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ESTABLISHMENT AND CHARACTERIZATION OF STABLY TRANSFECTED MDCK CELL LINE, EXPRESSING HBEST1 PROTEIN

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Abstract

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Bestrophin-1(Best1) is a transmembrane protein specifically expressed in the retinal pigment epithelial cells. It is localized on their basolateral plasma membrane. Malfunctions of the protein lead to retinal pathologies, named Bestrophinophaties. Best1 is thought to be a Ca^{2+}- activated Cl^- channel or a regulator of ion transport, or both. Some other functions in the maintaining of cellular homeostasis are also attributed to this protein. In order to obtain additional information about protein structure and functions we established a stably transfected MDCK II cell line expressing human Best1, conjugated with Myc and His- tags at C-terminus. The presence of stably expressed Best1 protein was demonstrated by immunofluoresce studies. Confocal microscopy images confirmed its basolateral localization. Additionally, no differences in the cell growth ratio and mitotic index of established cell line, in comparison with conventional MDCK cells were observed. Our studies suggest that Best1 does not influence cell growth and cell polarity in transfected cells, making our stably transfected cell line an appropriate model for investigations of Best1 functions.

Key words: Best1 protein, BVMD, MDCK II cells

Introduction

Bestrophins are a family of proteins with limited expression in the human organism. Best1 protein is found in the retinal epithelium cells localised at the basolateral plasma membrane (Marmorstein et al., 2000). Mutations in the bestrophin gene (*BEST1/VMD2*) lead to degenerative conditions known as Bestrophinopathies (Boon et al., 2009). It is assumed that Best1 forms a Ca^{2+}- activated Cl^- channel, but some other functions are still under discussion (Marmorstein et al., 2009). To better understand some of these functions we established a stably transfected MDCK II cell model system, expressing hBest1. In this model system, the Best1 protein is expressed and properly located at the basolateral cell surface, and cells do not show differences in growth characteristics (growth ratio and mitotic index) in comparison with nontransfected MDCK cells. Our studies suggest that a new established cell line is an appropriate model for further investigations of Best1 functions.

Materials and Methods

All reagents and chemicals were supplied by Sigma-Aldrich (Sofia, Bulgaria) unless otherwise stated. Polymount

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mounting buffer was supplied from Biovalley (Conches, France). Antibodies were obtained as follows: mouse IgG1 antibody (E6-6) against human Best1 from Novus Biologicals Inc. (Littleton, France), rabbit anti-ZO-1 from Invitrogen (Sofia, Bulgaria), HRP-linked anti-mouse and anti-rabbit IgG from Enzo, goat anti-mouse AlexaFluor 488 and 594, goat anti-rabbit AlexaFluor 633 from Invitrogen.

MDCK II (Madin-Darby Canine Kidney) cells were grown in DMEM (Sigma), in the presence of 1% Penicillin-Streptomycin solution and 10% Fetal bovine serum, at 310, 15°K, and 5% CO_2 at the gas phase. The transfection was performed using a vector (p. Receiver) containing the human *BEST1* gene, conjugated with Myc and His- tags at C-terminus (imaGenes GmbH, Berlin, Germany), by Effectene® transfection reagent (Qiagen, Bulgaria) according manufacturer's instructions (Doumanov et al., 2006). The transfected cells were selected with 5.10^{-4}kg.l^{-1} G418 for 14 days.

All experiments were performed on populations of transfectants to minimize the effect of clonal variation.

For immunofluorescence staining, cells were grown on the cover slips for 5 days until they reach the polarity state (Doumanov et al., 2006). Cells were washed twice with PBS,

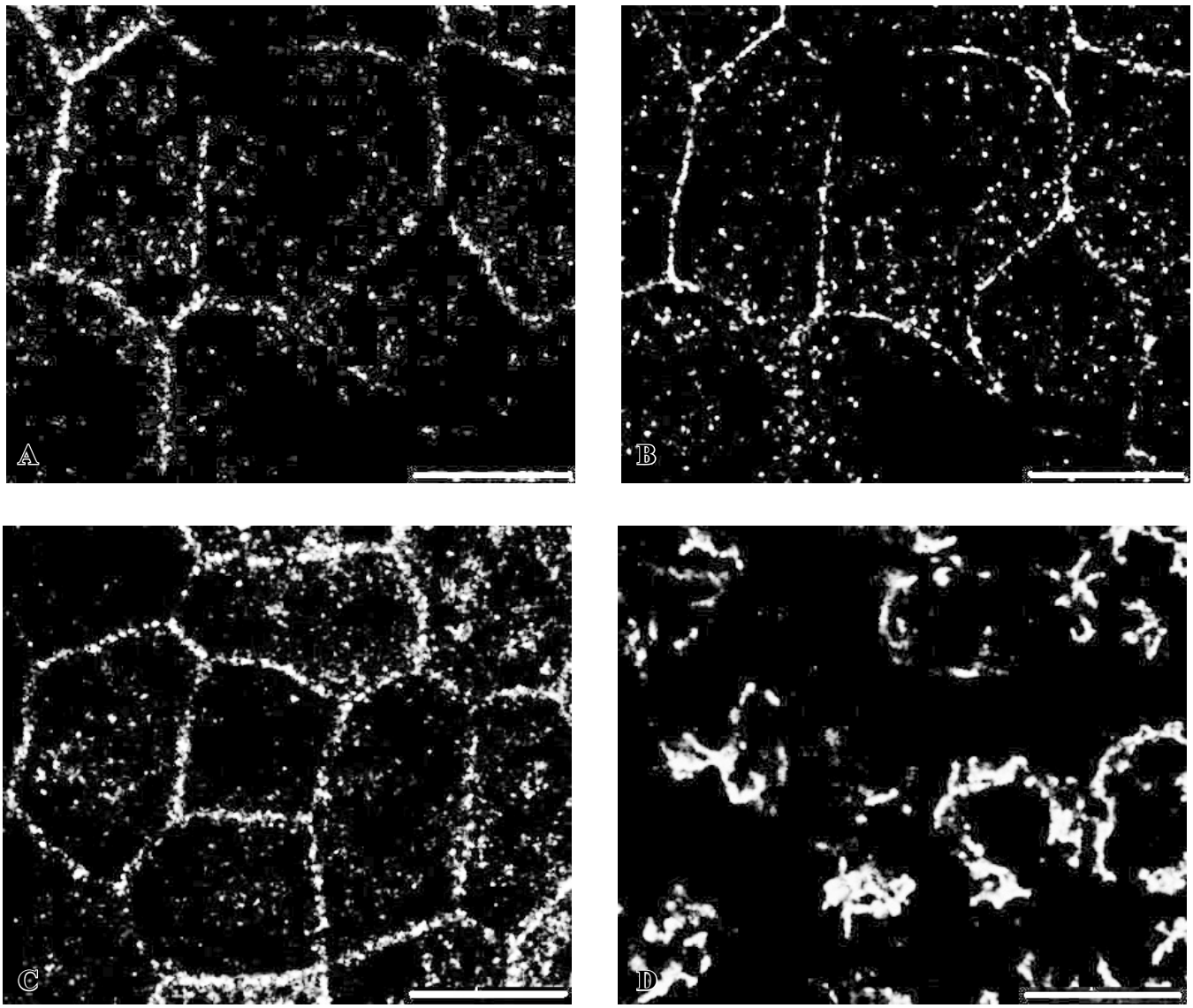

Fig. 1. Immunostaining of stably transfected MDCK II cells. Best1 (A and C) colocalised completely with the ZO-1 (B), and partially with GM130 (D), respectively. Scale bar = 10 µm

containing 1.10^{-4} mol.l^{-1} $CaCl_2$ and 1.10^{-3} mol.l^{-1} $MgCl_2$ (PBS/CM) on ice, fixed with absolute methanol for 15 minutes, blocked at room temperature for 30 minutes with PBS/CM, containing 1% BSA, washed twice in PBS/CM and incubated with a mouse anti-Best1 antibody, rabbit ZO-1 and rabbit GM130 for 1 hour at room temperature. Bound antibodies were detected using proper secondary antibodies for one hour at room temperature. For negative controls, samples were incubated with the secondary antibody alone.

For all confocal images, Leica TCS SP5 confocal microscope and LAS AF software (Leica Microsystems) with a HCX PL APO lambda blue 63.0x1.40 OIL objective and 4-time zoom enlargement were used. Y-series image data were acquired in 0.30-µm step size sequential scanning.

For growth curve, cells were seeded at two concentrations – 1.10^4 and 5.10^4 cells/ml. Cell number was calculated every 24 hours in a period of 7 days. For mitotic index permanent microscope slides were used with initial cell concentration of 5.10^4 cells/ml. Cells were fixed with 70% Ethanol for 15 min on every 24 hours for a period of 6 days, stained with Giemsa and mitotic and nonmitotic cells on randomly chosen areas of the slides were count. Mitotic index was calculated as the percentage of mitotic cells from the total cell population.

Results

BEST1 is a retina-specific gene expressed in RPE cells (Marquardt et al., 1998; Petrukhin et al., 1998). Our results indicate that MDCK cells do not express endogenously Best1 protein (data not shown). After transfection, a specific Best1 signal was obtained in all stably transfected cells (Figure 1A, C). Confocal microscopy images of polarized cells determined the exact location of Best1 on the cell membranes. As expected, protein was located on the basolateral surface (Figure 1A, C) and specific signal from the protein was localised as a rim-like structures.

Cell polarity state of the cells was proved by ZO-1, marking the tight junctions between the cells (Figure 1B). Colocalization with Golgi apparatus marker (GM130) confirmed that the Best1 is not retained in it, but properly trafficked to the plasma membrane (Figure 1D).

We do not observed differences in the cell growth characteristics and mitosis ratio of transfected with Best1 and nontransfected MDCK II cells (Figure 2).

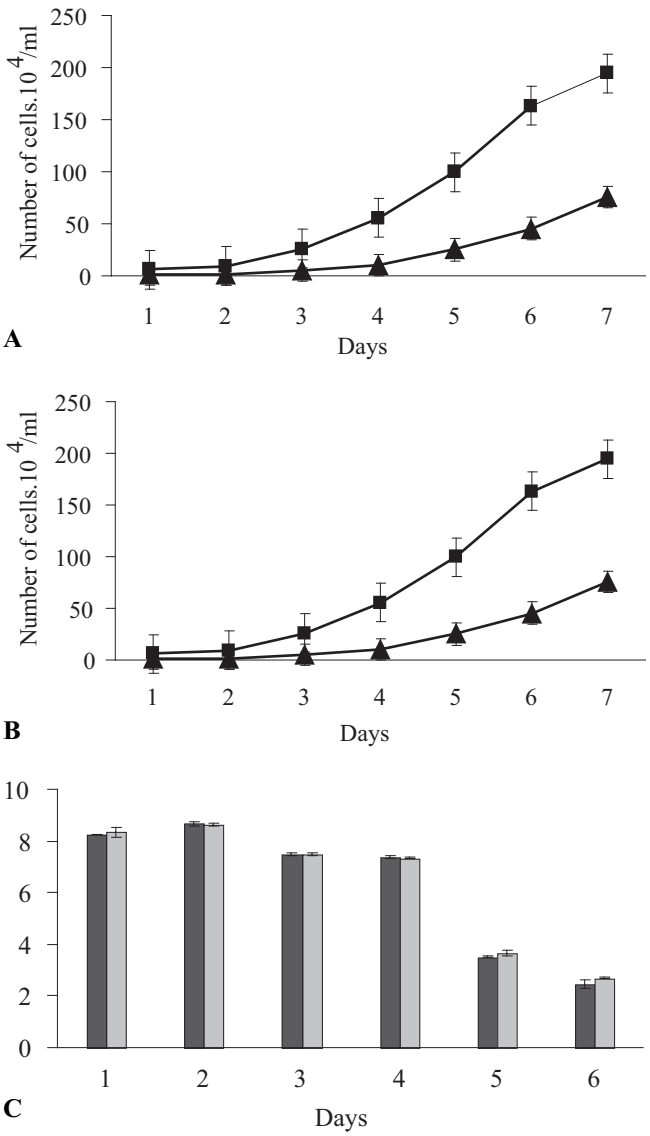

Fig. 2. Growth curves of transfected (A) and nontransfected (B) MDCK cells with initial cell concentration of 1.10^4 cells per ml (▲) and 5.10^4 cells per ml (■), n = 2. Mitotic indexes of transfected (dark colons) and nontransfected (bright colons) cells in 6-day period of cultivation (C)

Discussion

Purpose of this study was to establish and characterise a stably transfected MDCK cell line, expressing hBest1. Using conventional transfection techniques and selection of transfectants we obtained 100% transfected cells. The signal for the Best1 was present even after the 25th passaging, confirming the stability of transfection. Cellular localization of the protein was identical with those in the retinal epithelial cells, endogenously expressing it. Even it is exogenous protein for

the MDCK cells, Best1 do not affect their mitotic and growth characteristics.

Conclusions

We consider that newly established MDCK cell line, stably expressing hBest1 can be used as a proper model system for investigations of structure and functions of Best1 and as a source of protein for purification.

Acknowledgments

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