Eag1 and Bestrophin 1 are upregulated in fast growing colonic cancer cells

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Abstract: Ion channels like voltage gated ether á gogo (Eag1) K+ channels or Ca2+ activated Cl- channels have been shown to support cell proliferation. Bestrophin 1 (Best1) has been proposed to form Ca2+ activated Cl- channels in epithelial cells. Here we show that original T84 colonic carcinoma cells grow slow (T84-slow) and express low amounts of Eag1 and Best1, while spontaneously transformed T84 cells grow fast (T84-fast) and express high levels of both proteins. Both Eag1 and Best1 currents are upregulated in T84-fast cells. Eag1 currents were cell cycle dependent, with upregulation during G1/S transition. T84-slow but not T84-fast formed tight monolayers when grown on permeable supports. RNAi inhibition of Eag1 and Best1 reduced proliferation of T84-fast cells, while overexpression of Best1 turned T84-slow into fast growing cells. Eag1 and Best1 improve intracellular Ca2+ signaling and cell volume regulation. These results establish a novel role of bestrophins for cell proliferation.

An increasing number of studies demonstrate proliferative effects of membrane ion channels (1). Voltage gated K+ channels and other types of K+ channels are expressed in numerous types of tumors, where they may serve as diagnostic and prognostic markers and potential drug targets (2-4). Eag1 channels are probably necessary for progression through the G1 phase and G0/G1 transition of the cell cycle (5). A recent study demonstrates the hyperpolarizing effects of Eag1 and other Kv channels on the membrane voltage of T84 cells, which supports intracellular pH regulation and Ca2+ increase, necessary for proliferation (6).

Much less is known about the role of Ca2+ activated Cl- channels for cell proliferation (7). This may be due to the ongoing controversy regarding the molecular nature of Ca2+ activated Cl- channels (8). A family of putative Ca2+ activated Cl- channels (CLCA) has been identified, which also controls cell-cell adhesion, apoptosis and cell cycle. However, their structure and biophysical properties are poorly understood (9,10). Recent studies defined bestrophin proteins as bona fide Ca2+ activated Cl- channels. The Cl- currents generated upon expression of bestrophin show many of the properties found for native Ca2+ activated Cl- currents (11-13). However, bestrophins have also been proposed to function as regulators of voltage gated L-type Ca2+ channels (14). Our own ongoing work in epithelial cells supports both concepts in that bestrophins may form part of a Cl- channel complex or may couple intracellular Ca2+ signals to Cl- channels of unknown molecular identity (15).

In the present report we demonstrate that both voltage gated Eag1 K+ channels and bestrophin (Best1) Cl- channels support proliferation of
fast growing T₈⁴ colonic carcinoma cells. The fast growing T₈⁴ cell clone was obtained through a spontaneous transformation of slow-growing T₈⁴ cells. In contrast to slow growing T₈⁴ cells, transformed cells do not form polarized monolayers and show a remarkable upregulation of Eag1 and Best1 expression. We demonstrate that both currents are in charge of enhanced cell proliferation.

Material and Methods

Cell culture and proliferation studies: Human colorectal carcinoma epithelial T₈⁴ cells (ATCC, Rockville, MD, USA) were grown in DMEM/Ham's F-12 medium (1:1) supplemented with 10% fetal bovine serum, 2 mM glutamine, 100 units/ml penicillin and 100 µg/ml streptomycin (Invitrogen, Karlsruhe, Germany) at 5% CO₂/37°C. Cells were seeded on fibronectin (Invitrogen)/collagen (Cellon, Luxembourg) coated glass cover slips or permeable supports (snap-well, Costar). Typically these cells grow slowly as polarized monolayers (T₈⁴-slow). Due to spontaneous transformation, a T₈⁴ cell line was selected which grew remarkably faster (T₈⁴-fast). For proliferation assays cells were plated at a density of 2000 cells/0.35 cm² and incubated 2 days later with either niflumic acid (0,01–100mM) or astemizole (0,5–5000nM). Cell proliferation was assessed by 5-bromo-2´-deoxyuridine (BrdU) incorporation using an ELISA kit (Roche, Penzberg, Germany) and cell counting. The cell number was assessed after fixation in 3.7% formaldehyde / 0.5% Triton X-100 for 30 minutes at room temperature and after staining with Mayers hemalaun (Merck, Darmstadt, Germany) for 5 minutes. Digitized microscopic images were taken (Fluovert FS, Leitz, Germany) and nuclei were counted using imaging software (TINA 2.09g). Toxicity of the blockers was assessed using Trypan Blue (Sigma). Each experiment was performed at least in triplicate.

Cell cycle, FACS analysis, Caspase assay, RT-PCR: Cells were synchronized into early G1 by 24h serum starvation. Incubation in thymidine (2 mM), (Sigma) halted the cells at G1/S transition. 36h treatment with demecolcine (0.05 µg/ml) (Sigma) synchronized into M phase. Synchonization was verified by FACS (COULTER EPICS® XL-MCL, Beckmann, Miami, USA) using propidium iodide staining of the DNA (Sigma). Apoptosis was analyzed after 8h and 24 h incubation with the protein kinase C inhibitor staurosporine (1 µM), (Sigma) and detection of cleaved caspase-3 in Western blots using rabbit anti-human caspase-3 antibody (1:1000, Cell Signaling Technology, Inc., Danvers, USA). For RT-PCR total RNA was isolated using NucleoSpin RNA II columns (Macherey-Nagel, Düren, Germany). 1 µg total RNA was reverse transcribed for 1 h at 37°C using random primer and RT (M-MLV Reverse Transcriptase, Promega, Mannheim, Germany). For PCR the following primers were used: hEag1 (KCNIH1, NM_002238): 5'- CGCATGAACATCCTGAAAGAC-3' (s), 5'- TCTGTGGATGGGGCGATG-3' (as), 560 bp. hBest1 (VMD2, NM_004183): 5'- CTGCTCTGACTACATC-3' (s), 5'- GTGTCACACTGAGTGACGC-3' (as), 552 bp. The conditions were: 94°C / 2 min, 35 cycles of 94°C / 30 sec, 58°C / 30 sec and 72°C / 1 min. PCR products were visualized by loading on 2% agarose gels and verified by sequencing.

Downregulation of Best1 and Eag1 expression by RNAi: Three different batches (A,B,C) of duplexes of 21-nucleotide RNAi with 3'-overhanging TT were purchased from Invitrogen (Karlsruhe, Germany). The sense strands of the RNAi used to silence the Best1 gene were 5'AUUCUGUGACAAUCA-GUUUGU-3'(A), 5'-AUCUCAUCCACA-GCCACACGGGACA-3'(B), and 5'-UAAAUAAAGCGGAUGAUGAGAGC-3'(C). RNAi sequences for Eag1 are shown in (6). Fluorophore labeled RNAi and exposure to the transfection reagent lipofectamine 2000, (Invitogen) served as controls. After 48 hrs cells were further processed in proliferation assays and for western blotting.

Detection of Eag1 and Best1 by Western blotting: T84 cells were homogenized in lysis buffer (mmol/l: NaCl 150, Tris 50, DTT 100, 1% NP-40, and 1% protease inhibitor cocktail) (Sigma). Equal amounts of total protein (50 µg) were separated by 7% SDS-PAGE, transferred to Hybond-P (Amersham Biosciences, Freiburg, Germany) and incubated with either rabbit anti-hKv10.1 (Eag1) (Alomone labs, Jerusalem, Israel) or rabbit anti-hVMD-2 (Best1) (15) antibodies. Proteins were visualized using goat anti-rabbit IgG conjugated to horseradish peroxidase (Acris Antibodies, Hiddenhausen, Germany) and ECL.
Measurement of the intracellular Ca\(^{2+}\) concentration and cell volume: T\(_{84}\) cells were loaded with 2 µM Fura-2 AM (Molecular Probes, Eugene, USA) in Opti-MEM medium (GIBCO) with 2.5 mM probenecid (Sigma) for 1h at room temperature. Fluorescence was detected in cells perfused with ringer solution containing 2.5 mM probenecid (Sigma) at 37\(^\circ\)C, using an inverted microscope IMT-2 (Olympus, Nürnberg, Germany) and a high speed polychromator system (VisiChrome, Puchheim, Germany). Fura-2 was excited at 340/380 nm and emission was recorded between 470 and 550 nm using a CCD camera (CoolSnap HQ, VisiRon). \([\text{Ca}^{2+}]_i\) was calculated from the 340/380 nm fluorescence ratio (after background subtraction). The formula used to calculate \([\text{Ca}^{2+}]_i\) was 
\[ \text{Ca}^{2+} = K_d \times \frac{(R-R_{min})(R_{max}-R)}{(S_{f2}/S_{b2})} \]
where \(R\) is the observed fluorescence ratio. The values \(R_{max}\), \(R_{min}\) (maximum and minimum ratios) and the constant \(S_{f2}/S_{b2}\) (fluorescence of free and Ca\(^{2+}\)-bound fura-2 at 380 nm) were calculated, using 2 µmol/l ionomycin (Calbiochem), 5 µmol/l nigericin, 10µmol/l monensin (Sigma) and 5 mmol/l EGTA to equilibrate intracellular and extracellular Ca\(^{2+}\) in intact fura-2-loaded cells. The dissociation constant for the fura-2-Ca\(^{2+}\) complex was taken as 224 nmol/l.

Patch clamping: Cell culture dishes were mounted on the stage of an inverted microscope (IM35, Zeiss, Germany), and perfused continuously (37\(^\circ\)C) with Ringer solution. Patch-clamp experiments were performed in the fast and slow whole-cell configuration. Patch pipettes had an input resistance of 2–4 M\(\Omega\), when filled with a solution containing (mM) KCl 30, K-gluconate 95, Na\(_2\)HPO\(_4\) 1.2, Na\(_2\)HPO\(_4\) 4.8, EGTA 1, Ca-gluconate 0.758, MgCl\(_2\) 1.034, D-glucose 5, ATP 3. pH was adjusted to 7.2. The access conductance \(G_m\) was calculated from the measured current (\(I\)) and \(V_c\) values according to Ohm’s law.

Cell volume was measured directly by a Zeiss-Axiovert 200M/ApoTome using Axiosvision software or was assessed by fluorescence measurements in calcein (2 µM; Molecular Probes, USA) loaded cells at an excitation of 500 nm and emission of 520-550 nm. The experiments were done in the presence of 2.5 mM probenecid. The control isotonic solution (290 mOsm) was prepared by adding 120 mM mannitol. The hypotonic (170 mOsm) and control isotonic solution contained 85 mM NaCl.

Materials and statistical analysis: All compounds used were of highest available grade of purity. Astemizole, niflumic acid (NFA), DIDS, tetrapentylammonium (TPeA), carbachol, and ATP were all from SIGMA (Taufkirchen, Germany). Student’s t-test (for paired or unpaired samples as appropriate) and analysis of variance (ANOVA) was used for statistical analysis. P<0.05 was accepted as significant.

Results

Fast growing colonic carcinoma cells (T\(_{84}\)-fast) express Eag1 and Best1: T\(_{84}\)-fast cells typically grow in slowly expanding patches (T\(_{84}\)-slow). At passage number 73, the cell line spontaneously changed its growth pattern, i.e. the cells grew remarkably faster (T\(_{84}\)-fast) as single cells, non-polarized and above each other (Fig.1A,B). T\(_{84}\)-slow formed tight monolayers with a transepithelial resistance (R\(_{te}\)) of 2.2 ± 0.3 kΩ cm\(^2\) (n= 25), when grown on permeable supports, while T\(_{84}\)-fast did not (0.12 ± 0.07 kΩ cm\(^2\); n= 23). Thus, growth pattern or phenotype were not changed by the way the cells were grown. We examined apoptosis in both cell lines by Western blotting and analyzed uncleaved and cleaved caspase 3. Small amounts of cleaved caspase 3 were detected in T\(_{84}\)-slow but not in T\(_{84}\)-fast cells, after treatment with the PKC inhibitor staurosporin (1 µM) (Fig. 1C).

Semi-quantitative RT-PCR analysis was performed for voltage gated K\(^+\) channels (Eag1) and putative Ca\(^{2+}\) activated Cl\(^-\) channels (Best1), using β-actin as an internal standard. Densitometric analysis suggested transcript...
numbers for Eag1 and Best1 that were at least 10 times higher in T\textsubscript{84}\textsubscript{fast} cells. Thus much higher protein expression was found for Eag1 and Best1 in T\textsubscript{84}\textsubscript{fast} cells in Western blots (Fig. 1D). The membrane conductance properties were analyzed in T\textsubscript{84}\textsubscript{slow} and T\textsubscript{84}\textsubscript{fast} cells in whole cell patch clamp experiments and membrane voltages (Vm) were measured in the current clamp mode. T\textsubscript{84}\textsubscript{slow} cells (n = 8) were more hyperpolarized (-51.7 ± 5.6 mV) and had a lower baseline conductance (3.8 ± 0.7 nS) than T\textsubscript{84}\textsubscript{fast} cells (-36.6 ± 2.9 mV and 23.8 ± 2.9 nS, n = 8). The increased baseline conductance and depolarized Vm of T\textsubscript{84}\textsubscript{fast} cells was due to a higher activity of Cl\textsuperscript{-} channels, since replacement of extracellular Cl\textsuperscript{-} by gluconate (5Cl) shifted the I/V curve to more positive clamp voltages. This was not observed for T\textsubscript{84}\textsubscript{slow} cells (Fig. 1E).

**Eag1 controls proliferation of fast but not of slow growing T\textsubscript{84} cells:** Eag1 has been demonstrated to support proliferation of several different cell types (6;16). We therefore examined if high expression levels of Eag1 correlate with increased proliferation of T\textsubscript{84}\textsubscript{fast} cells. To that end T\textsubscript{84}\textsubscript{slow} and T\textsubscript{84}\textsubscript{fast} cells were treated with three different batches (A-C) of siRNA for Eag1. Incubation of the cells with either fluorescence labeled scrambled oligos or lipid (transfection reagent) and non-treated cells served as controls. Measurement of BrdU incorporation clearly indicates inhibition of proliferation of T\textsubscript{84}\textsubscript{fast} cells after treatment with siRNA for Eag1 (Fig. 2A). siRNA treatment of T\textsubscript{84}\textsubscript{slow} cells did not affect cell proliferation, suggesting a proliferative function of Eag1 only in T\textsubscript{84}\textsubscript{fast} cells. Notably, Eag1 expression was significantly upregulated by re-addition of 10% FCS in serum starved cells (data not shown). Using the inhibitor astemizole (5 µM), we examined the contribution of Eag1 to whole cell currents measured in T\textsubscript{84}\textsubscript{slow} and T\textsubscript{84}\textsubscript{fast} cells. Astemizole inhibited whole cell currents in both cell types, however, the effect was more pronounced in T\textsubscript{84}\textsubscript{fast} cells and thus astemizole-sensitive whole cell currents were significantly larger in T\textsubscript{84}\textsubscript{fast} cells (Fig. 2B,C). The normalized conductance/voltage relationship for Eag1 (astemizole sensitive whole cell conductances) was not different for T\textsubscript{84}\textsubscript{fast} (solid line) and T\textsubscript{84}\textsubscript{slow} (dashed line) cells (Fig. 2D).

A previous report supplied evidence that hyperpolarizing Eag1 currents assist in the increase of intracellular Ca\textsuperscript{2+} ([Ca\textsuperscript{2+}]\textsubscript{i}), when cells are stimulated with secretagogues (ATP, carbachol) or mitogens, and may thereby support proliferation. Both Ca\textsuperscript{2+} release from ER stores and Ca\textsuperscript{2+} influx through store operated Ca\textsuperscript{2+} channels depend on Kv channel function, as reported earlier (6). We compared increase in [Ca\textsuperscript{2+}]\textsubscript{i}, in Fura-2 loaded T\textsubscript{84}\textsubscript{slow} and T\textsubscript{84}\textsubscript{fast} cells upon stimulation with ATP (100 µM). ATP binds to purinergic P2Y\textsubscript{2} receptors and thereby induces a peak and plateau [Ca\textsuperscript{2+}]\textsubscript{i} increase in both cell lines (Fig. 2E). The peak [Ca\textsuperscript{2+}]\textsubscript{i} increase was doubled in T\textsubscript{84}\textsubscript{fast} when compared with T\textsubscript{84}\textsubscript{slow} cells, which suggests a role of Eag1 for Ca\textsuperscript{2+} signaling in T\textsubscript{84}\textsubscript{fast} cells (Fig. 2E,F). Enhanced Ca\textsuperscript{2+} signaling is not due to an increased expression of the abundant purinergic receptor P2Y\textsubscript{2} in T\textsubscript{84}\textsubscript{fast}. In contrast, P2Y\textsubscript{2} expression was slightly higher in T\textsubscript{84}\textsubscript{slow} cells (Fig. 2G).

**Eag1 activity in T\textsubscript{84}\textsubscript{fast} cells is cell cycle dependent:** In other cell types Eag1-activity varies during the cell cycle (16). Therefore we examined cell cycle dependence of Eag1 in T\textsubscript{84}\textsubscript{fast} cells. Cells were synchronized in early G1 (eG1), G1/S or M phase (c.f. methods), and synchronization was verified by FACS analysis (Fig. 3A). We found that astemizole sensitive whole cell currents were augmented in cells synchronized in G1/S, when compared to eG1 or M phase (Fig. 3B). T\textsubscript{84} cells also express other voltage gated K\textsuperscript{+} channels such as Kv1.5 or Kv3.4, which are blocked by the inhibitor TPeA (6). However, in contrast to Eag1, TPeA sensitive whole cell currents were not cell cycle dependent (data not shown). We further compared the increase in [Ca\textsuperscript{2+}]\textsubscript{i} in T\textsubscript{84}\textsubscript{fast} cells synchronized in eG1 and G1/S phase. Both peak and plateau [Ca\textsuperscript{2+}]\textsubscript{i} increase were significantly enhanced in G1/S-cells. Moreover, the Eag1-blocker astemizole inhibited peak and plateau [Ca\textsuperscript{2+}] increase in both cell cycle phases, but the effect of astemizole on plateau [Ca\textsuperscript{2+}]\textsubscript{i} was augmented in the G1/S phase (Fig. 3C). These results supply evidence for cell cycle regulated Eag1-currents in T\textsubscript{84}\textsubscript{fast} cells and cell cycle dependent effects of Eag1 on Ca\textsuperscript{2+} signaling.

**Best1 controls proliferation of fast but not of slow growing T\textsubscript{84} cells:** Because T\textsubscript{84}\textsubscript{fast} cells show much higher expression of Best1 when
compared with T₈₄-slow cells (Fig. 4A), we examined the effect of this putative Ca²⁺-activated Cl⁻ channel on cell proliferation. T₈₄-slow and T₈₄-fast cells were treated with three different batches (A-C) of siRNA for Best₁, which reduced Best₁ levels in T₈₄-fast cells. Expression levels in T₈₄-slow cells were already very low, so that more protein was loaded on the gel, as indicated by the β-actin signal (Fig. 4A,B). Incubation of the cells with either fluorescence labeled scrambled oligos or transfection reagent (lipid) and non-treated cells served as controls. Measurement of BrdU incorporation clearly indicates inhibition of proliferation of T₈₄-fast cells after treatment with siRNA for Best₁ (Fig. 4C). No effects of siRNA were seen in T₈₄-slow cells, suggesting a proliferative function of Best₁ only in T₈₄-fast cells.

In whole cell patch clamp experiments, the high baseline conductance found in T₈₄-fast cells could be partially inhibited by the blockers of Ca²⁺-activated Cl⁻ channels, NFA and DIDS (both 100 µM). Both inhibitors had no effect in T₈₄-slow cells (Fig. 5A). Stimulation with ATP (100 µM) to increase intracellular Ca²⁺ (Fig. 2D), activated a whole cell current in T₈₄-slow but not in T₈₄-fast cells (Fig. 5B). In T₈₄-slow cells, ATP activated primarily a K⁺ conductance, as indicated by the hyperpolarizing effect of ATP on the membrane voltage (Fig. 5C). Little effects of ATP were seen in T₈₄-fast cells. Correspondingly, replacement of extracellular Cl⁻ by impermeable gluconate (5Cl⁻) showed no effects in T₈₄-slow cells, but reduced the baseline conductance in T₈₄-fast cells (Fig. 5D). Taken together, these results suggest active Best₁ Cl⁻ channels in non-stimulated T₈₄-fast cells, which cause enhanced proliferation.

Increased proliferation and Cl⁻ conductance in Best₁-transfected T₈₄-slow cells: To further demonstrate that Best₁ contributes to proliferation of T₈₄ cells, we expressed human Best₁ in T₈₄-slow cells. As shown in Fig. 6A, transfection of 100 ng of exogenous Best₁ increased Best₁ expression in T₈₄-slow cells almost to the level found in T₈₄-fast cells. No change was seen in mock transfected cells. Notably, overexpression of Best₁ changed the growth pattern of T₈₄-slow cells towards that found for T₈₄-fast cells (Fig. 6B). Moreover, after expression of Best₁, a DIDS-sensitive whole cell current appeared in T₈₄-slow cells, which was not found in mock-transfected or parental cells (Fig. 6C). Measurement of the BrdU-incorporation indicated a significant increase in proliferation of Best₁-transfected T₈₄-slow cells, which was not observed for mock-transfected cells (Fig. 6D). In summary, both Eag₁ K⁺ channels and Best₁ Cl⁻ channels are upregulated in spontaneously transformed T₈₄ cells, where they augment proliferation.

**Eag₁ and Best₁ support intracellular Ca²⁺ signaling and volume regulation, respectively:** Eag₁ K⁺ channels (and to some degree Best₁) have a clear impact on intracellular Ca²⁺ signaling. This was demonstrated by stimulation with ATP (100 µM), which increased intracellular peak (ER- Ca²⁺ release) and plateau (Ca²⁺ - influx through SOC) in T₈₄-fast cells. Treatment with siRNA for Eag₁ significantly reduced peak and plateau Ca²⁺ increase (Fig. 7A). Volume measurements indicate stronger cell swelling and potent regulatory volume decrease (RVD) in T₈₄-fast, when compared to T₈₄-slow cells, as identified by direct volume measurements and indirect measurements of calcein fluorescence (Fig. 7B-E). T₈₄-fast cells treated with siRNA-Best₁ show reduced RVD (Fig. 7B). As both intracellular Ca²⁺ and cell volume control is essential for the mitotic cell cycle, these results may provide a mechanism for the proliferative role of Eag₁ and Best₁.

**Discussion**

Transformation of colonic epithelial cells increases proliferation and ion conductances: T₈₄ colonic carcinoma cells are well established (17;18). They grow slowly in patches and form tight and polarized monolayers, when cultured on permeable supports. T₈₄ cells were used for numerous electrophysiological studies and resemble a model for electrolyte transport in the colonic epithelium (17). They show many aspects of native epithelial cells such as a relatively hyperpolarized membrane voltage and Cl⁻ secretion by cAMP-dependent cystic fibrosis transmembrane conductance regulator (CFTR) channels. When stimulated by secretagogues, which increase the intracellular Ca²⁺ concentration such as ATP or carbachol, predominantly K⁺ channels are activated, hyperpolarizing the membrane voltage. This is comparable to colonic crypt cells, which also activate Ca²⁺-activated K⁺ channels upon
stimulation of basolateral muscarinic M3 and apical purinergic P2Y2 receptors (18;19). In contrast, T84 cells that had undergone spontaneous transformation were remarkably different: They proliferated much faster, were unable to polarize and to form tight monolayers when grown on permeable support, showed typical features of malignancy and had different membrane conductances.

Anderson and Welsh as well as Morris and Frizzell reported earlier that expression of ion channels in epithelial cells is significantly affected by the underlying substrate (20;21). Thus Ca2+ activated Cl− channels were prominent in non-differentiated HT29 colonic carcinoma cells but disappeared with polarization (22). Although T84-fast cells did not polarize when grown on permeable supports, a change in Best1 expression upon differentiation of filter grown HT29 cells may explain earlier results (22).

A change in membrane ion conductance has also been reported for spontaneously transformed Madin-Darby canine kidney (MDCK) cells. These fast growing MDCK-F cells express Ca2+ activated K+ channels that largely enhance cell migration (23). The fast growing T84 cells described in the present study, showed a strong increase in the expression of the Best1 Cl− channel, along with Eag1 potassium channels. Eag1 currents were detected despite a relatively high (100 nM) Ca2+ concentration in resting cells. However up to 1 µM [Ca2+]i are required for complete inhibition of Eag1 and thus a fraction (∼40%) of the Eag1 conductance is still detectable in T84-fast cells (24). Notably, the Cl− channels were already active in non-stimulated cells, i.e. without purinergic stimulation. Additional increase of intracellular Ca2+ by ATP or carbachol did not further increase Cl− conductance. A similar result was obtained when Best1 was overexpressed in T84-slow cells, and has also been observed when Best1 was expressed in Hek293 cells (8). It suggests that additional components may be required for Ca2+-regulation of Best1 currents, which may not be present in cancer cells. The presence of these DIDS-sensitive Cl− currents along with astemizole inhibited Eag1 K+ currents clearly augmented cell proliferation.

Ion channels induce malignancy and metastatic cell growth: Clonal selection of fast proliferating T84 cells (T84SF) has previously been reported (25). These cells demonstrate invasive and metastatic cell growth, when transferred into nude mice. Basal tyrosine phosphorylation and expression of src kinase were enhanced in these T84SF cells (25). Along this line, the src-inhibitor PP2 reduced cell growth, invasion and cell adhesion of T84SF cells (26). In Jurkat T lymphocytes, src kinase controls voltage-dependent Kv1.3 K+ channels, which also affect cell proliferation (27). Src kinase may also be up-regulated in fast growing T84 cells and may be responsible for changes in membrane conductance. It will be interesting to examine in subsequent studies the impact of Eag1 and Best1 on cell migration and tissue invasion, since we previously found that genomic amplification of Eag1 in human colorectal carcinoma is an independent marker of adverse prognosis (28).

How do ion channels determine malignancy? K+ and Cl− channels are essential for cell migration and metastasis of cancer (29). It has been shown that enhanced intracellular Ca2+ activates Kv channels during intestinal wound healing (30). Cell migration and formation of tumor metastasis is due to fluctuations in the activity of membrane transporters and ion channels, since they cause localized cell swelling and shrinkage (29). These changes in cell volume appear to be a prerequisite for cell migration and malignant invasion. There are only a few studies investigating the role of Cl− channels in cell migration. Cl− channels are probably necessary for cell movement, since K+ transport needs to be accompanied by a counterion (23). Notably, bestrophin has been shown to operate as a volume sensitive Cl− channel (31).

A novel function of bestrophin for cell proliferation? Cl− currents induced by expression of bestrophins share many of the properties attributed to Ca2+ activated Cl− channels, such as an anion selectivity of I− > Cl− and inhibition by NFA and DIDS (11-13;15). There is, however, an ongoing controversy whether bestrophins are actually channel forming proteins or rather regulators of ion channels. Moreover, other proteins have also been proposed as molecular candidates for the Ca2+ activated Cl− channel (reviewed in (8). The present results would support the role of Best1 as a Cl− channel. As known from previous studies, Ca2+ and volume regulated
Cl- channels support cell proliferation, and bestrophin is activated by increase in intracellular Ca$^{2+}$ as well as cell swelling (7;32). In an ongoing study with M1 collecting duct cells expressing high levels of Best1, we found inhibition of proliferation by the Cl- channel blockers DIDS and NFA (unpublished results). Thus bestrophins may provide the molecular basis for an understanding of the role of Ca$^{2+}$ and volume regulated Cl- channels in cell proliferation.

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**Figure legends**

**Fig. 1:** Slow and fast growing T84 cells.
A) Typical growth patterns of T84-slow cells as obtained from ATCC, and spontaneously transformed T84-fast cells. Bars indicate 50 µm. B) Proliferation curves for T84-slow and T84-fast cells, as obtained by cell counting (n = 4). C) Western blot for uncleaved and cleaved caspase-3 in T84-slow and T84-fast cells after 8 and 24 h incubation with 1 µM staurosporine (stauro). D) Western blot analysis of the expression of Eag1 and Best1 in slow and fast growing T84 cells. Actin indicates equal loading of the gel. Bar graph indicates large upregulation of ion channel expression in T84-fast cells. E) Whole cell currents measured in slow and fast growing T84 cells under control conditions (upper panels). Corresponding current/voltage relationships (n = 8 for both cell types; lower panels). Replacement of extracellular Cl̄ by gluconate (5Cl) shifted the i/v curve to more depolarized clamp voltages only in fast growing T84 cells, indicating the presence of a baseline Cl̄ conductance. RT-PCR and Western blots were performed at least in triplicates.

**Fig. 2:** Eag1 supports proliferation of fast growing T84 cells.
A) Proliferation of slow and fast growing T84 cells as measured by BrdU incorporation. Cells were treated with three different batches (A-C) of siRNA for Eag1. Incubation of the cells with fluorescence labeled scrambled oligos (Oligos) or transfection reagent only (Lipid) and non-treated cells (Control) served as controls. Assays were performed at least in triplicates. * indicate significant inhibition of proliferation by RNAi in T84-fast cells (ANOVA). B) Whole cell currents in T84-slow and T84-fast cells and effect of the Eag1 inhibitor astemizole (Aste; 5 µM). C) Summary of the calculated astemizole sensitive whole cell conductances (G_{Aste}) in T84-slow (n = 8) and T84-fast (n = 9) cells. G_{Aste} was significant in both cell lines and was enhanced in T84-fast cells (unpaired t-test). D) Normalized conductance / voltage relationship for Eag1 (i.e. astemizole sensitive whole cell conductance) measured in T84-fast (solid line) and T84-slow (dashed line) cells. E) Increase of the intracellular Ca^{2+} concentration ([Ca^{2+}]) by stimulation with ATP (100 µM) in T84-fast and T84-slow cells. F) Summary of the ATP induced peak and plateau increase in [Ca^{2+}] in slow (n = 59) and fast (n = 69) growing T84 cells. G) RT-PCR analysis of mRNA expression for P2Y2 receptors (764 bp) in T84-slow and T84-fast cells. When compared to the internal β-actin standard, expression of P2Y2 receptors was slightly enhanced in T84-slow. H₂O served as control. * indicates significant difference in peak [Ca^{2+}], increase in T84-fast cells.

**Fig. 3:** Eag1 operates as a cell cycle regulated channel in fast growing T84 cells.
A) FACS analysis of fast growing T84 cells synchronized in early G1 (eG1), G1/S transition and M phase (c.f. Methods). Experiments were performed at least in triplicates. B) Astemizole sensitive whole cell conductances (G_{Aste}) measured in T84-fast cells synchronized in eG1 (n = 14), G1/S (n = 9) and M (n = 9) phase. G_{Aste} was significant during all cell cycle phases but was enhanced during G1/S (ANOVA). C) Upper panel: Summary of the peak and plateau [Ca^{2+}] increase induced by CCH in T84-fast cells, synchronized into eG1 and effects of 0.5 µM astemizole (Aste, n = 73). Lower panel: Summary of the peak and plateau [Ca^{2+}] increase induced by CCH in T84-fast cells, synchronized into G1/S and effects of 0.5 µM astemizole (Aste, n = 64). The effect of astemizole was significant in all series (paired t-test) and was enhanced for plateau [Ca^{2+}] increase in G1/S when compared to eG1 (unpaired t-test).

**Fig. 4:** Best1 supports proliferation of fast growing T84 cells.
A) Western blot analysis of Best1 in T84-slow and T84-fast cells treated with three different batches (A-C) of siRNA for Best1. Incubation of the cells with fluorescence labeled scrambled oligos (Oligos) or non-treated cells (Control) served as controls. Actin indicates equal loading of the gels. B) Densitometric analysis of Best1 expression and ratio between Best1 and actin expression. C) Proliferation of slow and fast growing T84 cells as measured by BrdU incorporation. Assays were performed at least in triplicates. * indicate significant inhibition of proliferation by RNAi in T84-fast cells (ANOVA).

**Fig. 5:** T84-fast but not T84-slow cells have a Cl̄ conductance.
A) Summaries for the baseline whole cell conductances (G) and effects of the inhibitors of Ca\(^{2+}\)-activated Cl\(^{-}\) channels, niflumic acid (NFA; 10 µM) and DIDS (100 µM) in slow and fast growing T\(_{84}\) cells (n = 8 – 11). * indicate significant effects of NFA and DIDS (paired \(t\)-test). B) Whole cell currents measured in T\(_{84}\)-slow and T\(_{84}\)-fast cells. Stimulation of the cells with ATP (100 µM) activated a whole cell conductance only in T\(_{84}\)-slow but not in T\(_{84}\)-fast cells. C) Summaries for the whole cell conductances (upper panels) and membrane voltages (lower panels) measured in T\(_{84}\)-slow (n = 13) and T\(_{84}\)-fast (n = 14) cells, and effects of ATP (100 µM). * indicate significant effects on whole cell conductance and membrane voltages (paired \(t\)-test). D) Summaries for the whole cell conductances measured in T\(_{84}\)-slow (n = 9) and T\(_{84}\)-fast (n = 8) cells, and effect of replacement of extracellular Cl\(^{-}\) by gluconate (5Cl) and ATP (100 µM). * indicate significant activation of conductance by ATP in T\(_{84}\)-slow cells and significant inhibition by 5Cl (paired \(t\)-test).

**Fig. 6: Expression of Best1 in T\(_{84}\)-slow cells enhances Cl\(^{-}\) currents and proliferation.**

A) Western blot analysis of human Best1 expression in T\(_{84}\)-slow cells after transfection of 50 or 100 ng of Best1 cDNA, or mock (50 and 100 ng) transfection, and comparison with the level of Best1 expression in T\(_{84}\)-fast cells and non-transfected T\(_{84}\)-slow cells. Actin indicates equal loading of the gel. B) Growth pattern of mock transfected (- Best1) and Best1 transfected (+Best1) T\(_{84}\)-slow cells. Bars indicate 50 µm. C) Upper panel: Whole cell currents measured in mock-transfected and Best1-transfected T\(_{84}\)-slow cells and effect of DIDS (100 µM). Lower panel: Summaries of the whole cell conductances and effects of DIDS on mock transfected (n = 7) and Best1 transfected (n = 7) T\(_{84}\)-fast cells. * indicates significant inhibition of whole cell conductance by DIDS (paired \(t\)-test). D) Proliferation of mock transfected (50 and 100 ng) and Best1 transfected (50 and 100 ng) cells. Non-transfected cells (Control) and cells exposed the transfection reagent only (Lipids) served as controls. * indicate significant increase in proliferation (ANOVA).

**Fig. 7: Eag1 and Best1 support intracellular Ca\(^{2+}\) signaling and volume regulation:** A) Summary of intracellular peak (left) and plateau (right) Ca\(^{2+}\) increase in T\(_{84}\)-fast cells, upon stimulation with ATP (100 µM). Cells were treated with scrambled siRNA or siEag1 and siBest1, respectively (n = 24 – 57). B) Hypotonic (170 mosmol) cell swelling assessed by volume measurement in T\(_{84}\)-slow and T\(_{84}\)-fast cells (upper trace) and T\(_{84}\)-fast cells treated with siRNA-Best1 or control transfection (GFP) (n = 5 each time point). C) Change in calcein fluorescence due to hypotonic cell swelling. D,E) Summaries for maximal change in fluorescence intensity and slope of recovery (from swelling) of T\(_{84}\)-slow and T\(_{84}\)-fast cells (n = 12). * indicate significant difference (paired \(t\)-test).
Fig. 1

A  

T_{84}\text{-slow}  

T_{84}\text{-fast}  

B  

\begin{align*}
\text{cell number} & = \begin{cases} 4 & \text{T}_{84}\text{-slow} \\ 3 & \text{T}_{84}\text{-fast} \end{cases} \\
\text{day} & = \begin{cases} 0 & \text{T}_{84}\text{-slow} \\ 2 & \text{T}_{84}\text{-fast} \end{cases}
\end{align*}

C  

\text{Caspase-3 uncleaved}  

\text{Caspase-3 cleaved}  

D  

\begin{align*}
\text{T}_{84}\text{-slow} & : \text{Eag1}  \\
\text{T}_{84}\text{-fast} & : \text{Best1}
\end{align*}

E  

\begin{align*}
\text{T}_{84}\text{-slow} & : \text{Actin}  \\
\text{T}_{84}\text{-fast} & : \text{Eag1, Best1}
\end{align*}
Fig. 2

A

\[ \text{BrdU incorporation (\%)} \]

\begin{align*}
\text{siEag1 A} & \quad \text{siEag1 B} & \quad \text{siEag1 C} \\
\text{Oligos} & \quad \text{Lipids} & \quad \text{Control}
\end{align*}

\begin{align*}
\text{T}_84\text{-slow} & \quad \text{T}_84\text{-fast}
\end{align*}

B

\begin{align*}
\text{T}_84\text{-slow} & \quad \text{T}_84\text{-fast} \\
\text{con} & \quad \text{Aste} \\
\text{0.5 nA} & \quad \text{200 msec}
\end{align*}

C

\[ G_{\text{Aste}} \text{ (nS)} \]

D

\[ G/G_{\text{max}} \]

E

\[ [\text{Ca}^{2+}]_i \text{ (nM)} \]

F

\[ \Delta [\text{Ca}^{2+}] \text{ (nM)} \]

\begin{align*}
\text{peak} & \quad \text{plateau} \\
\text{peak} & \quad \text{plateau}
\end{align*}

G

\[ \beta\text{-actin} \quad \text{hP2Y}_2 \]

\[ P2Y_2 / \beta\text{-actin} \]

\[ \text{T}_84\text{-fast} \quad \text{T}_84\text{-slow} \]

\[ \text{ATP} \quad \text{H}_2\text{O} \]
Fig. 3

A

relative fluorescence intensity

relative cell number

eG1  G1/S  M

B

$G_{Ast}$ (nS)

$\Delta [\text{Ca}^{2+}]_i$ peak (nM)

$\Delta [\text{Ca}^{2+}]_i$ plateau (nM)

eG1  G1/S  M

C

eG1

G1/S

$\Delta [\text{Ca}^{2+}]_i$ peak (nM)

$\Delta [\text{Ca}^{2+}]_i$ plateau (nM)

control  Aste

con  Aste

#  *

#  *
Fig. 4

$T_{84}$-slow

A

kD

siBest1 A  siBest1 B  siBest1 C  scrambled

Best1

actin

B

hBest1 : actin

C

BrdU incorporation (%)

siBest1 A  siBest1 B  siBest1 C  Oligos  Lipids  Control

$T_{84}$-fast

kD

siBest1 A  siBest1 B  siBest1 C  scrambled

Best1

actin

hBest1 : actin

BrdU incorporation (%)

siBest1 A  siBest1 B  siBest1 C  Oligos  Lipids  Control
Fig. 5

A  \( T_{84}\)-slow  \( T_{84}\)-fast

\[
\begin{array}{c}
\text{G (µS)} \\
\text{NFA} \\
\text{DIDS} \\
\end{array}
\]

B

\text{con}  \quad \text{ATP}

C

\[
\begin{array}{c}
\text{G (nS)} \\
\text{ATP} \\
\text{Vm (mV)} \\
\end{array}
\]

D

\[
\begin{array}{c}
\text{G (nS)} \\
\text{ATP} \\
\text{5Cl} \\
\end{array}
\]
Fig. 6

A

T84 slow

| kD | Control | mock (50) | Best1 (50) | Best1 (100) | Control |
|----|---------|-----------|------------|-------------|---------|
| 75 |          |           |            |             |         |

hBest1

actin

B

T84-slow - Best1

T84-slow + Best1

C

con

DIDS

D

G (nS)

- Best1

+ Best1

DIDS

200 msec

0.5 nA

DIDS

BrdU incorporation (%)

Control

Lipids

Mock 50

Mock 100

Best1 50

Best1 100
Fig. 7

A

B

C

D

E

1.04
1.12
1.2
T84-fast
T84-slow

0 min
1 min
4 min
6 min

Hypotonic

0246
time (min)

[Ca^{2+}]_i (nM)

scrbld
siEag1
siBest1

*
**

scrbld
siEag1
siBest1

0 min 1 min 6 min

Hypotonic

2 min

100 units

T84-slow
T84-fast

△ fluorescence intensity

T84-slow
T84-fast

slope of recovery

T84-slow
T84-fast
Eag1 and Bestrophin 1 are upregulated in fast growing colonic cancer cells
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