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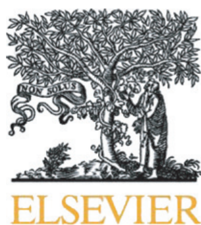

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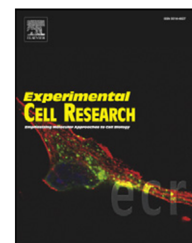

Research Article

Downregulation of tight junction-associated MARVEL protein marvelD3 during epithelial–mesenchymal transition in human pancreatic cancer cells

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ABSTRACT

The novel tight junction protein marvelD3 contains a conserved MARVEL (MAL and related proteins for vesicle trafficking and membrane link) domain like occludin and tricellulin. However, little is yet known about the detailed role and regulation of marvelD3 in normal epithelial cells and cancer cells, including pancreatic cancer. In the present study, we investigated marvelD3 expression in well and poorly differentiated human pancreatic cancer cell lines and normal pancreatic duct epithelial cells in which the hTERT gene was introduced into human pancreatic duct epithelial cells in primary culture, and the changes of marvelD3 during Snail-induced epithelial–mesenchymal transition (EMT) under hypoxia, TGF-β treatment and knockdown of FOXA2 in well differentiated pancreatic cancer HPAC cells. MarvelD3 was transcriptionally downregulated in poorly differentiated pancreatic cancer cells and during Snail-induced EMT of pancreatic cancer cells in which Snail was highly expressed and the fence function downregulated, whereas it was maintained in well differentiated human pancreatic cancer cells and normal pancreatic duct epithelial cells. Depletion of marvelD3 by siRNAs in HPAC cells resulted in downregulation of barrier functions indicated as a decrease in transepithelial electric resistance and an increase of permeability to fluorescent dextran tracers, whereas it did not affect fence function of tight junctions. In conclusion, marvelD3 is transcriptionally downregulated in Snail-induced EMT during the progression for the pancreatic cancer.

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Introduction

The tight junction, the most apically located of the intercellular junctional complexes, inhibits solute and water flow through the paracellular space (termed the “barrier” function) [1,2]. It also separates the apical from the basolateral cell surface domains to establish cell polarity (termed the “fence” function) [3,4]. Recent evidence suggests that tight junctions also participate in signal transduction mechanisms that regulate epithelial cell proliferation, gene expression, differentiation and morphogenesis [5].

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Tight junctions are formed by not only the integral membrane proteins claudins, occludin, and JAMs, but also many peripheral membrane proteins, including the scaffold PDZ-expression proteins zonula occludens (ZO)-1, ZO-2, ZO-3, multi-PDZ domain protein-1 (MUPP1), membrane-associated guanylate kinase with inverted orientation-1 (MAGI)-1, MAGI-2, MAGI-3, cell polarity molecules ASIP/PAR-3, PAR-6, PALS-1 and PALS-1 associated tight junction (PATJ), as well as the non-PDZ-expressing proteins cingulin, symplekin, ZONAB, GEF-H1, aPKC, PP2A, Rab3b, Rab13, PTEN and 7H6 [6–8]. These tight junction proteins are regulated by various cytokines and growth factors via distinct signal transduction pathways [9,10]. Recently, tricellulin was identified as the first marker of the tricellular tight junction in epithelial cells, and its loss affects the organization of the tricellular tight junction and the barrier function of epithelial cells [11,12]. JNK is involved in the regulation of tricellular tight junctions, including tricellulin expression and the barrier function during normal remodeling of epithelial cells, and prevents disruption of the epithelial barrier in inflammation in human pancreatic duct epithelial cells [13].

The transcription factor Snail has a key role in epithelial–mesenchymal transition (EMT) during development and in tumor progression by negative regulation of adherens and tight junctions such as E-cadherin, claudins and occludin [14–17]. EMT is characterized by a loss of cell–cell contact and apicobasal polarity, which are hallmarks of dysfunction of the tight junction fence [18,19]. The repression of tricellulin is also related to Snail-induced EMT in human gastric carcinoma [20].

Both occludin and tricellulin (marvelD2) contain the tetra-spanning MARVEL (MAL and related proteins for vesicle trafficking and membrane link) domain that is present in proteins involved in membrane apposition and concentrated in cholesterol-rich micro-domains [21]. The novel tight junction protein marvelD3 contains a conserved MARVEL domain like occludin and tricellulin [22,23]. However, little is known about the detailed role of marvelD3 in epithelial tight junctions and how it is regulated, including Snail signaling.

In the present study, we examined marvelD3 expression in well and poorly differentiated human pancreatic cancer cell lines and normal pancreatic duct epithelial cells, and the changes of marvelD3 during Snail-induced EMT occurring as a result of hypoxia, TGF-β treatment and knockdown of forkhead box transcription factor A2 (FOXA2) in well-differentiated pancreatic cancer HPAC cells, in which occludin and tricellulin were highly expressed.

Materials and methods

Antibodies, activators and inhibitors

A rabbit polyclonal anti-marvelD3 antibody was obtained from AVIAN System Biology (San Diego, CA). A goat polyclonal anti-FOXA2 (M-20) antibody was obtained from Santa Cruz Biotechnology Inc. (Santa Cruz, CA). Rabbit polyclonal anti-tricellulin (c-term), anti-occludin, anti-JAM-A, anti-ZO-1, anti-claudin-1, anti-claudin-4, anti-claudin-7, and mouse monoclonal anti-occludin (OC-3F10) antibodies were obtained from Zymed Laboratories (San Francisco, CA). A rabbit polyclonal anti-actin antibody was obtained from Sigma-Aldrich (St Louis, MO). Alexa 488 (green)-conjugated anti-rabbit IgG and Alexa594 (red)-conjugated anti-mouse IgG antibodies were purchased from Molecular Probes Inc. (Eugene, OR).

Cultures of cell lines and treatment

Human pancreatic cancer cell lines PANC-1 and HPAC were purchased from ATCC (Manassas, VA). PANC-1 cells and HPAC cells were maintained with DMEM (Sigma-Aldrich) supplemented with 10% dialyzed fetal bovine serum (FBS, Invitrogen; Carlsbad, CA). The medium for all cell lines contained 100 U/ml penicillin and 100 μg/ml streptomycin, and all cells were plated on 35- and 60-mm culture dishes (Corning Glass Works, Corning, NY) that were coated with rat tail collagen (500 μg of dried tendon/ml in 0.1% acetic acid), and incubated in a humidified 5% CO_2 incubator at 37 °C.

The HPAC cells were treated with 20 ng/ml TGF-β1 or incubated in a 2% CO_2:2% O_2 incubator balanced with nitrogen.

For RNA interference studies, small interference RNA (siRNA) duplexes targeting the mRNA sequences of human marvelD3 and human FOXA2 were purchased from Invitrogen (Carlsbad, CA; Table 1). A scrambled siRNA sequence (BLOCK-iT Alexa Fluor fluorescent, Invitrogen) was employed as control siRNA. At one day before transfection, the HPAC cells were plated in medium without antibiotics such that they would be half confluent at the time of transfection. The cells were transfected with 100 nM siRNAs using Lipofectamine RNAiMAX (Invitrogen) as a carrier according to the manufacturer's instructions.

Isolation and culture of human pancreatic duct epithelial (HPDE) cells

Human pancreatic tissues were obtained from patients with pancreatic or biliary tract diseases who underwent pancreatic resection in the Sapporo Medical University hospital. Informed consent was obtained from all patients, and the study was approved by the ethics committee of Sapporo Medical University. Human pancreatic tissues were minced into pieces 2 to 3 mm^3 in volume and washed with phosphate-buffered saline (PBS) containing 100 U/ml penicillin and 100 μg/ml streptomycin (Lonza Walkersville, Walkersville, MD) three times. These minced tissues were suspended in 10 ml of Hanks' balanced salt solution with 0.5 μg/ml DNase I and 0.04 mg/ml Liberase Blenzyme 3 (Roche, Basel, Switzerland) and then incubated with bubbling of mixed O_2 gas containing 5.2% CO_2 at 37 °C for 10 min. The dissociated tissues were subsequently filtrated with 300-μm mesh followed by filtration with 70-μm mesh (Cell Strainer; BD Biosciences, San Jose, CA). After centrifugation at 1000 g for 4 min, isolated cells were cultured in bronchial epithelial basal medium (BEBM; Lonza Walkersville) containing 10% fetal bovine serum (FBS; CCB, Nichirei Bioscience, Tokyo, Japan) and supplemented with BEGM® SingleQuots® (Lonza Walkersville, including 0.4% bovine pituitary extract, 0.1% insulin, 0.1% hydrocortisone, 0.1% gentamicin, amphotericin-B [GA-1000], 0.1% retinoic acid, 0.1% transferrin, 0.1% triiodothyronine, 0.1% epinephrine, 0.1% human epidermal growth factor), 100 U/ml penicillin and 100 μg/ml streptomycin on 60-mm culture dishes (Corning Life Science, Acton, MA), coated with rat tail collagen (500 μg of dried tendon/ml of 0.1% acetic acid). Following the above protocol, tissue dissociation and cell isolation were repeated for the same sample a maximum of seven times. The cells were placed in a humidified 5% CO_2:95% air incubator at 37 °C.

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