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miR-210 regulates the interaction between pancreatic cancer cells and stellate cells

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ABSTRACT

There is accumulating evidence that pancreatic stellate cells (PSCs) promote the progression of pancreatic cancer. microRNAs (miRNAs) are small non-coding RNAs acting as negative regulators of gene expression at the post-transcriptional level. This study aimed to clarify the role of miRNAs in the interaction between PSCs and pancreatic cancer cells. Pancreatic cancer cells were mono-cultured or indirectly co-cultured with PSCs. miRNAs were prepared, and Agilent's miRNA microarray containing probes for 904 human miRNAs was used to identify differentially expressed miRNAs. miR-210 was identified as an upregulated miRNA by co-culture with PSCs. Conditioned media of PSCs activated ERK and Akt, but not hypoxia-inducible factor-1α pathway. PSCs-induced miR-210 upregulation was inhibited by inhibitors of ERK and PI3K/Akt pathways. Inhibition of miR-210 expression decreased migration, decreased the expression of vimentin and snai-1, and increased the membrane-associated expression of β-catenin in Panc-1 cells co-cultured with PSCs. In conclusion, our results suggest a novel role of miR-210 in the interaction between PSCs and pancreatic cancer cells.

1. Introduction

Pancreatic ductal adenocarcinoma is a highly malignant phenotype characterized by rapid progression, early metastasis, and a limited response to chemotherapy and radiotherapy [1–3]. The abundant desmoplastic/stromal reaction is a characteristic feature of pancreatic cancers [3,4]. It has been recognized that the cells responsible for the production of the desmoplastic reaction in pancreatic cancer are pancreatic stellate cells (PSCs) [4–10]. In normal pancreas, PSCs are quiescent and can be identified by the presence of vitamin A-containing lipid droplets in the cytoplasm. In response to pancreatic injury or inflammation, they are transformed ("activated") from their quiescent phenotype into myofibroblast-like cells, which express α-smooth muscle actin, actively proliferate, and produce extracellular matrix components such as type I collagen [4–10]. For more than a decade, evidence has been accumulating that activated PSCs play a pivotal role in the development of pancreatic fibrosis in chronic pancreatitis and pancreatic cancer [4–10]. microRNAs (miRNAs) are small, non-coding RNAs consisting of 17–25 nucleotides that regulate gene expression by binding loosely complementary sequences in the 3′-untranslated region of target mRNAs to repress translation or induce mRNA cleavage [11,12]. miRNAs regulate a variety of cell functions such as cell proliferation, development, apoptosis, differentiation, and carcinogenesis [11,12]. Although previous studies have clarified the roles of miRNAs in pancreatic cancer [13–17], no studies have addressed the role of miRNAs in the interactions between PSCs and pancreatic cancer. To address this issue, we compared the miRNA expression profiles between mono-cultured cancer cells and those co-cultured with PSCs. We here show that PSCs induce the expression of miR-210 in pancreatic cancer cells.

2. Materials and methods

2.1. Materials

Mouse anti-hypoxia-inducible factor (HIF)-1α antibody was purchased from Novus Biologicals (Littleton, CO). Mouse anti-vimentin and rabbit anti-Snai1 antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Mouse anti-β-catenin antibody was purchased from BD Biosciences (Franklin Lakes, NJ). Rabbit antibodies against ERK (total and phosphorylated at Tyr^{202}/Tyr^{204}) and Akt (total and phosphorylated at Ser^{473}) were purchased from Cell Signaling Technologies (Bevery, MA). Rabbit

Abbreviations: BrdU, 5-bromo-2′-deoxyuridine; DAPI, 4′, 6-diamidino-2-phenylinodole; EMT, epithelial-mesenchymal transition; HIF, hypoxia-inducible factor; miRNA, micro RNA; OD, optical density; PSCs, pancreatic stellate cells; PSC-CM, conditioned medium of hPSC21-S/T cells.

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anti-GAPDH antibody was purchased from R&D Systems (Minneapolis, MN). U0126, LY294002, and wortmannin were purchased from Merck Millipore (Billerica, MA). Other reagents were purchased from Sigma–Aldrich (St. Louis, MO) unless specifically described.

2.2. Cell culture

The human pancreatic cancer cell lines Panc-1 and MIAPaCa-2 were obtained from the American Type Culture Collection (Manassas, VA). The human pancreatic cancer cell line SUIT-2 was obtained from the Japanese Collection of Research Bioresources (Ibaraki, Osaka, Japan). Panc-1 and SUIT-2 cells were grown in RPMI1640 medium supplemented with 10% FBS, penicillin sodium, and streptomycin sulfate. MIAPaCa-2 cells were maintained in DMEM supplemented with 10% FBS, penicillin sodium, and streptomycin sulfate.

The immortalized human PSC line hPSC21-S/T was established by retrovirus-mediated gene transfer of simian virus 40 T antigen and human telomerase reverse transcriptase into the human PSCs isolated from the resected pancreas tissue of a patient undergoing operation for pancreatic cancer [18]. Cells were maintained in Ham's F-12/DMEM (1:1) supplemented with 10% FBS, penicillin sodium, and streptomycin sulfate. This study was approved by the Ethics Committee of Tohoku University School of Medicine.

The conditioned media of hPSC21-S/T cells was harvested following 72-h incubation, centrifuged at 3000 revolution/min for 10 min, filtered through 0.22-μm filters and concentrated 10-fold through 3000 molecular weight cut-off filters (Centriprep YM3; Merck Millipore) according to the manufacturer's instruction. The concentrated media was designated as PSC-CM.

2.3. Hypoxic treatment

Cells were usually incubated under normoxia (21% O_2, 5% CO_2, and 74% N_2). However, for some experiments, Panc-1 cells were incubated in a hypoxic incubator with 1% O_2, 5% CO_2, and 94% N_2. The oxygen level in the culture chambers was continuously monitored. Culture medium was preconditioned to the correct O_2 level in the hypoxic experiments.

2.4. Indirect co-culture of pancreatic cancer cells and PSCs

Pancreatic cancer cells (1×10^5 cells/well) were seeded in 6-well culture plates (BD Biosciences) in RPMI1640 medium supplemented with 10% FBS, penicillin sodium, and streptomycin sulfate. hPSC21-S/T cells (1×10^5 cells/culture insert) were seeded into culture inserts of 1.0 μm pore size (BD Biosciences) in Ham's F-12/DMEM (1:1) supplemented with 10% FBS, penicillin sodium, and streptomycin sulfate. Next day, the culture insets seeded with hPSC21-S/T cells were placed into 6-well plates containing pancreatic cancer cells and incubation was continued up to 3 days in RPMI1640 medium supplemented with 1% FBS, penicillin sodium and streptomycin sulfate.

2.5. miRNA microarray

Total RNAs including miRNAs were prepared using the miRNA RNeasy preparation kit (Qiagen, Valencia, CA). The Agilent's miRNA microarray (Human miRNA microarray Release 14.0; Agilent Technologies, Santa Clara, CA) containing probes for 904 human miRNAs was used to identify differentially expressed miRNAs between mono-cultured Panc-1 cells and those co-cultured with hPSC21-S/T cells. Data analysis was performed using the GeneSpring GX software version 12.5 (Agilent Technologies).

2.6. Quantitative real-time PCR for miRNA

Quantitative real-time PCR was performed using Taqman® Fast Universal PCR Master Mix (Life Technologies, Carlsbad, CA) and the StepOnePlus 7300 Real-Time PCR System (Life Technologies). Primers and probes were predesigned by the manufacturer (Life Technologies). The assay ID numbers were as follows: #000512 for hsa-miR-210 and #001093 for RNU6B. PCR was performed at 95 °C for 10 min, followed by 40 cycles of 95 °C for 15 s and 60 °C for 1 min.

The absolute number of copies was standardized by a sample standard curve. The values of hsa-miR-210 were normalized to RNU6B and expressed as those of the mono-cultured cells.

2.7. Inhibition of miR-210 expression

The stable miR-210 knockdown cell line was established by introducing tough decoy miRNA-blocking expression vector (pBAsi-Neo, Takara Bio, Otsu, Japan) which targets hsa-miR-210 using Lipofectamine 2000 (Life technologies) in Panc-1 cells. The control cell line was established by introducing tough decoy miRNA-blocking expression negative control vector (Takara Bio). Transfected cells were subjected to the limiting dilution in G418 containing (1 mg/ml) normal growth medium.

Transient inhibition of miR-210 expression was performed using the anti-miR-210 inhibitor (Cat#: AM10516; Life Technologies). Control experiments employed a negative control (Cat#: 17010; Life Technologies), Transfection was performed using the 4D-Nucleofector™ (Lonza, Basel, Switzerland) according to the manufacturer's instructions.

2.8. Western blotting

Cells were lysed, and total cell lysates (~100 μg) were fractionated on a 10% SDS–polyacrylamide gel. They were transferred to a nitrocellulose membrane (Bio-Rad, Hercules, CA), and the membrane was incubated overnight at 4 °C with rabbit anti-phospho-specific ERK antibody. After incubation with peroxidase-conjugated goat anti-rabbit *IgG* antibody, proteins were visualized using an enhanced chemiluminescence kit (GE Healthcare, Chalfont St Giles, United Kingdom). The levels of total ERK, Akt (total and phosphorylated at Ser^{473}), snai-1, and GAPDH were determined in a similar manner.

2.9. Immunofluorescent staining

Immunofluorescent staining of vimentin and β-catenin was performed as previously described [19]. Pancreatic cancer cells were seeded (1×10^5 cells/well) on cover slips placed on the bottom of 6-well plates. After 48 h of indirect co-culture with hPSC21-S/T cells, the pancreatic cancer cells were fixed in methanol at −20 °C. After blocking with 10% normal goat serum, the cells were incubated with mouse anti-vimentin or anti-β-catenin antibody at 1:100 dilution overnight at 4 °C. After washing, the cells were incubated with Alexa $Fluora^{546}$-labeled goat anti-mouse *IgG* antibody (Life Technologies) for 1 h. After washing, the cells wereanalyzed for fluorescence using an all-in-one type fluorescent microscope (Bio-Zero BZ-9000; Keyence, Osaka, Japan). Nuclei were counterstained with 4′, 6-diamidino-2-phenylinodole (DAPI). Expression of HIF-1α was examined in a similar manner.

2.10. Cell proliferation assay

Cell proliferation was assessed using a commercial kit (Roche Applied Science, Penzberg, Germany). This is a colorimetric immunoassay based on the measurement of 5-bromo-2′-deoxyuridine