Bestrophin 1 Promotes Epithelial-to-mesenchymal Transition of Renal Collecting Duct Cells

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ABSTRACT

Bestrophin 1 (Best1) controls intracellular Ca$^{2+}$ concentration, induces Ca$^{2+}$-activated Cl$^{-}$ conductance, and increases proliferation of colon carcinoma cells. Here, we show that expression of Best1 in mouse renal collecting duct (CD) cells causes i) an increase in cell proliferation, ii) a loss of amiloride-sensitive Na$^{+}$ absorption, iii) induction of Ca$^{2+}$-dependent Cl$^{-}$ conductance (CaCC), and iv) epithelial-to-mesenchymal transition. During conditions of high proliferation or when we exposed CD cells to serum or TGF–β1, we observed upregulation of Best1, increased CaCC, redistribution of the epithelial-to-mesenchymal transition marker β-catenin, and upregulation of vimentin. In contrast, suppression of Best1 by RNAi inhibited proliferation, reduced CaCC, and downregulated markers of EMT. CaCC and expression of Best1 were independent of the cell cycle but clearly correlated to cell proliferation and cell density. During renal inflammation in LPS-treated mice or after unilateral ureteral obstruction, we observed transient upregulation of Best1. These data indicate that repression of cell proliferation, CaCC, and expression of Best1 occurs during mesenchymal-to-epithelial transition once CD cells polarize and terminally differentiate. These results may suggest a role for Best1 in renal fibrosis and tissue repair.

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Principal cells of the renal collecting duct (CD) reabsorb Na$^{+}$ through epithelial Na$^{+}$ channels (ENaC), while stimulation of luminal purinergic receptors enhances intracellular Ca$^{2+}$ and inhibits ENaC by means of hydrolysis of phosphatidylinositol bisphosphate (P1P2). In intact micro-perfused CDs, no evidence was found for a luminal Ca$^{2+}$-dependent Cl$^{-}$ conductance (CaCC). In contrast, CaCC was readily detectable in M1 mouse CD cells and primary cultured renal epithelial cells. This raises the question as to what degree available CD culture models represent native renal tubular function. Discrepancies between properties in intact tubules and isolated epithelial cells are well recognized and may be caused by epithelial-to-mesenchymal transition (EMT) occurring during primary cell culture.

Transition from an epithelial to a mesenchymal phenotype during cell culture of epithelial cells is well known and has been examined previously. Also during development, both EMT and the reverse process, namely mesenchymal-to-epithelial transition (MET) are fundamental processes. EMT contributes to degeneration of mature epithelial structures and to generation of fibroblasts associated with accumulation of extracellular matrix during renal inflammation (reviewed in). EMT also takes place during morphogenesis, wound healing and tissue repair, and is observed during tumor progression. The most important stimulus for EMT is TGF-β. In fact, TGF-β is able to induce a genetic program of cell plasticity that involves key pathways and regulators of epithelial dedifferentiation, cytoskeletal reorganization, and proliferation.

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We recently detected an EMT-like process in T84 colonic carcinoma cells, which induced enhanced proliferative activity of this cell line.12 Notably, ion channels, such as the cell cycle regulated hEag1 potassium channel as well as the putative Ca\(^{2+}\)-dependent Cl\(^-\) channel bestrophin 1 (Best1), were upregulated during EMT. Both channels supported proliferation in fast growing T84 cells.12

Best1 was shown to form a Ca\(^{2+}\)-activated Cl\(^-\) channel but, surprisingly, also regulates intracellular Ca\(^{2+}\) signaling.13–15 We identified Best1 as a component important for the activation of endogenous Ca\(^{2+}\)-activated Cl\(^-\) channels in airway cells and other epithelial tissues.16–18 A novel family of Ca\(^{2+}\)-activated Cl\(^-\) channels (TMEM16) has been identified recently, with biophysical properties strikingly similar to endogenous CaCC.19–21 Strong evidence was presented that TMEM16A is, in fact, the endogenous Ca\(^{2+}\)-activated Cl\(^-\) channel, and studies are underway to examine the role of Best1 in activation of TMEM16A.19–21 Here, we asked whether expression of Best1 is upregulated during dedifferentiation of collecting duct cells, and if Best1 induces proliferation, EMT, and enhanced Ca\(^{2+}\)-activated Cl\(^-\) conductance.

**RESULTS**

Coincidence of CaCC and EMT in CD Cells

M1 CD cells grown on permeable supports for 3 d in the presence of 10% bovine serum formed tight monolayers (R\(_{\text{te}}\) = 228 ± 23 Ω cm\(^2\); n = 37), when mounted in a perfused micro Ussing chamber, and expressed amiloride sensitive Na\(^+\) channels (ENaC), as indicated by amiloride (10 μM)-induced positive voltage deflection (Figure 1A). Application of luminal ATP (100 μM) activated a luminal Ca\(^{2+}\)-dependent Cl\(^-\) conductance (CaCC; negative voltage deflection) and inhibited ENaC. When M1 cells were grown for 12 d in the absence of serum, they formed higher resistance monolayers (R\(_{\text{te}}\) = 874 ± 56 Ω cm\(^2\); n = 28) and showed augmented amiloride sensitive short circuit currents (I\(_{\text{sc-Amil}}\)\(_{\text{m}}\)). Under these conditions, ATP no longer activated CaCC but only induced a transient K\(^+\) secretion, as indicated by positive voltage deflections (Figure 1, B through D). As these properties are similar to those of native CD,2 M1 cells may undergo MET upon polarization of filter membranes. In fact, expression of the marker for EMT, vimentin, was reduced in polarized cultures, while E-cadherin expression was enhanced (Figure 1, E and F). In contrast, acute application of 1% serum (FBS) to polarized M1 cells inhibited I\(_{\text{sc-Amil}}\) and enabled ATP to activate CaCC (Figure 2). Thus, proliferation and loss of polarization of collecting duct cells correlates with upregulation of CaCC and probably with EMT.

Induction of EMT Enhances Expression of Best1 and CaCC

TGF-β1 has been described as a major factor for EMT.9 We incubated highly polarized M1 cells with TGF-β1 (5 ng/ml, 5 h) and observed a remarkable decrease in amiloride sensitive Na\(^+\) absorption and induction of Ca\(^{2+}\)-dependent Cl\(^-\) secretion, similar to the treatment with FBS (Figure 3, A through D). Inhibition of ENaC by TGF-β1 was slightly attenuated by 15.3 ± 2.1% (n = 5) upon treatment with siRNA for Best1. Notably, incubation with TGF-β1 for 6 h upregulated expression of Best1 in CD cells (Figure 3E). In contrast, other cytokines like IL1-β (100 ng/ml), IL-8 (10 ng/ml), IFN-γ (100 ng/ml), and TNF-α (100 ng/ml) did not activate CaCC but abolished amiloride sensitive transport as reported earlier (data not shown).22 Moreover, activation of CaCC by TGF-β1 was not affected by inhibition of PI3K (Ly294002; 10 μM), MAPK kinase or Erk1,2 (SB203840; 25 μM, U0126; 5 μM), or interfering with the NO pathway (SNAP; 30 μM, L-NAME; 100 μM) (data not shown). Redistribution and cytosolic location of β-catenin is a hallmark of EMT and is often observed during tumor invasion.23 Redistribution of β-catenin to the cytosol was also observed in proliferative M1 cells (70% density) or after treat-
ment with FBS or TGF-β1 (Figure 3F). Moreover, exposure to FBS induced expression of the EMT marker vimentin but decreased E-cadherin expression (data not shown). Thus, TGF-β1 promotes EMT, increased Best1-expression, and activation of CaCC.

Glucocorticoids such as dexamethasone are known to induce expression of epithelial Na\(^+\)/H\(^+\) channels and have been reported to reduce cell proliferation. We compared transport properties of M1 cells that had been grown in the presence of low (0.05 μM) or high (1 μM) dexamethasone concentration (Figure 4, A and B). High dexamethasone concentrations induced a large ENaC and a luminal ATP activated K\(^+\) conductance as demonstrated by inhibition of the K\(^+\) conductance with 5 mM Ba\(^{2+}\) (Figure 4, C and D). Both ENaC and K\(^+\) conductance were reduced in the absence of dexamethasone, while 4,4′-disothiocyanatostilbene-2,2′-disulfonylic acid (DIDS) -inhibited CaCC was readily detectable (Figure 4, C and D). Moreover, dexamethasone-induced expression of E-cadherin (Figure 4E) reduced expression of the EMT marker vimentin, as indicated by semiquantitative RT-PCR (Figure 4F). I\(_{\text{sc-ATP}}\) in dexamethasone-treated cells was due to a Ba\(^{2+}\)-sensitive K\(^+\) conductance, while I\(_{\text{sc-ATP}}\) in the absence of dexamethasone was due to a DIDS-sensitive Cl\(^-\) conductance (Figure 4, G and H). Western blot analysis of Best1 clearly indicated downregulation of Best1-expression by dexamethasone (Figure 4I).

CaCC and Best1 Are Expressed Only in Highly Proliferating CD Cells

Best1 has been shown previously to induce both proliferation and CaCC in colonic carcinoma cells. We found pronounced Best1-expression in cytosolic compartments of highly proliferative M1 cells at a cell density of approximately 70%, which was almost completely downregulated in confluent monolayers (Figure 5A). Expression of Best1 reached a peak during the log phase of proliferation but was turned off after cells reached confluence (Figure 5B). Patch clamp experiments indicated largely reduced activation of CaCC at high cell density, indicating a clear correlation with the expression of Best1.
between cell density, Best1 expression, and CaCC (Figure 5, C and D). Notably, the reduced ATP response in confluent monolayers was not due to a loss of P2Y2 receptors (Supplement 1A).

**Bestrophins Support Proliferation of M1 Cells**

To further examine whether Best1 affects proliferation of CD cells, we used RNAi to suppress expression of Best1 and the paralog Best2, respectively (Figure 6A). Cell proliferation was assessed by BrdU incorporation and cell counting, and was suppressed by approximately 40% with either Best1-RNAi or Best2-RNAi (Figure 6, A and C). Notably, expression of the EMT marker vimentin was reduced in Best1-siRNA treated cells (Figure 6B). No evidence was found for induction of apoptosis by RNAi, as indicated by a lack of caspase-3 cleavage (Figure 6D). Activation of CaCC by ATP (I_{sc-ATP}) was attenuated in RNAi-treated cells, as demonstrated in Ussing chamber recordings (Figure 7, A and B). This was further confirmed by reduced iodide-quenching of the fluorescence signal from the halide sensitive dye YFP, in Best1-RNAi- and Best2-RNAi-treated cells (Figure 7, C and D). In additional experiments, overexpression of Best1 increased cell proliferation by 18 ±

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**Figure 4.** Dexamethasone suppresses Ca^{2+}-dependent Cl^- secretion in M1 cells. (A and B) Original recordings of transepithelial voltages in M1 cells grown on permeable supports in the absence or presence of dexamethasone (5 \( \mu \)M). Summary of amiloride-sensitive I_{sc} (C) and ATP-activated short circuit currents (I_{sc} after ATP-stimulation − I_{sc} under control conditions = I_{sc-ATP}) (D) in the absence (black bars) or presence (white bars) of dexamethasone. (E and F). Dexamethasone induces expression of E-cadherin but reduces expression of the EMT marker vimentin (\( n = 2 \) for each RT-PCR). (G) Positive voltage deflections induced by ATP (I_{sc-ATP}) in Dexa-treated cells are due to Ba^{2+}-sensitive K^+ conductance. # indicates significant difference between absence and presence of dexamethasone (unpaired t test), \( n \) = number of monolayers. (I) Western blot indicates downregulation of Best1 by dexamethasone (\( n = 2 \)).

**Figure 5.** Expression of Best1 and Ca^{2+}-activated Cl^- currents are proliferation dependent. (A) Immunohistochemistry of Best1 in mouse M1 cells grown at 70% density (log phase; upper panels) and to complete confluence (lower panels) (\( n = 5 \) for each series; scale bar = 50 \( \mu \)m). (B) Expression of Best1 (western blots in duplicates) during 7 d of culture and cell proliferation (\( n = 3 \)). (C) Activation of whole cell Cl^- currents by ATP in cells grown to 70% and 100% confluence, respectively. Cells were voltage clamped from −50 to +50 mV. (D) Summary of the ATP-induced whole cell Cl^- conductances at 70% and 100% density. # indicates significant difference between high and low density (unpaired t test). \( n \) = number of experiments.
4.5%, as measured by cell number \((n = 3)\). Taken together, bestrophins support proliferation of CD cells and are essential for generating a Ca\(^{2+}\)-activated Cl\(^{-}\) conductance. Notably, common CaCC (and Best1) inhibitors, such as DIDS, also inhibited cell proliferation by approximately 30%, supporting the role of Best1 and CaCC for cell proliferation (Supplement 1B).

Since expression of Best1 was largely dependent on cell proliferation, we asked whether expression of Best1 was cell cycle-dependent. Yet synchronization of the cells into early G1, G1/S, and M-phase detected a perinuclear staining during eG1, which changed into a cytosolic staining during G1/S (Supplement 3A). This finding was supported by Western blotting, which showed an increase of Best1 in the perinuclear fraction of the cell lysate during eG1 (Supplement 3B).

Expression of Best1 is controlled by cell density and a soluble factor and upregulated during tissue repair. As pointed out, expression of Best1 is downregulated in dense and polarized monolayers. Notably, M1 cells at the edge of a cell island demonstrated particularly strong staining for Best1 (Figure 8A). β-catenin staining was typically broad, but M1 cells changed to a membrane restricted β-catenin expression when grown in a media that was substituted by 50% media from confluent monolayers (Figure 8B). Under these conditions both cell proliferation and Best1-expression were largely reduced (Figure 8C). In contrast, when grown in a medium that was frequently refreshed (2 times per day) Best1-expression was no longer downregulated when reaching confluence and proliferation was enhanced (Figure 8D).

These results suggest that EMT is induced by soluble factors such as TGF-β1, while MET occurs during polarization possibly engaging autocrine mechanisms. EMT in renal epithelial cells could be important for the replacement of aged cells and during posttraumatic tissue repair. In fact, in a scratch assay using densely grown M1 cells, we detected upregulation of Best1 in the proliferating cells facing the tissue defect (Figure 9A). Moreover, intraperitoneal injection of LPS, which is known to induce renal inflammation and inhibition of the epithelial Na\(^+\) channel ENaC, caused upregulation of tubular Best1 expression and increase in Best1-mRNA as detected by real-time RT-PCR (Figure 9, B and C). Western blot analysis confirmed a transient increase in renal Best1-expression in LPS-treated animals, while expression of other ion channels such as CFTR did not change (Supplement 4A). In another model for renal damage using unilateral ureter obstruction (UUO), we also found significant upregulation of Best1 by 65 ± 8.2%, as demonstrated by semiquantitative RT-PCR \((n = 5\) animals for each series) (Figure 9D). These data indicate that Best1 is upregulated during EMT transition. Since EMT is considered a contributor of renal fibrosis, attenuating expression or function of Best1 could be of therapeutic potential.
DISCUSSION

Here, we supply evidence that expression of Best1 in mouse renal collecting duct (CD) cells increases cell proliferation, causes Ca\(^{2+}\)-dependent Cl\(^{-}\) conductance and EMT, but is inversely correlated to amiloride-sensitive Na\(^{+}/H\(^{+}\) absorption. The data indicate that collecting duct cells retain their ability to express Ca\(^{2+}\)/H\(^{+}\)-activated Cl\(^{-}\)channels. Thus, in acutely isolated intramedullary CD cells, and various CD cell lines such as IMCD-K2, IMCD-K3, M1, and MDCKII, nolactide-sensitive CaCC were identified.\(^5,28-30\) This is in contrast to data obtained from isolated perfused CD tubules that are regarded as the gold standard for epithelial transport studies. In isolated perfused CD tubules no evidence was found for a significant role of CaCC in either the luminal or basolateral membrane.\(^2,5,31,32\) The present results suggest that CD cells in culture undergo a rapid dedifferentiation program that can be characterized as EMT. Depending on the growth conditions, M1 cells transform either into rapidly proliferating nonpolarized cells or into a highly polarized nonproliferating epithelium. Thus, M1 cells undergo MET when grown under polarized conditions in a serum-free medium substituted with dexamethasone.

Notably, glucocorticoids and aldosterone-induced transcription factors such as ATF3 impinge directly on the activity of transport proteins and cellular differentiation.\(^33-36\) Interestingly, a related transcription factor, ATF2, was shown to be activated through TGF-\(\beta\). In the present study, we found that dexamethasone is able to antagonize the effects of pro-inflammatory cytokines, such as TGF-\(\beta\). EMT is a process whereby renal tubular cells lose their epithelial characteristics and gain a fibroblast-like phenotype. This process is induced by TGF-\(\beta\) mediated activation of the transcription factor E2A and has a potential role in renal fibrosis.\(^38\) However, as cellular responses to TGF-\(\beta\) are variable, TGF-\(\beta\) may cause either growth arrest or EMT, depending on the cell type and context of stimulation.

TGF-\(\beta\) can signal through NF\(\kappa\)B to suppress Na\(^{+}\) transport in principal cells of the CD by downregulating the serum and glucocorticoid-regulated kinase SGK1, apart from other mechanisms for inhibition of ENaC in renal and airway epithelial cells.\(^27,39,40\) Notably, membrane expression of TGF-\(\beta\)-strongly depends on cell polarization, which may explain why in the present study only cells at the edge of a monolayer expressed Best1 and proliferated, since they are able to expose their basolateral TGF-\(\beta\) receptors to serum factors.\(^41\) Similar observations were done in a colonic carcinoma cell line (T84), that changed from a polarized growth pattern to fast growing cells with augmented intracellular Ca\(^{2+}\) signaling.\(^12\) Thereby, ER-localized bestrophins may enhance cell proliferation and couple receptor mediated intracellular Ca\(^{2+}\) signaling to membrane localized Ca\(^{2+}\)/H\(^{+}\)-dependent ion channels such as TMEM16A.\(^18-21\) As Ca\(^{2+}\)/H\(^{+}\)-activated Cl\(^{-}\)channels and probably Best1 expression are common features of epithelial cells undergoing dedifferentiation during primary cell culture,\(^42\) the present results suggest a novel role for both during EMT and probably embryonic development (Supplement 4B).

CONCISE METHODS

Animals, Cell Culture, cDNAs, siRNAs, and Transfection

Mice (C57Bl/6) received NaCl (control) or LPS (Escherichia coli, serotype 0111:B4; Sigma, St. Louis, MO) (10 mg/kg intraperitoneal), and were killed 6, 12, and 24 h (\(n = 5\) per group) after injection.\(^22\) Mice were treated...
with unilateral ureteral obstruction (UUO) in the laboratory of Prof. Dr. Mack (University of Regensburg, Germany) as described previously. M1 cells (mouse collecting duct cells) were grown at 37°C in 5% CO₂ as described previously. M1 cells were grown on permeable supports or proliferation and Best1-expression in cells grown in media that was frequently (twice per day) replaced (n = 2 for each series).

**Figure 8.** Cell density controls expression of Best1. (A) Immunohistochemistry of Best1 in mouse M1 cells. When grown to 70% density, cells at the edge of an island show high levels of Best1 expression, while cells in the center of islands and monolayers grown to 100% confluence downregulate Best1 expression (n = 5 for each series). (B) β-catenin localization in cells grown in normal media (control media) or cells exposed to conditioned medium from confluent monolayers (substituted media) (n = 3 for each series). Membrane-limited expression of β-catenin suggests MET in cells exposed to conditioned medium (scale bars = 20 μm). (C) Reduced cell proliferation and Best1-expression in cells grown in media substituted by supernatant (50 vol%) from confluent M1 cultures. (D) Enhanced cell proliferation and Best1-expression in cells grown in media that was frequently (twice per day) replaced (n = 2 for each series).

**Figure 9.** Upregulation of Best1 during tissue repair. (A) Immunocytochemistry of Best1 in a scratch assay with densely grown M1 cells. Upregulation of Best1 in proliferating cells facing the monolayer defect, at lower (upper panel, scale bar = 100 μm) and higher (lower panel, scale bar = 20 μm) magnification (n = 5). (B) Immunohistochemistry showing time dependent expression of renal Best1 expression in mice treated with LPS (intraperitoneal injection of 10 mg/kg; n = 5 mice). (C) Summary of real time RT-PCR showing time dependent increase of renal Best1-expression after LPS injection. * indicate significant difference between control and 14 h, and 14 h and 24 h after LPS injection, respectively (unpaired t test). (n) = number of animals. (D) Semiquantitative RT-PCR indicates upregulation of Best1-expression in renal epithelial cells after UUO (n = 4 animals for each series).
plastic dishes to highly polarized monolayers in the absence or presence of serum (FBS) or TGF-β1. To verify EMT, cells were stained for β-catenin and semiquantitative RT-PCR was performed to examine expression of vimentin and E-cadherin, using β-actin as an internal standard. We purchased three different batches of duplexes of 25-nucleotide RNAi from Invitrogen (Karlsruhe, Germany). The sense strands of the RNAi used to silence the Best1 gene were 5'-UCCAGUCAUGACGUUGUCUCAUGGG-3', and for Best2 gene were 5'-UUUGCUCCGA AACAUCACGCUCC-3', scrambled sequence RNAi ds-oligomer, not homologous to any known gene (BLOCK-IT™ Fluorescence Oligo) served as control. We transfected M1 cells using Lipofectamine™2000 (Invitrogen).

Western Blotting and Antibodies
M1 cells were homogenized in lysis buffer (mmol/L: NaCl 150, Tris 50, DTT 100, 1% NP-40, and 1% protease inhibitor cocktail) (Sigma), separated by 7% SDS-PAGE, and transferred to Hybond-P (Amer sham Biosciences, Freiburg, Germany). Primary antibodies were either rabbit anti-mouse best1, rabbit anti-mouse best2, or rabbit anti caspase 3 antibody (Davids Biotechnology, Regensburg, Germany). Proteins were visualized using goat anti-rabbit IgG conjugated to horseradish peroxidase (Acris Antibodies, Hiddenhausen, Germany) and ECL (Amer sham Biosciences).

Immunohistochemistry
M1 cells were fixed for 2 h with 4% paraformaldehyde in 0.1 M cadoxylate buffer, pH 7.4. Sections were incubated overnight at 4°C with rabbit anti-mouse Best1 antibodies diluted 1:10,000 in Tris buffer containing Triton X-100 (0.8%) and goat serum to prevent nonspecific binding. Sections were incubated with horseradish peroxidase linked goat anti-rabbit secondary antibodies (Amer sham Pharmacia Biotech, Freiburg, Germany). The avidin biotin peroxidase complex (ABC) technique was used to visualize the labeling with 3,3-diaminobenzidine. The ABC technique involves application of a biotin-labeled secondary antibody followed by the addition of the ABC. Sections were counterstained with Mayer’s hematoxylin. Alternatively a fluorescence-labeled secondary antibody was used (diluted 1:1,000; Acris Antibodies, Hiddenhausen, Germany).

Patch Clamping, Ussing Chamber, and Iodide Quenching
We performed patch-clamp experiments in the fast whole-cell configuration as described recently. In intervals, membrane voltages (Vj) were clamped in steps of 10 mV from −50 to +50 mV relative to resting potential. We calculated the membrane conductance Gm from the measured current (I) and Vj values according to Ohm’s law. M1 cells grown to confluence on permeable supports (Millipore, Schwal bach, Germany) were mounted into a perfused micro Ussing chamber. The luminal and basolateral surfaces of the epithelium were perfused continuously with buffer solution at a rate of 5 to 10 ml/min (chamber volume 2 ml). We carried out all experiments at 37°C under open circuit conditions. We determined transepithelial resistance (Rte) by applying short (1-s) current pulses (ΔI = 0.5 μA) and continuously recorded the corresponding changes in transepithelial voltage (Vte) and basal Vte. Values for the Vte were referred to the serosal side of the epithelium. We calculated Rte according to Ohms law (Rte = ΔVte/ΔI) and the equivalent short-circuit current (Isc) according to Ohms law from Vte and Rte (Isc = Vte / Rte). We induced YFP1152L fluorescence by excitation (wave length 500 nm) using a poly-chromatic illumination system for microscopic fluorescence measurement (VisiChrome; Visitron Systems, Puchheim, Germany) and light emission was measured at 535 ± 15 nm with a photomultiplier detector (SF, Zeiss, Munich, Germany). We induced quenching of YFP1152L fluorescence via I− influx by replacing 20 mM extracellular Cl− with I−.

Proliferation Assays and Statistics
We seeded M1 cells at a density of 5000 cells/0.35 cm² on 96-well plates (Sarstedt, Nuembrecht, Germany). We quantified cell proliferation by measuring 5-bromo-2'-deoxyuridine (BrdU) incorporation and performed cell counting as described recently. To measure the effect of RNAi treatment on cell proliferation, we seeded M1 cells at a density of 10,000 cells/0.35 cm² in 96-well plates and treated wells with the siRNA. We used BLOCK-IT™ Fluorescence Oligos as controls. After 48 h, we quantified cell proliferation by measuring BrdU incorporation and by cell number. All compounds used were of the highest available grade of purity and were from Sigma (Taufkirchen, Germany) or Calbiochem (Darmstadt, Germany). The t test (for paired or unpaired samples as appropriate) was used for statistical analysis. P < 0.05 was accepted as significant.

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DISCLOSURES
None.

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