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Transient receptor potential channel TRPM8 is over-expressed and required for cellular proliferation in pancreatic adenocarcinoma

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Abstract

The roles of transient receptor potential (TRP) cation channels in pancreatic tumorigenesis are essentially unknown. Here, we focus on the TRP melastatin-subfamily (TRPM) members. Expression of the thermally regulated transmembrane Ca^{2+} -permeable channel TRPM8 is consistently up-regulated in human pancreatic adenocarcinoma cell lines and tissues. TRPM8-deficient pancreatic cancer cells have reduced ability of proliferation and cell cycle progression with elevated levels of cyclin-dependent kinase inhibitors. These results indicate that TRPM8 is aberrantly over-expressed in pancreatic adenocarcinoma and required for cellular proliferation, and they support further investigation of the potential of TRPM8 as a clinical biomarker and therapeutic target in pancreatic adenocarcinoma.

Keywords

transient receptor potential (TRP); TRPM8; ion channel; proliferation; pancreatic cancer

1. Introduction

Pancreatic cancer is a deadly malignancy, and its incidence is rising [1]. Until effective interventions for prevention and early detection are available, new therapy based upon an improved understanding of the molecular biology and genetics of pancreatic tumorigenesis is urgently needed. Growing evidence indicates that the transient receptor potential (TRP) ion channels play important roles in human cancer [2,3]. Determining the functional roles of the TRP ion channels in tumor growth and underlying mechanisms may help identify novel biomarkers and molecular targets in pancreatic cancer.

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5. Conflicts of interest

None declared.

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TRP is a superfamily of cation channels that plays important roles in diverse physiological processes by sensing physical stimuli in the cellular environment and responding by modulation of intracellular ion currents [4,5]. The various TRP members have common characteristics, including a transmembrane channel that exhibits relative ionic selectivity. Among the TRP families of channels, the TRP melastatin-subfamily (TRPM) is not well characterized regarding its physiological functions and pathological roles. However, expression of several of the TRPM members is altered in a variety of human tumors. For instance, TRPM1 and TRPM7 have been implicated in melanoma [6,7]; TRPM5 in Wilms' tumors and rhabdomyosarcomas [8]; TRPM7 in neuroblastoma, head and neck cancer, and mammary and gastric carcinoma [9–12]; and TRPM8 in prostate carcinoma [13,14] and neuroendocrine tumor [15]. Yet the roles of the TRP ion channels in pancreatic adenocarcinoma are largely unexplored.

The goal of this study is to identify the role of TRP ion channels, with an initial focus on the melastatin-subfamily members, in pancreatic cancer. We analyzed the mRNA levels of the eight members of the TRPM subfamily, and only the expression of *TRPM8* is consistently elevated in all the human pancreatic adenocarcinoma cell lines examined. In agreement with this finding, *TRPM8* is over-expressed in human pancreatic adenocarcinoma tissues. RNA interference-mediated silencing of *TRPM8* reduced the ability of the cells to proliferate and progress through the cell cycle. These effects are associated with up-regulated expression of the cyclin-dependent kinase inhibitors *p21^{CDKN1A}* and *p27^{CDKN1B}*. Results of this study indicate that TRPM8 plays a proliferative role in the pathogenesis of pancreatic adenocarcinoma.

2. Materials and methods

2.1. Tissues and cell cultures

Human pancreatic adenocarcinoma tissues were obtained from the Tissue Procurement Core Facility of the University of Iowa (with approval of the Institutional Review Board). The human pancreatic adenocarcinoma cell lines BxPC-3, Capan-1, HPAF-II, MIA PaCa-2, PANC-1, Panc 02.03, and PL45 were obtained from the American Type Culture Collection (ATCC®) and cultured according to ATCC instructions. The human pancreatic ductal epithelial cell line H6c7 was generously provided by Dr. Ming-Sound Tsao at the University of Toronto and cultured as described [16]. All experiments were performed using culture medium, except where indicated. The cells were used within 20 passages of the frozen stocks in liquid nitrogen from which the cells were periodically recovered.

2.2. Quantification of TRPM8 mRNA

Total RNA was extracted from each cell line using RNeasy® Mini Kit (Qiagen). First-strand cDNA was generated using SuperScript® reverse transcriptase and random primers (Invitrogen™). The cDNA was amplified with TaqMan® (Applied Biosystems™) and analyzed by real-time polymerase chain reaction (PCR) (ABI Prism 7500, Applied Biosystems™). The sequences of the primers used were designed based on human cDNA sequences (GenBank accession numbers: *TRPM8*: AY090109) as follows:

- *TRPM8*: 5'-ACTCAGAAGGCTGAGGTACA-3', 5'-TTCAGTCGGAGTCTCACTCT-3'

The mRNA levels of *TRPM8* were determined with a standard curve using known concentrations of *TRPM8* mRNA.

2.3. Immunocytochemistry and immunohistochemistry of TRPM8

The paraffin-embedded pancreatic adenocarcinoma cells or tumor sections were deparaffinized and treated with citrate buffer pH 6 pre-heated in a steamer to unmask antigen. The sections were treated with 3% hydrogen peroxide to quench endogenous peroxidase activity, and then incubated with rabbit anti-human TRPM8 polyclonal antibodies (Lifespan Biosciences) at 1:50 dilution for 60 min, and this was followed by incubation with horseradish peroxidase-conjugated anti-rabbit IgG (EnVision™+ System, Dako) for 30 min. The signals were detected by color reaction using 3,3'-diaminobenzidine (Dako), counterstained with hematoxylin (Richard-Allan Scientific), and mounted using Permount (Sigma®). Negative controls included the cells or tissues being incubated in the presence of pre-immune serum and without anti-TRPM8 antibodies in order to rule out non-specific binding by the secondary antibodies. The expression of TRPM8 was examined under a compound microscope (Olympus BX51). The images were captured using a digital camera (Olympus DP71), analyzed using DP Manager software (Olympus), and constructed using Adobe® Photoshop® 7. The images shown are representative of matched specimens from five patients.

2.4. RNA interference-mediated gene silencing

The cell lines PANC-1 and BxPC-3 were grown to 70–80% confluency, trypsinized, and resuspended at 10^6 cells in 100 μ l of Nucleofector® Solution containing 600 nM of small interfering RNA (siRNA) directed against human *TRPM8* (sc-95009; Santa Cruz Biotechnology), 600 nM of non-targeting control siRNA (sc-37007; Santa Cruz Biotechnology, Inc.), or no siRNA. Transfection was performed using Nucleofector II (Amaxa®/Lonza) according to the manufacturer's instructions. The transfected cells were incubated at 37 °C for further analysis. To verify knock down of *TRPM8*, total RNA was extracted after 48 h of transfection and analyzed using real-time PCR as described above.

2.5. Cellular and nuclear morphology

PANC-1 and BxPC-3 cells transfected with anti-*TRPM8* siRNA or non-targeting control siRNA were seeded at 10^5 cells in 4 ml medium per well of a 6-well plate (Corning Incorporated) or 5×10^4 cells in 2 ml medium per well of a 2-chambered glass slide (Lab-Tek®, Nunc™), and incubated for 48 h. For the cells grown in the 6-well plate, images were captured under an inverted light microscope with phase contrast (Olympus IX81) at 20 $\times$ magnification. Those cells grown on glass slides were fixed with 95% ethanol in water (PANC-1) or 4% paraformaldehyde in PBS (BxPC-3), washed with Tris-buffered saline, permeabilized with 0.1% Tween-20, and then mounted with Vectashield® with 4',6-diamidino-2-phenylindole (DAPI) (Vector Laboratories, Inc.). The images were acquired under a compound microscope (Olympus BX51) with ultraviolet emission at 40 $\times$ magnification using a digital camera (Olympus DP71). The images were analyzed using DP Manager software (Olympus Corp.) and constructed with Adobe® Photoshop® 7.0.

2.6. Proliferation assay

The effects of siRNA directed against *TRPM8* on cellular proliferation were quantified using the CellTiter 96® AQueous One Solution Cell Proliferation Assay (Promega) based on absorbance of the chromogen, 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfonylphenyl)-2H-tetrazolium, inner salt (MTS) according to the manufacturer's instructions. Briefly, PANC-1 or BxPC-3 cells were transfected with anti-*TRPM8* siRNA or non-targeting control siRNA. The transfected cells were seeded at 2×10^4 in 100 μ l medium per well of a 96-well plate (Corning Incorporated) for MTS assay, or at 5×10^5 in 4 ml medium per well of a 6-well plate for cell counting. The cells were incubated for a total of 72 h with fresh culture medium being replaced at the final 24 h. Proliferation was analyzed using the MTS assay or by counting cells using trypan blue for exclusion of dead cells.

2.7. Cell cycle analysis

Serum-deprived PANC-1 and BxPC-3 cells (for 24 h) were transfected with anti-*TRPM8* siRNA or non-targeting control siRNAs, seeded at 5×10^5 in 4 ml medium in each well of a 6-well plate, and incubated for 48 h. The DNA contents were analyzed by flow cytometry using a FACScan™ (Becton, Dickinson and Company) as described [17]. For accurate analysis of cell cycle distribution curves in the viable cells, the non-specific sub-G₀/G₁ events were gated out.

2.8. Apoptosis assay

PANC-1 and BxPC-3 cells transfected with anti-*TRPM8* siRNA or non-targeting control siRNA were incubated for 72 h and analyzed for apoptosis using a FACScan™ flow cytometer (Becton, Dickinson and Company) as described [17].

2.9. Relative quantification of *p21^{CDKN1A}* and *p27^{CDKN1B}* mRNA

PANC-1 and BxPC-3 cells transfected with anti-*TRPM8* siRNA or non-targeting control siRNA were seeded at 5×10^5 in 4 ml medium per well of a 6-well plate, and incubated for 48 h. Total RNA was extracted and reverse transcribed as described above in 2.2. The cDNA was then amplified and quantified using SYBR® Green PCR Master Mix (Applied Biosystems™) and ABI PRISM 7700 Sequence Detection System (Applied Biosystems™). The sequences of the primers used were designed based on human cDNA sequences (GenBank accession numbers: *p21^{CDKN1A}*: NM_000389; *p27^{CDKN1B}*: BC001971; *GAPDH*: NM_002046) as follows:

- *p21^{CDKN1A}*: 5'-CATTTTAAGATGGTGGCAGT-3', 5'-AGTGCCAGGAAAGACAACTA-3';
- *p27^{CDKN1B}*: 5'-GTCCATTTATCCACAGGAAA-3', 5'-ATGGTTTTTCCATACACAGG-3';
- *GAPDH*: 5'-GAGTCAACGGATTTGGTCGT-3', 5'-TTGATTTTGGAGGGATCTCG-3'.

The relative mRNA levels of *p21^{CDKN1A}* or *p27^{CDKN1B}* were determined as compared with those of *GAPDH*.

2.10. Statistics

The difference between the experimental group and control was analyzed using the Student's *t*-test. Statistical significance was considered at a *P*-value < 0.05.

3. Results

3.1. TRPM8 is over-expressed in human pancreatic adenocarcinoma

To identify the role of TRP ion channels in pancreatic cancer with an initial focus on TRPM, we examined mRNA expression of the eight members of the TRPM family in human pancreatic adenocarcinoma cell lines by real-time PCR. Only the mRNA levels of *TRPM8* are consistently elevated in the seven cell lines examined, and analysis of its expression and role becomes the focus of this study. TRPM8 is a thermally regulated Ca²⁺-permeable channel that can be activated by cold sensation [18,19]. In the pancreatic adenocarcinoma cell lines, *TRPM8* mRNA levels are variably elevated from 26% to 1.17-fold, as compared with that in the immortalized but non-cancerous human pancreatic ductal epithelia H6c7 (Fig. 1). Two of the cell lines, PANC-1 and BxPC-3, were further examined for expression of TRPM8 protein, and they expressed relatively strong immunoreactivity against TRPM8 (Fig. 2). The expression of TRPM8 was then determined in human pancreatic adenocarcinoma tissues in surgically

resected specimens. Consistent with the data of the cell lines, ductal adenocarcinoma in all the specimens being examined expressed relatively strong immunoreactivity against TRPM8, whereas normal pancreatic ducts expressed trace level of TRPM8 (Fig. 3). Note that normal pancreatic acinar cells expressed TRPM8 to a considerable extent. These data indicate that *TRPM8* is aberrantly over-expressed in pancreatic adenocarcinoma and suggest that TRPM8 plays a functional role in pancreatic cancer cells.

3.2. TRPM8 is required for proliferation of pancreatic adenocarcinoma cells by regulating cell cycle progression

To determine the functional role of TRPM8 in pancreatic adenocarcinoma, we employed siRNA directed against *TRPM8* to knock down its expression in the cell lines PANC-1 and BxPC-3. In the siRNA-treated cells, expression of *TRPM8* mRNA was reduced by 31% in PANC-1 and by 39% in BxPC-3, as indicated by quantitative real-time PCR. The morphology of the siRNA-transfected cells was examined under an inverted microscope with phase contrast. As compared with the control, the cells in the anti-*TRPM8* siRNA treatment group were enlarged and flattened, and they contained multiple micronuclei (Fig. 4A). Consistent with their appearance in the phase-contrast micrographs, the nuclei of the TRPM8-deficient cells were multi-lobed or partially fragmented, as revealed by DAPI staining of DNA (Fig. 4B). These cellular and nuclear features are suggestive of replicative senescence and non-apoptotic cell death such as mitotic catastrophe (20).

The effect of RNA interference-mediated depletion of *TRPM8* on cellular proliferation was determined by the MTS assay and by counting cells. The TRPM8-deficient PANC-1 cells exhibited significantly reduced proliferation by 59% using the MTS assay and by 35% using cell count (Fig. 5). Similarly, BxPC-3 cells transfected with anti-*TRPM8* siRNA showed significantly reduced proliferation by 55% and 30% using the MTS assay and cell count, respectively (Fig. 5). Consistent with the results of the proliferation assays, flow cytometric analysis of TRPM8-deficient PANC-1 cells indicated increased proportion of cells in the G₀/G₁ phases by 30% with a decrease of 27% and 6% of cells in the S and G₂/M phases, respectively (Fig. 6). Likewise, BxPC-3 cells transfected with anti-*TRPM8* siRNA showed an increase of 29% G₀/G₁-phase cells, and a decrease of 19% and 45% S- and G₂/M-phase cells, respectively (Fig. 6).

Examination of the molecular markers of cell cycle progression by real-time PCR revealed that the cyclin-dependent kinase inhibitors mRNA was up-regulated by anti-*TRPM8* siRNA (Fig. 7). In TRPM8-deficient PANC-1, the *p21^{CDKN1A}* and *p27^{CDKN1B}* mRNA levels were increased by 22% and 66%, respectively. In BxPC-3 cells transfected with anti-*TRPM8* siRNA, the *p21^{CDKN1A}* and *p27^{CDKN1B}* mRNA levels were elevated by 35% and 27%, respectively. The mRNA levels of *cyclin G1* and *cyclin B1* were not significantly altered by anti-*TRPM8* siRNA (data not shown). Comparing the cells treated with anti-*TRPM8* siRNA to those with non-targeting control siRNA, there is no significant change in the proportion of cells undergoing apoptosis as analyzed by flow cytometry (data not shown). These results indicate that TRPM8 is required for proliferation of pancreatic cancer cells by maintaining cell survival and promoting cell cycle progression.

4. Discussion

The TRP family members are cellular sensors of environmental stimuli, and they respond by modulation of cellular homeostasis of cations. Certain TRP channels are aberrantly expressed in malignant tumors, and they contribute to proliferation and survival of the cancer cells. The role of TRP channels in pancreatic cancer is mostly unknown, and identification of their functional significance may help shed new light into its pathogenic mechanism. This study provides evidence that TRPM8 is aberrantly over-expressed in pancreatic adenocarcinoma,

and it is required for uncontrolled proliferation of pancreatic cancer cells. These findings indicate a contributory role of the thermally regulated ion channel TRPM8 in pancreatic oncogenesis and suggest its potential as a diagnostic/prognostic biomarker and a therapeutic target in pancreatic adenocarcinoma.

Of all the TRPM channels, *TRPM8* has been shown to be normally expressed in a relatively distinct pattern, with the highest levels of mRNA in the prostate, but virtually undetectable in all other human tissues except the liver [21]. In prostate carcinoma, expression of *TRPM8* is up-regulated in androgen-dependent differentiated epithelia, but its expression diminishes as the cells become de-differentiated and androgen-independent [13,14,22]. Moreover, in prostate cancer epithelia, TRPM8 is expressed in the plasma membrane and endoplasmic reticulum, and there is a relationship between its subcellular location/channel activity and the androgen dependence of the cells [14,23,24]. Our data show that expression of TRPM8 is elevated in pancreatic adenocarcinoma relative to normal pancreatic ductal epithelia. In this study, a limited number of surgically resected pancreatic adenocarcinoma specimens representing localized tumors were examined. Detailed examination of a large number of pancreatic intra-epithelial and invasive neoplasia and correlation with the clinicopathologic features will be necessary to determine the significance of TRPM8 expression in the initiation and progression of pancreatic tumor. Moreover, high-resolution examination will be required to determine the subcellular localization of TRPM8 and the associated functions in pancreatic adenocarcinoma.

The mechanism underlying the up-regulated expression of *TRPM8* in pancreatic cancer cells is unclear. In prostate cancer cells, androgen response element is present in the promoter region of *TRPM8* [14,22]. It is possible that androgen may stimulate expression of *TRPM8* in pancreatic adenocarcinoma through androgen response elements in the *TRPM8* promoter region. Of note, androgen receptor is variably expressed in human pancreatic adenocarcinoma cell lines [25]. Alternatively, epigenetic mechanisms—such as histone modifications or DNA methylation of the promoter region of the *TRPM8* gene—may account for the altered expression of *TRPM8* in pancreatic adenocarcinoma. Future studies using androgen or its antagonist and analysis of the promoter region of *TRPM8* will help elucidate the mechanism underlying the altered expression of *TRPM8* in pancreatic adenocarcinoma.

TRPM8 is required for maintaining proliferation by promoting survival and cell cycle progression of pancreatic adenocarcinoma cells, and the mechanisms underlying the proliferative role of TRPM8 are under active investigation. It has been shown that TRPM8 is required for proliferation and survival in androgen-dependent prostate carcinoma [14,22]. The channel activity of TRPM8 in sensory neurons can be stimulated by cold temperature and by coolness-inducing agents such as menthol, resulting in increased intracellular Ca^{2+} level [18, 19]. Furthermore, the pro-proliferative and pro-survival roles of TRPM8 in prostate carcinoma-derived epithelia involve release of Ca^{2+} from endoplasmic reticulum and activation of store-operated channels at the plasma membrane [23,24]. Hypothetically, the proliferative role of TRPM8 in pancreatic adenocarcinoma cells is mediated by as yet unidentified signaling pathways that lead to a transient increase in intracellular Ca^{2+} level, which then leads to modulation of the genes involved in cellular proliferation. Future studies by modulating the TRPM8 channel activity and Ca^{2+} homeostasis will help elucidate the signaling mechanisms that mediate the proliferative role of TRPM8 in pancreatic cancer cells. Of interest, the heat-sensitive TRP vanilloid receptor 1 (TRPV1) is over-expressed in pancreatic adenocarcinoma cells and the involved neurons, providing a link to pain sensation associated with pancreatic cancer [26]. Thus, a potential relationship between cold sensation and pathogenesis of pancreatic adenocarcinoma via TRPM8 warrants further investigation.

In conclusion, TRPM8 is aberrantly over-expressed in pancreatic adenocarcinoma and required for proliferation of pancreatic cancer cells. Results of this study suggest that TRPM8

contributes to uncontrolled growth and progression of pancreatic tumor. Ongoing studies aim to determine the mechanism that mediates the proliferative role of TRPM8 in pancreatic adenocarcinoma cells by investigating the effects of modulating TRPM8 expression and channel activity on Ca^{2+} homeostasis and the signaling pathways involved. Research efforts in parallel focus on exploring the potential of TRPM8 as a diagnostic and prognostic biomarker as well as a molecular target for therapy in pancreatic adenocarcinoma.

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Fig. 1. *TRPM8* mRNA is over-expressed in human pancreatic adenocarcinoma cell lines. Quantification of *TRPM8* mRNA by real-time PCR. Each value represents the relative *TRPM8* mRNA level in each cell line as compared to that in H6c7. This experiment was performed independently twice with consistent results. MIA, MIA PaCa-2.

Fig. 2. TRPM8 protein is strongly expressed in human pancreatic adenocarcinoma cell lines. Immunocytochemistry using anti-TRPM8 antibodies. Bright field images of PANC-1 and BxPC-3 cells expressing TRPM8 to different extents. Negative controls for immunostaining were performed in the absence of anti-TRPM8 antibodies.

Fig. 3. TRPM8 protein is over-expressed in human pancreatic adenocarcinoma. Immunohistochemistry using anti-TRPM8 antibodies. The normal pancreatic tissue and pancreatic adenocarcinoma were obtained from different regions of the pancreas from the patient. These images are representative of the normal-tumor pairs of pancreatic tissues from five patients. Tissue sections incubated in the absence of anti-TRPM8 antibodies were included as negative controls.

Fig. 4. RNA interference-mediated silencing of *TRPM8* induces alterations of cellular and nuclear morphology suggestive of proliferative arrest. PANC-1 and BxPC-3 cells transfected with siRNA directed against *TRPM8* or non-targeting control siRNA. **A.** Phase contrast micrographs of *TRPM8*-deficient cells with multiple micronuclei (red arrowheads). **B.** Fluorescent micrographs of DAPI-stained *TRPM8*-deficient cells with abnormal nuclear morphology (white arrows). These experiments were repeated two times with essentially the same results.

Fig. 5.

SiRNA-induced knock down of *TRPM8* reduces the ability of pancreatic adenocarcinoma cells to proliferate. PANC-1 and BxPC-3 cells treated with siRNA anti-*TRPM8* were analyzed by the MTS assay and by counting cells. Proliferation of the *TRPM8*-deficient cells is expressed as a percentage of that in control siRNA transfected cells. Each value represents the mean of 6 independent cell samples $\pm$ standard deviation. * $P < 0.05$ indicates statistical significance.

Fig. 6.

TRPM8-deficient pancreatic adenocarcinoma cells have impaired ability to progress through the cell division cycle. Serum-deprived PANC-1 and BxPC-3 cells were transfected with anti-*TRPM8* siRNA or non-targeting control siRNA. Forty-eight hours later, the cells were analyzed for DNA content by flow cytometry. The proportion of cells in each phase of the cell cycle is indicated. Non-specific sub-G₀/G₁ events were gated out for accurate analysis of cell cycle distribution curves in the viable cells. This experiment was repeated with similar results.

Fig. 7.

Anti-*TRPM8* siRNA up-regulates expression of *p21^{CDKN1A}* and *p27^{CDKN1B}*. Total RNA extracted from PANC-1 and BxPC-3 cells 72 h following transfection with siRNA directed against *TRPM8* or non-targeting control siRNA was analyzed for *p21^{CDKN1A}* and *p27^{CDKN1B}* mRNA levels by quantitative real-time PCR. Each value represents the relative *p21^{CDKN1A}* or *p27^{CDKN1B}* mRNA level in the anti-*TRPM8* siRNA transfected cells as compared to that in control siRNA transfected cells. This experiment was independently performed twice, and the same pattern of changes in *p21^{CDKN1A}* and *p27^{CDKN1B}* mRNA was observed.