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Altered SK3/KCa2.3-mediated migration in adenomatous polyposis coli (*Apc*) mutated mouse colon epithelial cells

Marie Potier [a,1], Truong An Tran [b,1], Aurelie Chantome [a], Alban Girault [a], Virginie Joulin [c], Philippe Bougnoux [a], Christophe Vandier [a,*], Fabrice Pierre [d]

[a] Inserm U921, Nutrition Croissance et Cancer, 10 Boulevard Tonnelle, 37032 Tours Cedex, France
[b] Inserm U619, Hôpital Bretonneau, 2 Boulevard Tonnelle, 37044 Tours Cedex 9, France
[c] CNRS-FRE 2939, Institut Gustave Roussy, 94805 Villejuif, France
[d] Université de Toulouse, UMR1089 Xenobiotiques, INRA, ENVT, 23 chemin des Capelles, 31076 Toulouse, France

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ABSTRACT

Lost of adenomatous polyposis coli gene (*Apc*) disturbs the migration of intestinal epithelial cells but the mechanisms have not been fully characterized. Since we have demonstrated that SK3/KCa2.3 channel promotes cancer cell migration, we hypothesized that Apc mutation may affect SK3/KCa2.3 channel-mediated colon epithelial cell motility. We report evidence that SK3/KCa2.3 channel promotes colon epithelial cells motility. Following Apc mutation SK3/KCa2.3 expression is largely reduced leading to a suppression of the SK3/KCa2.3 channel mediated-cell migration. Our findings reveal a previously unknown function of the SK3/KCa2.3 channel in epithelial colonic cells, and suggest that Apc is a powerful regulator SK3/KCa2.3 channel.

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1. Introduction

In colon carcinogenesis, mutations in the adenomatous polyposis coli (*Apc*) gene are considered to be one of the earliest events in the initiation and promotion of colorectal cancer [1]. Germline mutations in *Apc* gene also cause inherited familial adenomatous polyposis (FAP) syndrome, characterized by the early onset of hundreds to thousand of adenomatous polyps throughout the colon.

The *Apc* protein has different functions in maintaining ordered growth of intestinal epithelial cells and is a classical tumor suppressor protein [2]. The *Apc* gene product indirectly regulates the transcription of a number of critical cell proliferation, cell cycle control, apoptosis and tumor progression genes. *Apc* function has been linked to the Wnt signal transduction pathway, where it normally functions to target the transcription factor β-catenin for degradation [3]. Several mouse models (Apc Delta716, Apc Delta1309, Apc Delta474, Apc *Min*) have been established to investigate carcinogenesis caused by Apc mutations [4]. *Apc* also interacts with numerous actin- and microtubule-associated proteins. *In vivo*, the loss of Apc function disturbs the migration of intestinal epithelial cells [5]. Persuasive evidence that Apc could regulate cell migration by remodelling actin cytoskeleton has been provided [6]. To understand the effective role of Apc mutation in the disturbance of migration, it is essential to have an adapted cellular model. Recent intestinal cell lines derived from C57BL/6J mice of the two genotypes ($Apc^{+/+}$ and $Apc^{Min/+}$) were obtained [7]. $Apc^{Min/+}$ cell line, heterozygous for a germline nonsense mutation in the tumor suppressor gene *Apc*, retains the disordered actin cytoskeleton network. As reported *in vivo*, we have observed *in vitro*, that Apc mutation disturbs cell migration. We found that the $Apc^{Min/+}$ epithelial cells were less motile than $Apc^{+/+}$ epithelial cells but the mechanisms were not fully characterized.

Ionic channels have been involved in epithelial tumorigenesis and numerous studies demonstrate a contribution of potassium channels to proliferation of cancer cells. Colonic changes in $Apc^{Min/+}$ are accompanied by an increase activity of voltage-dependant potassium channels Eag-1, Elk-1, Erg-1 and of the large conductance Ca2+-activated potassium channel BKCa [8,9]. All of theses channels support proliferation of colonic cancer cells and any role for potassium channel in cell motility have not been reported.

Recently, we have demonstrated that a small-conductance Ca2+-activated K+ channels family, SK3 also named KCa2.3 channel, is a mediator of epithelial cancer and melanoma cell motility [10,11]. There are three isoforms of SKCa α subunits, named SK1/KCa2.1, SK2/KCa2.2, SK3/KCa2.3 which associate to form homo- or hetero-tetramers [12,13]. SK3/KCa2.3 protein was found to be

Abbreviations: Apc, adenomatous polyposis coli; sK3, small-conductance Ca2+-activated K+ channel isoform 3.

* Corresponding author. Fax: +33 247366226.
E-mail address: christophe.vandier@univ-tours.fr (C. Vandier).

[1] Marie Potier and Truong An Tran contributed equally to this work.

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expressed in vascular and visceral smooth muscle-rich tissues [14]. In these muscles and in cancer cells the activation of SK3/KCa2.3 channel induces hyperpolarisation which respectively regulates muscle tone and cell motility [11,15–17].

Since SK3 channel promotes epithelial cancer cell migration and Apc mutation disturbs colon epithelial cell motility and expression of other potassium channels involved in cell motility [8,9,18] we hypothesize that Apc mutation may modify the SK3/KCa2.3 channel-mediates colon epithelial cells motility.

In this report, we take advantage of a cellular model established from the Apc+/+ and *ApcMin/+* mouse to compare the expression and the function of SK3/KCa2.3 channel in normal and *Apc* mutated mouse colon epithelial cells.

2. Materials and methods

2.1. Cell lines

$Apc^{+/+}$ and $Apc^{Min/+}$ colon epithelial cells were established as previously described [7]. Both cell lines express the heat-labile SV40 large T antigen (AgT tsa58) under the control of an interferon γ-inducible promoter. At 33 °C with interferon γ, the temperature-sensitive SV40 large T antigen is active and drives cell proliferation. At 37 °C the temperature-sensitive mutation yields an inactive protein, and cells behave as non-proliferating colonic epithelial cells. Cells were seeded on 24-well plates. Cells were cultured overnight after seeding at the permissive temperature of 33 °C in DMEM (Invitrogen, France) supplemented with 10% fetal calf serum (Sigma–Aldrich, France), 1% penicillin/streptomycin, 2% glutamin (Invitrogen), 10 ng/mL epidermal growth factor (Sigma–Aldrich) and 10 U/mL interferon γ (Becton–Dickinson, France). The experiments were performed after 24 h at 37 °C without interferon γ, in order to inhibit the SV40 transgene and to limit cell proliferation. At non-permissive temperature, the cell line can be maintained in culture for 8 days, which is comparable to normal epithelial cells.

The presence or absence of *Min* mutation in these both cell lines was confirmed by checking the consequence of *Apc* mutation on actin networks by immunofluorescence microscopy.

2.2. Cell proliferation and cell migration in vitro

Cell proliferation was determined using the tetrazolium salt reduction method [19]. Cells were seeded on 24-well plates and grown for 48 h. Drugs were then added for 24 h at concentrations that had no effect on cell proliferation. Cell migration was analyzed in 24-well plates receiving 8-μm pore size polyethylene terephtalate membrane cell culture inserts (Becton–Dickinson), as already described [19].

2.3. Wound-healing assays

Cells were seeded in culture dishes and grown until confluence in DMEM supplemented with 10% foetal calf serum. The monolayer was scratched with a sterile yellow pipette tip. The migration of cells to the cleared area was inspected under a microscope. Pictures were taken directly at the time of scratching and after 24 h.

2.4. Western blot experiments

Western blot experiments were performed as described [10]. Briefly, proteins were electrotransferred onto polyvinylidene fluoride membranes that were incubated with antibodies directed against SK3/KCa2.3 protein (1:1000; Sigma–Aldrich) followed by incubation with a horseradish peroxidase-conjugated anti-rabbit IgG (1:5000; Tebu-Bio, France).

2.5. Synthesis and transfection of small interfering RNA directed against SK3/KCa2.3

A SK3 specific siRNA were designed as already described [20]: first set, hSK3-ex1-sense 5′-GAAAGCGACUGAGUGACUAdTdT-3′ and hSK3-ex1-antisense 5′-UAGUCACUCAGUCGCUUCdTdT-3′, located in exon 1; The negative control siRNA (scramble) used had the following sequence 5′-AUAACUGUAUCGAAUGUUAUGAGCC-3′. Transfections were performed as previously described [20].

2.6. Electrophysiology

All voltage-clamp experiments were performed using the perforated patch whole-cell recording technique. Electrodes (5–7 MΩ) were made using GC150F-15 borosilicate glass capillaries (Harvard Apparatus, UK). The junction potentials between the electrode and the bath were cancelled using the voltage pipette offset control of the amplifier. Electrode capacitances were electronically compensated. Membrane capacitance was calculated by integrating the capacitive current measured during a 10 mV voltage step and current-density was obtained by dividing the averaged steady-state by the respective cell capacitance.

Pipette tips were filled by dipping the tip of the pipette into the amphotericin B-free filling solution, before backfilling with amphotericin B-containing solution. After the gigaseal between the pipette and the cell was achieved, the electrical access to the cytoplasm was monitored by applying 10 mV pulses for 10 ms from a holding potential of −70 mV and monitoring the capacitive transient. Typically, access was gained within 5 min and once access was stable the experiments started.

Cells were voltage clamped at −70 mV and global currents were measured during a 10 mV increment pulse protocol between −70 and +90 mV (average of the last 50 ms of 500 ms duration pulse every 6 s) from a holding potential of −70 mV. Currents were sampled at 5 kHz and filtered at 1 kHz. Apamin-sensitive currents were determined by subtracting the current-density amplitudes obtained in the presence of apamin from those obtained under control conditions.

2.7. Solutions and drugs

The physiological saline solution (PSS) in mM: NaCl 140, $MgCl_2$ 1, KCl 4, NaH_2PO_4 0.33 $CaCl_2$ 2, D-glucose 11.1, and HEPES 10, adjusted to pH 7.4 with NaOH. The pipette filling intracellular solution used for perforated patch whole-cell recording contained (mM): Glutamic acid 122, KCl 25, $MgCl_2$ 1, EGTA 1, HEPES 10 (pH 7.2 adjusted with KOH). Perforated patch filling solutions also contained amphotericin B (240–360 μg ml^{-1}).

Apamin were added to the PSS or culture media at the concentrations indicated in the Figure legends. All drugs and chemicals were purchased from Sigma–Aldrich (St. Quentin, France).

2.8. Immunofluorescence microscopy

Cells seeded on glass coverslips were fixed in 2% formaldehyde in culture medium for 20 min. After three 5-min washes in PBS containing 1 mM $CaCl_2$ and 1 mM $MgCl_2$ (PBS+), cells were permeabilized with 0.05%/0.05% Triton X-100/Tween 20 in PBS+ for 3 min at room-temperature. After three washes in PBS+, nonspecific binding sites were blocked by incubating the cells with 3% BSA in PBS+ for 30 min. The cell monolayer was incubated with Rhodamine-labeled phalloidin (Sigma-Alrich) to label actin and DAPI to

label nucleus. Imaging was performed by using an Olympus BX60 fluorescence microscope.

2.9. Data acquisition and analysis

Experiments were conducted using an Axopatch-200B (Axon Instruments Inc.) amplifier and currents were filtered with a Bessel low-pass filter. Records were digitised with a 1322-A Digidata A/D converter (Axon Instruments Inc.) and stored on disk in a computer using the pClamp 8.1 software (Clampex, Axon Instruments). The analyses were done using analysis of the patch-clamp data was performed using Clampfit 8.1 and Origin 7.0 softwares (Microcal Software, Northampton, MA).

Data are expressed as means ± SEM (n is the number of observations) and were tested for significant differences ($p < 0.05$) with paired t tests or one-factor ANOVA with *post hoc* tests, as appropriate. SigmaStat (version 3.0.1a, Systat Software, Inc.) was used for statistical analysis.

3. Results

3.1. SKCa channels mediate migration of Apc+/+ cells but not of Apc Min/+ cells

Previous results suggest that Apc could regulate the actin cytoskeletal network, cell morphology and migration [6]. Using our cellular model established from the Apc+/+ and *ApcMin/+* mouse we first compare the actin network. Indeed, the consequence of the *Min* mutation on actin networks has been confirmed by immunofluorescence microscopy. As it can be observed, loss of *Apc* gene product leads to the disorganization of the actin cytoskeletal integrity visualized by phalloidin, as it classically associated with mutation of *Apc* gene (Fig. 1, case B), whereas in $Apc^{+/+}$, actin cytoskeletal integrity is preserved (Fig. 1, case A).

We recently demonstrated that SK3/KCa2.3 channel promotes breast cancer and melanoma cell migration [10,11]. We speculated that these channel might be differentially involved in the migratory ability of colon epithelial cells following Apc mutation. No specific KCa2.3 channel blocker was available, so we tested apamin, a blocker of all SKCa channels. Apamin decreased the number of migrated Apc+/+ cells without affecting their cell proliferation/viability (Fig. 2A). In contrast, apamin had no effect on ApcMin/+ cell migration (Fig. 2B). The role of SKCa channels on cell migration was also assessed by a monolayer wound-healing assay. Cultures of confluent cells grown on culture dishes were scratched to create a denuded area, and then the cells at the wound edges were allowed to migrate into the denuded area over a 24 h period. After 24 h, the scraped areas were largely reduced (Fig. 2C). In the presence of 10 nM apamin, migration of Apc+/+ was reduced, while this blocker didn't affect migration of ApcMin/+ colon cancer cells. These data showed that SKCa channels promote the cell migration of Apc+/+ colon cells but not of Apc Min/+ colon cells.

3.2. SKCa channels are activated in Apc +/+ cells but not in Apc Min/+ cells

To measure SKCa channel activity we performed patch-clamp experiments. Using 10 mV voltage steps, we calculated cell capacitance, reflecting the cell size. No statistical difference ($p = 0.4$) was observed between Apc+/+ cells (39.7 ± 20.4 pF; $n = 10$) and ApcMin/+ cells (41.1 ± 19.9 pF; $n = 7$). Apc+/+ cells display outward currents with no apparent time-dependence as expected for SKCa currents (data not shown). As illustrated in Fig. 3A the current-density in ApcMin/+ cells was markedly lower than in Apc+/+ cells. The part of SKCa channels in net outward current in both cells was further examined and quantified using apamin (Fig. 3). The magnitude of apamin-sensitive current was obtained by subtracting the net outward current observed in normal PSS solution from the outward current recorded in the presence of apamin, thereby obtaining the apamin-sensitive currents. In Fig. 3B it was clearly demonstrated that Apc+/+ cells expressed an apamin-sensitive current in contrast to ApcMin/+ cells in which apamin had not effect on net outward current amplitude. This suggests that SKCa channels are activated in Apc+/+ cells but not in ApcMin/+ cells.

3.3. SK3/KCa2.3 protein is differentially expressed in Apc+/+ cells compare to ApcMin/+ cells

Increased expression of potassium channels has been reported in colon cancer [8,9]. To investigate the expression of SK3/KCa2.3 protein in Apc+/+ and ApcMin/+ cell, western blot analyses were performed. As shown in Fig. 3C, both Apc+/+ and ApcMin/+ cells expressed SK3/KCa2.3 protein, with a protein level lower in ApcMin/+ compared to Apc+/+ cells.

3.4. SK3/KCa2.3 mRNA knock-down decreased migration of Apc+/+ cells but not of ApcMin/+ cells

To further demonstrate the contribution of SK3/KCa2.3 channel in Apc+/+ cell migration, SK3/KCa2.3 mRNA were knocked-down by transiently transfecting cells with siRNA locating in exon 1 of KCNN3 human gene, or with scrambled-siRNAs as a negative control. The knock-down of SK3/KCa2.3 markedly reduced the number of Apc+/+ migrating cells to an approximately same amount than apamin, but did not affect ApcMin/+ cells (Fig. 4). Our data demonstrate that endogenous SK3/KCa2.3 channel is necessary for Apc+/+ cell migration but not for ApcMin/+ migration.

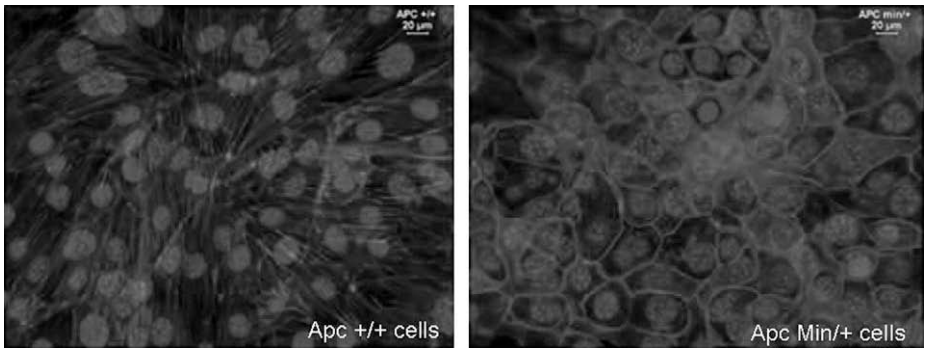

Fig. 1. Loss of Apc function leads to the disorganization of the actin cytoskeleton. Immunofluorescent staining of $Apc^{+/+}$ and $Apc^{Min/+}$ cells in the presence of phalloidin (confocal fluorescence microscopy). (A) $Apc^{+/+}$ cells, (B) $Apc^{Min/+}$ cells.

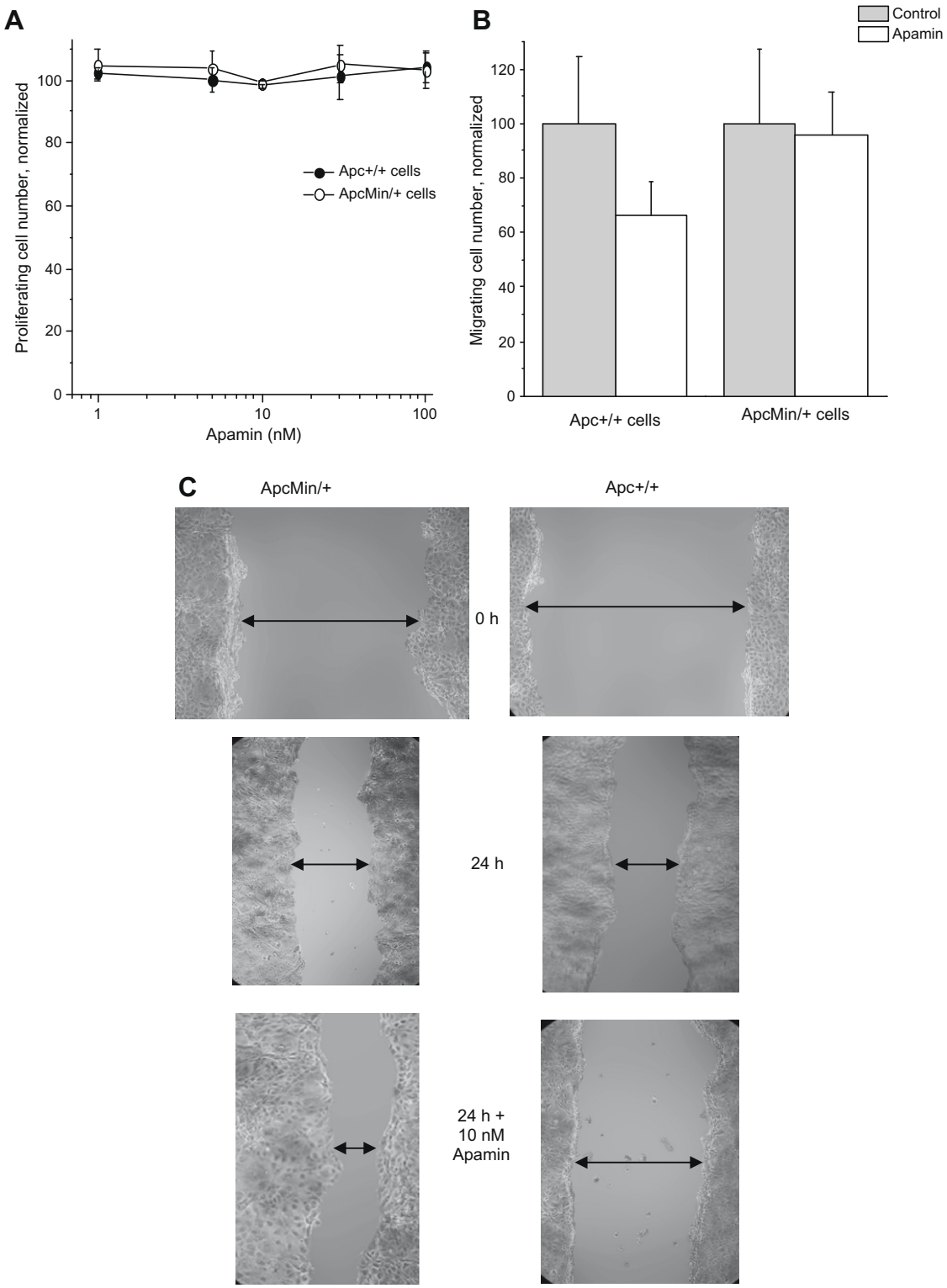

Fig. 2. Involvement of SKCa channels on Apc+/+ and ApcMin/+ mouse colon epithelial cell survival, proliferation and migration. (A) Dose-dependent effect of apamin on cell survival and proliferation in Apc+/+ and ApcMin/+ mouse colon epithelial cells. Cell proliferation was determined using the tetrazolium salt reduction method. Cells were seeded on 24-well plates and grown for 48 h. Drugs were then added for 24 h. The normalized cell number corresponded to the ratio of total number of cells in presence of apamin/total number of cells in control experiments. (B) Histograms showing the effect of apamin on Apc+/+ and ApcMin/+ murine colon epithelial cells. Cells were seeded at 60,000 cells in a cell culture insert in DMEM with 5% FBS ± apamin. The lower compartment of the insert contained DMEM with 10% FBS as a chemoattractant ± apamin. After 24 h, cells of the lower compartment were stained with hematoxylin and counted. The normalized cell number corresponded to the ratio of total number of migrating cells in presence of apamin/total number of migrating cells in control experiments. The selected drug concentrations have no effect on cell proliferation and viability. Results from two separate experiments performed in triplicate are expressed as mean ± SEM. * Significantly different from control at $p < 0.05$. (C) Wound-healing assays were performed with Apc+/+ and ApcMin/+ cells after 24 h in the presence or in the absence of apamin. Representative images are shown at the indicated times. The magnification is ×200. The lines indicate the clear region of the scraped area.

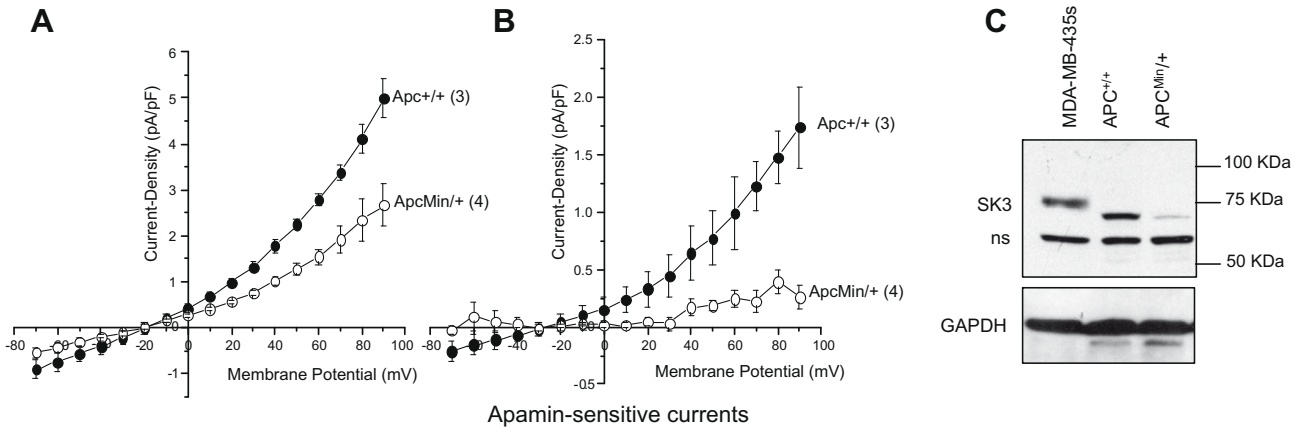

Fig. 3. Effects of apamin on SKCa currents of Apc+/+ and ApcMin/+ mouse colon epithelial cells and SK3/KCa2.3 protein expression d in Apc+/+ and ApcMin/+. (A) Current density-voltage relation obtains in control and in Apc+/+ and ApcMin/+ murine colon epithelial cells. The current density-voltage relation was obtained by dividing the averaged steady-state currents elicited between −70 mV to +90 mV (recorded during the latest 50 ms of the pulse) by the respective cell capacitance. Membrane capacitance was calculated by integrating the capacitive current measured during a 10 mV voltage step. (B) Apamin-sensitive current density-voltage relations obtained during voltage steps in Apc+/+ or ApcMin/+ murine colon epithelial cells. The magnitude of apamin-sensitive currents were obtained by subtracting the net outward current observed in normal PSS solution from the outward current recorded in the presence of apamin. Results represent the mean ± SEM. $^{*}p < 0.05$; indicated significant difference compare to control cells. The numbers in brackets indicated the number of cells. (C) Representative Western blot pattern of SK3 protein expression in Apc+/+ compared to ApcMin/+ murine colon epithelial cells. Lysates of murine colon epithelial cells were prepared in lysis buffer (SDS 5%, protease inhibitors 1%, PMSF 200 mM). Cells extracts were subjected to electrophoresis on SDS–polyacrylamide gel under reducing conditions and the signal was detected by ECL. Results were provided in triplicate.

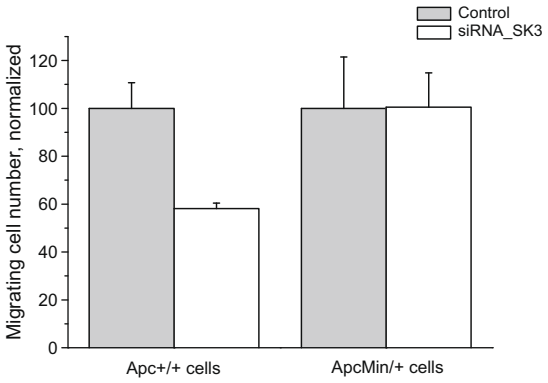

Fig. 4. SK3/KCa2.3 mRNA knock-down decreases migration of Apc+/+ but not of ApcMin/+ mouse colon epithelial cells. Histograms showing the effect on Apc+/+ but not of ApcMin/+ mouse colon epithelial cell migration after 48 h siRNA transfection. Cells were transfected with siRNA-lipofectamine complexes for 48 h. A scrambled-siRNA was used as negative control. SiRNA oligonucleotide sequences are listed in the Materials and methods. Results from two separate experiments performed in triplicate are expressed as mean ± SEM. Normalization of cell number performed as described in the legend of Fig. 2.

4. Discussion

In the present study we report that SK3/KCa2.3 protein is produced in murine colon epithelial cells and that its expression is largely reduced following Apc mutation. Furthermore, we show that SK3/KCa2.3 channel promotes colon epithelial cells motility as already observed in melanoma or epithelial breast cancer cells [10,11]. The reduction of SK3/KCa2.3 protein expression observed in $Apc^{Min/+}$ epithelial cells compared to *Apc+/+* epithelial cells was accompanied by the suppression of the SK3/KCa2.3 channel activity and a partial reduction of cell migration.

This suppression of SK3/KCa2.3 channel-mediated-cell migration could explain the decrease in motility that we already observed in $Apc^{Min/+}$ epithelial cells, compared to *Apc+/+* epithelial cells [7].

Although this does not allow concluding that SK3/KCa2.3 channel are necessary for colonic cancer development, it nevertheless suggests that this ion channels play a supportive role. Reducing cancer cell motility by loss of SK3/KCa2.3 channel activity would increase the probabilities for the initiated cell to acquire additional genetic alterations leading to neoplasia, and would promotes proliferation of cancer cells in the initiation part of colorectal cancer. This is consistent with the enhanced expression of other potassium channel family supporting the proliferation of cancer cell and already observed following Apc mutation. Indeed, an increase in Eag-1, Erg-1 and Elk-1 and BKCa currents were demonstrated in the colonic epithelium of APC Min/+ epithelium [8,9]. This increase of potassium channel activity was accompanied by an increase of Erg-1, Elk-1 and beta subunits of BKCa channel expression following Apc mutation in colonic cells [8,9] and by an increase of KCNQ1 expression in parietal gastric cells [18]. We observed such a correlation between potassium channel protein expression and activity with SK3/KCa2.3 channel. This suggest that the decrease of SK3/KCa2.3 channels mediated motility is mainly due to a reduction in the expression of SK3/KCa2.3 protein at the plasma membrane level (using patch clamp technique we observed no apamin-sensitive current following Apc mutation). We already observed, using siRNA, that a small reduction of SK3/KCa2.3 protein expression totally abolished SK3/KCa2.3 channel activity thereby the motility mediated by this channel [10].

ApcMin/+ cells are less motile than Apc+/+ cells and exhibit a disordered actin cytoskeletal network ([7] and Fig. 1). Persuasive evidence that Apc could regulate cell migration by remodelling the actin cytoskeleton has been provided [6]. SK3/KCa2.3 protein was found to be localized in lamellipodia as well as filopodial structures and close to actin [21,22]. Moreover, it was clearly demonstrated that actin is necessary for KCa channels to be addressed and expressed in the plasma membrane [23].

The mechanism by which the Apc mutation reduces SK3/KCa2.3 channel mediated colonic epithelial cell motility remains to be elucidated. Interesting papers demonstrated a link between potassium channel activity and β-catenin/Akt pathway [24,25]. This suggests that a relationship between β-catenin and SK3/KCa2.3 protein expression may actually be of an opposite nature whereby the reduction of the transcription factor β-catenin results in increase of SK3/KCa2.3 protein. Interestingly, a clear link between β-catenin degradation and calpain activation through its activation

following an increase of intracellular calcium concentration was demonstrated in human colon carcinoma cells [26,27]. Note that this increase of internal calcium concentration would activate SK3/KCa2.3 channel.

As a general conclusion, SK3/KCa2.3 protein expression is largely decreased in colonic epithelial cells carrying the defective Apc gene thereby this suppress the SK3/KCa2.3 channel mediated cell motility. Thus Apc is a powerful regulator of potassium channel function that highlights the potential use of potassium channel modulator as a novel avenue for research on the treatment of colon cancers.

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