble Agar was placed over the bottom layers. Samples were monitored for 14 days; colonies were then counted and photographed. Results represent mean number of colonies (± standard error) (N=4). This experiment was repeated and confirmed, and data were analyzed using Student's t-test for unequal variance.

In vivo tumorigenicity studies

Animal studies were performed following approval from the UCLA Animal Research Committee. The MSV-Vector, MSV-NaK-ß$_1$-cl1, and MSV-NaK-ß$_1$-cl$_2$ cells were harvested, washed, and resuspended in sterile phosphate buffered saline (PBS). One million cells were injected subcutaneously into the hind limb of 8-week-old Fox Chase C.B-17 severe combined immunodeficiency (SCID) Beige mice (model number: CBSCBG-MM; Taconic, Hudson, NY) (n=8). Animals were observed three times a week until formation of a palpable tumor. Tumors then were measured once per week until the end of the observation period (60 days). Tumors were harvested and tissue separated for immunoblotting and immunhistochemistry. Samples for immuno-histochemistry were fixed overnight in 10% neutral buffer formalin and processed into paraffin blocks by standard procedures.

Cell aggregation

MSV-Vector, MSV-NaK-ß$_1$-cl1, and MSV-NaK-ß$_1$-cl2 cells were trypsinized and resuspended in warm media. 2x10^6 cells were placed onto 10-cm Petri dishes coated with 1% agarose in sterile water and were gently shaken for 24 hours in a tissue culture incubator (5% CO_2, 37°C). After 24 hours, the media and cells were collected, spun down, and prepared for immunoblotting.

MSV cell pellets

Confluent plates of MSV-Vector cells were treated with either 10 µM PD98059 or vehicle (DMSO) for one hour. Plates then were washed in PBS and fixed in 10% neutral buffered formalin. Plates were scraped and cells were spun down, and the resulting cell pellet was prepared in HistoGel (Richard-Allen Scientific, Kalamazoo, MI) according to the manufacturer's instructions. The cell/gel pellets then were processed into paraffin blocks by standard procedures.

Immunoblot analysis

Total protein cell lysates or tumor tissue lysates were prepared in a lysate buffer containing 20 mM Tris (pH 7.5); 150 mM NaCl; 1 mM EDTA; 1 mM EGTA; 1% Triton X-100; 2.5 mM sodium pyrophosphate; 1 mM ß-glycerolphosphate; 1 mM sodium vanadate; 1 mM phenylmethylsulfonyl fluoride; and 5 mg/ml of antipapain, leupeptin, and pepstatin. In addition, tumor tissues were homogenized with a polytron homgenizer and sonicated. Twenty-five micrograms of cell or tumor lysates were separated by SDS-PAGE and transferred

onto nitrocellulose membrane. Blots were blocked in 5% nonfat milk in Tris buffered saline (TBS)/0.1% Tween 20 (TBST). Primary antibodies were diluted in either 5% bovine serum albumin/TBST or 5% nonfat milk/TBST and incubated overnight at 4°C. Secondary antibodies were diluted in 5% nonfat milk/TBST, and blots were developed with ECL plus (GE Healthcare, Piscataway, NJ). The rabbit polyclonal antibodies to phosphorylated and total ERK1/2 were purchased from Cell Signaling Technology (Danvers, MA), and mouse monoclonal antibodies to Na,K-ATPase α and ß-subunits have been described previously (Rajasekaran et al., 2001).

Immunohistochemistry

Five-micron sections were cut from paraffin blocks, floated onto charged slides, and dried. Sections were incubated at 60°C for 30 minutes, deparaffinized in xylene, and rehydrated in graded ethanol. Antigen retrieval was performed as described previously (Espineda et al., 2003). Endogenous peroxidase was blocked by incubation of sections in 3% hydrogen peroxide in water for 10 minutes. Sections were blocked in 4% FBS in TBST, and the primary antibodies were incubated at a dilution of 1:200 (NaK-ß$_1$) or 1:100 (phosphorlyated ERK 1/2) in 4% FBS/TBST overnight at 4°C. Sections were washed in TBST and incubated with donkey anti-mouse biotinylated (Fab)'2 fragment (Jackson Imunoresearch, Westgrove, PA) or donkey anti-rabbit biotinylated IgG for 1 hour at room temperature. ABC complex and DAB development were performed as described previously (Espineda et al., 2003). Sections were counterstained with Harris hemotoxylin, dehydrated, and coverslipped. Sections minus the primary antibody were used as controls.

Fully quantitative image analysis of NaK-ß$_1$ and extracellular regulated kinase (ERK)1/2 levels

Image analysis-based scoring of phosphorylated ERK1/2 (P-ERK1/2) and NaK-ß$_1$ immunohistochemistry was performed with the use of Soft Imaging System Software (Soft Imaging Systems, Inc., Lakewood, CO) according to the previously described method (Mellinghoff et al., 2005). Tumors from all eight of the mice immunostained for ERK1/2 and NaK-ß$_1$ were blindly analyzed by this approach (KY). Representative images from P-ERK1/2, NaK-ß$_1$ immunostained, and control slides were imaged. Using Soft Imaging analysis (Soft Imaging Systems Inc.), borders between individual cells were approximated using a separator function with the parameters of smooth and fine/coarse, 2 and 10 respectively, as previously reported (Mellinghoff et al., 2005). Total cell number separated by this filter function ranged from 500 to 1000 per immunostained slide, which was used for quantification. Quantitative analysis of DAB staining was done using an HSI color algorithm based on hue, saturation, and intensity. Saturation of reaction product in the separated cells was quantified in the red-brown hue range (<120, >300) to exclude the negative staining areas with hematoxylin nuclear staining. To compare the staining intensity between samples, mean saturation of total cells in the red-brown hue range on each image was quantified and calculated. Individual mean saturation values of P-ERK1/2 (n=8) samples were normalized to their corresponding mean saturation value of NaK-ß$_1$ subunit to generate the saturation ratio and were used to determine mean saturation ratio for each cell line (MSV-Vector, MSV-NaKß$_1$-cl1, and MSV-NaKß$_1$-cl2). Results represent mean saturation ratio for all samples (± standard error). Data were analyzed by Student's t-test for unequal variance.

Results

We used an *in vitro* anchorage-independent growth assay and an *in vivo* xenograft assay to test whether repletion of NaK-ß$_1$ expression suppresses tumorigenicity of MSV-MDCK cells. Two independent clones, MSV-NaK-ß$_1$-cl1 and MSV-NaK-ß$_1$-cl2, which express 3.6- and 5.2-fold more NaK-ß$_1$ compared with vector transfected control MSV-MDCK (MSV-Vector) cells (Fig. 1A), were used for these experiments. After 14 days in soft agar, MSV-Vector cells developed into large colonies, while MSV-NaK-ß$_1$-cl1 and cl2 remained as either single cells or small cell aggregates (Fig. 1B). The MSV-Vector cells produced 20 (± 3) colonies compared with 1 (± 0) for MSV-NaK-ß$_1$-cl1 (P=0.005) and one (± 0) for MSV-NaK-ß$_1$-cl2, respectively (P=0.005) (Fig. 1C). This result indicated that repletion of NaK-ß$_1$ in MSV-MDCK cells significantly inhibits anchorage-independent growth of MSV-MDCK cells.

MSV-MDCK cells readily form tumors in nude mice (U et al., 1985). To test whether NaK-ß1 expression reduces the tumorigenic potential of MSV-MDCK cells, we injected MSV-Vector, MSV-NaK-ß$_1$-cl1, and NaK-ß$_1$-cl2 cells into SCID mice. Cells were injected subcutaneously into the flanks of SCID mice, eight mice per group, and monitored for tumor formation. As shown in Figure 2A and Table 1, seven of eight mice injected with MSV-vector cells had palpable tumors (average diameter: 1.5 mm, ± 0.22 mm) by day 28. In contrast, no tumors were detected at day 28 in either group of mice

Table 1. Tumor burden of mice injected with MSV-Vector and MSV-NaK-ß$_1$ cell lines.

Sample	28 Days	40 Days	53 Days	60 Days
MSV-Vector	7/8	8/8	8/8	8/8
MSV-NaK-ß$_1$-cl1	0/8	5/8	8/8	8/8
MSV-NaK-ß$_1$-cl2	0/8	4/8	7/8	8/8

The tumor size was monitored with calipers at the indicated time points and tumor bearing animals per total number of animals used in study are indicated. (MSV=Moloney sarcoma virus)

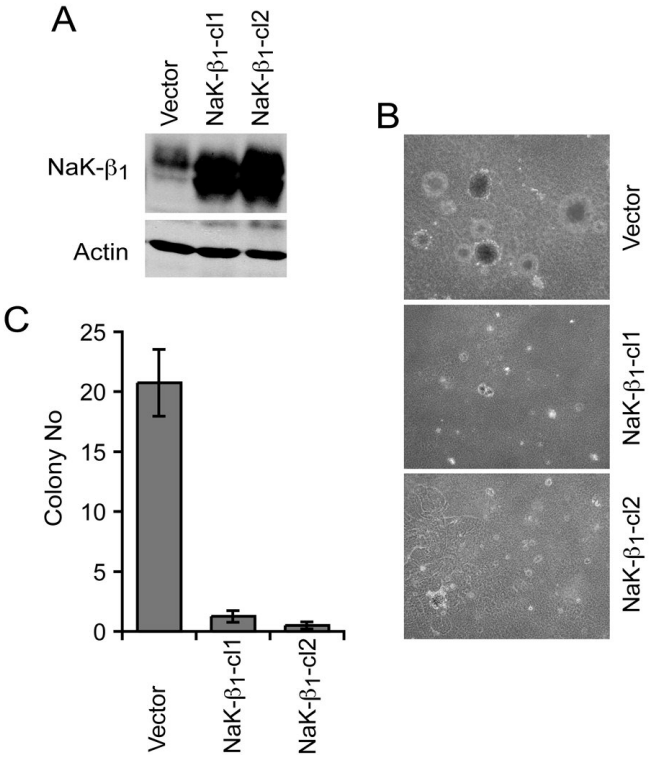

Fig. 1. Characterization of NaK-ß$_1$ levels and anchorage independent growth in MSV-MDCK cell lines: **A.** Immunoblot of NaK-ß$_1$. Cell lysates (50 µg) were separated by SDS-PAGE and immunoblotted for NaK-ß$_1$ and actin (loading control). **B.** Phase contrast images showing anchorage independent growth of MSV-Vector and NaK-ß$_1$ expressing cell lines. Representative images of MSV-Vector, MSV- NaK-ß$_1$-cl1, and cl2 cells at day 14 are shown. MSV-Vector cells develop into large colonies, while both MSV- NaK-ß$_1$ -cl1 and cl2 cells formed small colonies. x 320. **C.** Quantification of anchorage independent growth. Colonies from MSV-Vector, MSV-NaK-ß$_1$ cl1 and cl2 (n=4 per cell line) from two independent determinations were counted and the mean was calculated. Colonies of 0.5 mm or larger diameter were counted and quantified. Bars represent standard error. (MDCK=Madin-Darby canine kidney; MSV=Moloney sarcoma virus)

injected with NaK-ß$_1$-expressing cells (P<0.001). Tumors eventually appeared in all mice but initially grew at a much slower rate in the two NaK-ß$_1$ groups. At day 40, mean tumor diameters for MSV-NaK-ß$_1$-cl1 and MSV-NaK-ß$_1$-cl2 groups were 32% and 21%, respectively, of the MSV-Vector group (both P=0.001). By day 53, tumor sizes in the MSV-NaK-ß$_1$-cl1 group were similar to those in the MSV-Vector group. However, mean tumor diameters in mice receiving MSV-NaK-ß$_1$-cl2, which expresses the highest levels of NaK-ß$_1$, were only 45% and 66% of the diameters in the MSV-Vector group at days 53 and 60, respectively

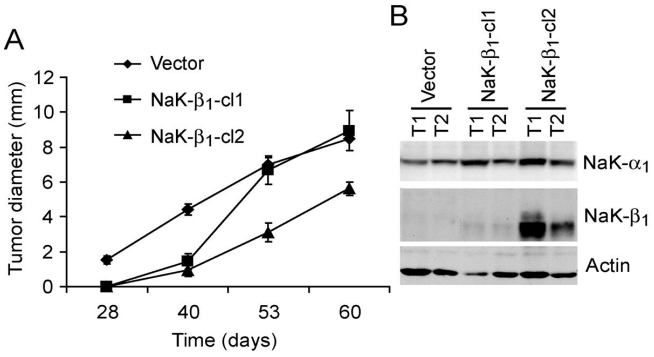

Fig. 2. Tumorigenicity in MSV-Vector and NaK-ß$_1$ expressing cell lines: **A.** Tumor growth of MSV cell lines. SCID mice were injected with MSV-Vector, MSV-NaK-ß$_1$-c11 and cl2 cells as described in Materials and methods. Tumors were measured with calipers and data points represent mean tumor diameter from eight mice for indicated time points. Bars represent standard error. **B.** Immunoblot of NaK-α_1 and NaK-ß$_1$ levels in MSV-Vector and MSV-NaK-ß$_1$ cl1 and cl2 tumors. Tumor lysates (50 µg) were separated by SDS-PAGE and immunoblotted for NaK-α_1 and ß$_1$. Two samples from each group are shown. Actin was used as a loading control. (MSV=Moloney sarcoma virus; SCID=severe combined immunodeficiency)

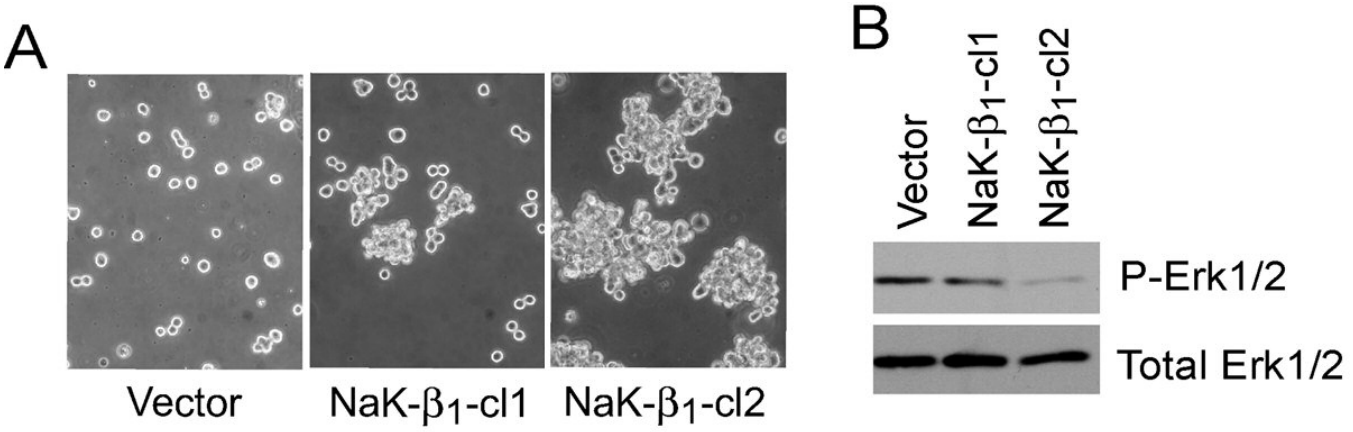

Fig. 3. Cell aggregation and ERK1/2 activation in MSV-Vector and NaK-ß$_1$ expressing cell lines. **A.** Phase contrast images showing cell-cell aggregation: Note lack of cell aggregation in MSV-vector cells compared with the large aggregates present in NaK-ß$_1$ expressing cells. x 300. **B.** Immunoblot analysis of active of ERK1/2. Protein lysate (50 µg) from MSV-Vector, MSV-NaK-ß$_1$ c11 and cl2 cells following aggregation for 24 hours were immunoblotted for phosphorylated ERK1/2 and total ERK1/2. (ERK=extracellular regulated kinase; MSV=Moloney sarcoma virus).

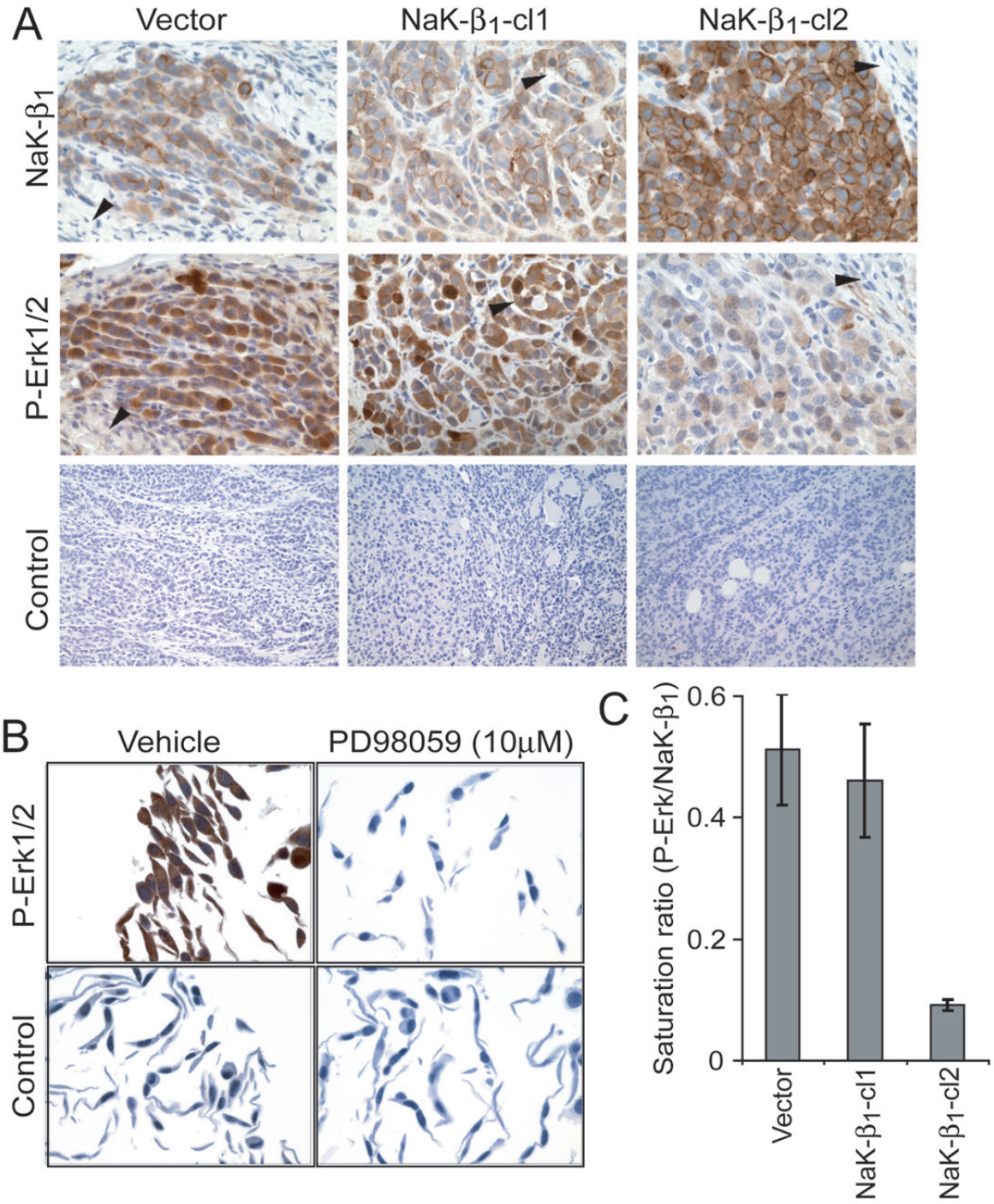

Fig. 4. A. Immunohistochemical staining and quantification of NaK-ß$_1$ and phosphorylated ERK1/2 levels in MSV-Vector, MSV-NaK-ß$_1$ cl1 and cl2 tumors: Serial sections of MSV-Vector, MSV-NaK-ß$_1$ cl1 and cl2 tumors were stained for phosphorylated ERK1/2 and NaK-ß$_1$. Representative images of stained sections are shown. Arrowheads denote the landmarks in each stained serial section to identify similar regions of tissue stained with either NaK-ß$_1$ or phosphorylated ERK1/2 (P-ERK1/2). Magnification 1200x for immunostained samples and magnification for control 450x to show lack of background staining. **B.** Specificity of phosphorylated ERK1/2 antibody. MSV-Vector cells were incubated with either 10μM PD98059 or vehicle and were processed into paraffin blocks and stained. Top panels- P-ERK1/2 staining of MSV-Vector cells incubated with vehicle or 10μM PD98059. Bottom panels-control staining of vehicle and 10μM PD98059 treated MSV-Vector paraffin cell pellets. x 1200. **C.** Quantification of immonohistochemical staining in MSV-Vector, MSV-NaK-ß1 clones 1 and 2 tumors. Mean saturation ratio was quantified and calculated. Bars represent standard error. ERK: extracellular regulated kinase; MSV: Moloney sarcoma virus.

(P<0.01). Immunoblot analysis of tumor tissues at day 60 revealed that NaK-ß$_1$ levels in MSV-NaK-ß$_1$-cl1 tumors were similar to the low NaK-ß$_1$ levels of MSV-Vector tumors (Fig. 2B, middle panel). In comparison, MSV-NaK-ß$_1$-cl2 tumors had significantly higher NaK-ß$_1$ levels (Fig. 2B). Although the ß-subunit levels were drastically reduced in both clones in the *in vivo* xenografts models, levels of NaK-α_1 remained more or less the same in these tumors (Fig. 2B, top panel). Moreover, immunohistochemical analysis using antibodies to NaK-α_1 (data not shown) and NaK-ß$_1$ subunit (Fig. 4A) were consistent with the immunoblot data.

Transformation by the Moloney sarcoma virus results in expression of the v-mos oncogene (Topol and Blair, 1995). Constitutive expression of v-mos activates ERK 1/2 (Maxwell and Arlinghaus, 1985; Topol and Blair, 1995; Topol et al., 1995). The ERK1/2 (p44 and p42 MAPK, respectively) controls cell growth and differentiation and has long been a focus for cancer therapeutics. The ERK1/2 is activated by threonine and tyrosine phosphorylation in response to mitogens such as epidermal growth factor (EGF), insulin, and phorbol esters (Cooper et al., 1982; Rossomando et al., 1989). When activated, ERK1/2 phosphorylates various downstream substrates involved in many cellular responses, such as cell proliferation, cell differentiation, cell survival, and cell motility (Kohno and Pouyssegur, 2006). Cell-cell adhesion regulates ERK1/2 activation (Conacci-Sorrell et al., 2003; Laprise et al., 2004). Since NaK-ß$_1$ has adhesive function, we hypothesized that repletion of NaK-ß$_1$ in MSV-MDCK cells might affect ERK1/2 activation. Incubation of MSV-Vector, MSV-NaK-ß$_1$-cl1, and MSV- NaK-ß$_1$–cl2 cells under conditions that prevent adhesion to the substratum produced large cell aggregates in MSV- NaK-ß$_1$–cl1 and cl2 cells, whereas MSV-Vector cells did not (Fig. 3A). The size of the aggregates positively correlated with the levels of NaK-ß$_1$ expressed in these cells (Fig. 1A). This adhesion assay confirmed the cell-cell adhesion function of NaK-ß$_1$ as reported earlier (Rajasekaran et al., 2001). Analyses of activated ERK1/2 levels from these cell aggregates were found to be inversely proportional to the NaK-ß$_1$ expression levels. MSV-NaK-ß$_1$-cl1 and cl2 cells had 18% and 73% less activated ERK1/2, respectively, when compared with MSV-vector cells (Fig. 3B). The total levels of ERK1/2 were similar in all three cell lines.

These results indicated that the level of NaK-ß$_1$ inversely correlates with the levels of phospho-ERK1/2 in MSV-MDCK cells. To further confirm this finding, we investigated the association of NaK-ß$_1$ and activated ERK1/2 in tumor tissues. We used a fully quantitative immunohistochemical analysis of MSV-Vector, MSV-NaK-ß$_1$-cl1, and MSV-NaK-ß$_1$-cl2 tumor tissues to validate the levels of phosphorylated ERK1/2 and the NaK-ß$_1$. As a control for specificity of the phosphorylated ERK1/2 antibody, we treated MSV-MDCK cells with either 10 µM PD98059 or vehicle (DMSO), which was fixed in formalin, processed, and stained for phosphorylated ERK1/2 identically to the tumor tissues. PD98059 inhibits MEK, an upstream activator of ERK1/2 that is directly activated by the v-mos gene product (Pham et al., 1995). As shown in Figure 4B, vehicle-treated cells showed intense nuclear and cytoplasmic staining, whereas this staining was absent in cells treated with 10 µM PD98059 (Fig. 4B). Immunohistochemical staining of phosphorylated ERK1/2 in tumor tissues exhibited similar intensities between MSV-Vector and MSV- NaK-ß$_1$-cl1 tumors, whereas MSV- NaK-ß$_1$-cl2 tumors revealed lesser staining intensity (Fig. 4A). NaK-ß$_1$ staining intensity showed an inverse pattern, with MSV- NaK-ß$_1$–cl2 displaying the highest intensity (Fig. 4A). Quantitative image analysis of staining intensity from tumor samples revealed comparable phosphorylated ERK1/2 saturation ratios between MSV-Vector (0.51, ±0.091) and MSV-NaK-ß$_1$-cl1 (0.46, ±0.093) tumors (P=0.706). The MSV-NaK-ß$_1$-cl2 tumors had a significantly lower saturation ratio (0.09, ±0.008) (Fig. 4C, P=0.002). This result indicated that increased NaK-ß$_1$ expression in MSV-NaK-ß$_1$-cl2 tumor tissues is associated with decreased phosphorylated ERK1/2 levels, which may have contributed to the latency and slow growth of these tumors.

Discussion

In this study, we demonstrated that repletion of NaK-ß$_1$ in MSV-MDCK cells inhibits anchorage-independent growth and suppresses tumorigenicity in *in vivo* xenograft models. We also showed that NaK-ß$_1$-mediated cell-cell adhesion in MSV-MDCK cells is associated with reduced phospho-ERK1/2 levels. Using immunohistochemistry and fully quantitative image analysis, we also provided evidence that the levels of NaK-ß$_1$ negatively correlate with the phospho-ERK1/2 levels in tumor tissues. Thus, these results suggest that NaK-ß$_1$might suppress tumor growth by reducing the levels of phospho-ERK1/2 levels in MSV-MDCK cells. To our knowledge, this is the first report implicating NaK-ß$_1$ in the tumor-suppressor function in epithelial cells.

Although the levels of NaK-ß$_1$ in MSV-NaK-ß$_1$-cl1 cells were lower than that of cl2 cells, both similarly inhibited the anchorage-independent growth. However, in the xenograft assay, the cl1 cells formed tumors much more rapidly than the cl2 cells. The duration of the experiment and the levels of NaK-ß$_1$ maintained in the cells might have contributed to this effect. The anchorage-independent assay was done within 14 days, whereas the tumorigenic assay was completed only after 60 days. When MSV-NaK-ß$_1$-cl2 were grown in the absence of selection marker G418 for 15 days, there was hardly any change in the levels of NaK-ß$_1$; however, after 90 days, its expression was similar to vector-transfected cells (Rajasekaran et al., 2001). Therefore, it is possible that in the absence of selection pressure, the

NaK-ß$_1$ expression is reduced with time, leading to increased tumor growth in MSV- NaK-ß$_1$-cl1 cells when compared with cl2 cells. Although the levels of NaK-ß$_1$ in cl2 cells were reduced with time, at day 60, these cells contained higher levels ofNaK-ß$_1$ than cl1 cells. This higher level of NaK-ß$_1$ may have contributed to the attenuated growth of the tumor. Thus, these results suggest that high levels of NaK-ß$_1$ are necessary to inhibit anchorage-independent growth and tumorigenicity.

Several studies have shown that the NaK-ß$_1$ expression is necessary to stabilize and transport NaK-α_1 to the plasma membrane (Ackermann et al., 1990; Geering, 1990; McDonough et al., 1990; Noguchi et al., 1990). However, the highly reduced levels of NaK-ß$_1$ in tumor tissues were not accompanied by diminished levels of NaK-α_1. We previously observed a similar phenomenon in poorly differentiated carcinoma cell lines (i.e., a drastic reduction of NaK-ß$_1$ in these cells was not accompanied by similar reduction in the levels of NaK-α_1) (Espineda et al., 2004). These results suggest that NaK-α_1 and ß$_1$ subunits are differentially regulated in cancer cells and that NaK-α_1 remains stable even in the presence of low amounts of NaK-ß$_1$. It is well known that NaK-ß$_1$ is necessary for efficient translation, transport, and stability of NaK-α_1. However, how these functions are accomplished in the presence of low NaK-ß$_1$ levels in tumor tissues remains to be determined.

One marked finding reported here is the inverse correlation of ERK1/2 activity and NaK-ß$_1$ levels. We showed that the levels of NaK-ß$_1$ negatively correlate with the levels of activated ERK1/2. Interestingly, we observed this effect only when the cells were grown in three-dimensional conditions such as aggregation, anchorage-independent growth, and xenograft assays and not when the cells were grown on culture dishes. Although the mechanism by which NaK-ß$_1$ levels modulate ERK1/2 activity is not known, this result suggests that loss of NaK-ß$_1$ epithelial cells might be associated with the activation of ERK1/2 in carcinoma cells. Experiments to validate this notion continue in our laboratory.

In an earlier study, we showed that both the MSV-NaK-ß$_1$-cl1 and cl2 used in this study are invasive in an *in vitro* collagen gel invasion assay (Rajasekaran et al., 2001). However, both of these cell lines showed inhibition of anchorage-independent growth and were less tumorigenic in nude mice (current study). These results indicate that tumorigenicity and invasive behavior are regulated by different mechanisms and that NaK-ß$_1$ has a more potent tumor-suppressor function compared with its invasion suppression in MSV-MDCK cells.

Interestingly, the suppression of tumorigenicity by NaK-ß$_1$ is similar to the tumor-suppressor function reported for E-Cadherin, a cell-cell adhesion molecule expressed in epithelial cells (Frixen et al., 1991; Birchmeier, 1995; Christofori and Semb, 1999). The injection of v-Ras-transformed MDCK cells expressing E-cadherin into nude mice also formed tumors with a period of latency (Mareel et al., 1991), a characteristic observed in our study. These cells also had decreased expression of E-cadherin in tumors, similar to the reduced NaK-ß$_1$ found in our MSV-MDCK clones. Likewise, two independent studies with human colon carcinoma cell lines (SW480 and Caco-2/15) have shown that the E-cadherin cell-cell adhesion function decreases ERK1/2 activation (Conacci-Sorrell et al., 2003; Laprise et al., 2004). Since MSV-MDCK cells do not express detectable levels of E-cadherin (Rajasekaran et al., 2001), the cell-cell adhesion function, the suppression of tumorigenicity, and the decrease of ERK1/2 activation observed in the MSV- NaK-ß$_1$ cl1 and cl2 cell lines are due primarily to the expression of NaK-ß$_1$. Whether the NaK-α_1 has any role in the tumor-suppressor function is not known. However, because the levels of NaK-α_1 are similar in all the tumors, it is unlikely that this protein plays a role in tumor suppression. These results indicate that the adhesive function of NaK-ß$_1$ could modulate ERK1/2 activity and thus suppress tumor growth. These studies strongly suggest that the NaK-ß$_1$ is a multifunctional protein, and loss or reduced expression of this protein in carcinoma cells is associated with their tumorigenicity. Since inhibition of anchorage-independent growth and tumorigenicity are bona fide features of tumor-suppressor proteins (Koi et al., 1989; Boylan and Zarbl, 1991; Mikheev et al., 2004), we suggest that the NaK-ß$_1$ has potential tumor-suppressor function in epithelial cells.

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