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Overexpression of microRNA-345 Affects the Invasive Capacity of Pancreatic Ductal Adenocarcinoma Cell Lines by Suppressing MUC1 and TJP2 Expression

Shigenobu Tatsumi [1,2], Tomomi Fujii [1,*], Aya Sugimoto [1], Yoko Sekita-Hatakeyama [1], Kohei Morita [1], Tomoko Uchiyama [1], Hiroe Itami [1], Maiko Takeda [1], Masaharu Yamazaki [2], Masayuki Sho [3] and Chiho Ohbayashi [1]

[1] Department of Diagnostic Pathology, Nara Medical University School of Medicine, Nara 634-8521, Japan; statsu@naramed-u.ac.jp (S.T.); a_sugimoto@naramed-u.ac.jp (A.S.); yhatakeyama@naramed-u.ac.jp (Y.S.-H.); moritak@naramed-u.ac.jp (K.M.); uchiyama0403@naramed-u.ac.jp (T.U.); hritami@naramed-u.ac.jp (H.I.); maikot@naramed-u.ac.jp (M.T.); ohbayashi@naramed-u.ac.jp (C.O.)
[2] Department of Central Clinical Laboratory, Nara Medical University Hospital, Nara 634-8521, Japan; masayama@naramed-u.ac.jp
[3] Department of Surgery, Nara Medical University School of Medicine, Nara 634-8521, Japan; m-sho@naramed-u.ac.jp
* Correspondence: fujiit@naramed-u.ac.jp; Tel.: +81-744-29-8910

Abstract: The majority of pancreatic carcinomas are pancreatic ductal adenocarcinomas (PDAC), and the presence of non-invasive pancreatic intraepithelial neoplasia or intraductal papillary mucinous neoplasm, as an associated lesion, is considered important. These microscopic hyperplastic or grossly papillomatous lesions exhibit varying degrees of morphological atypia and may develop into invasive carcinomas. In this study, we investigated whether mucin-1 (MUC1) is involved in the progression of pancreatic carcinoma and examined the mechanisms by which microRNAs regulate MUC1 expression in vitro. In PDAC cell lines, suppression of MUC1 expression reduced cell proliferation and invasion; PDAC cell lines transfected with an miR-345 precursor suppressed the expression of MUC1, and reduced cell proliferation and invasion. Tight junction protein 2 (TJP2), a putative target of miR-345, is regulated by MUC1. The suppression of TJP2 expression reduced cell proliferation by inducing apoptosis. These results suggest that MUC1 and TJP2, the putative target molecules of miR-345, are critical in maintaining the invasive potential of pancreatic carcinoma cells, and regulating their expression may prevent the progression of non-invasive pancreatic intraductal lesions to invasive carcinomas. This study provides new insights for the development of novel molecular targeted therapies for pancreatic carcinomas.

Keywords: PDAC; MUC1; TJP2; miR-345; cell proliferation; invasion

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1. Introduction

Pancreatic ductal adenocarcinoma is a lethal disease and the fourth leading cause of cancer-related deaths in Japan. Pancreatic intraepithelial neoplasia (PanIN) and intraductal papillary mucinous neoplasm (IPMN) are important potential precancerous lesions. PanINs are defined as microscopic atypia of glandular epithelial cells and IPMNs as histologically atypical epithelia with gross papillary lesions [1,2]. Although these lesions may progress to become invasive adenocarcinomas, a clear assessment of this potential is difficult. Prognostic predictions, and early therapeutic actions are needed, to prevent the progression of these lesions into aggressive invasive carcinoma.

Mucins (MUC), especially MUC1, -2, -4, and -5AC, are reliable biomarker candidates for tumor diagnosis and prognosis [3–7]. MUC1 is upregulated in pancreatic ductal adenocarcinoma and is associated with an increased degree of dysplasia in PanINs. De novo expression of MUC1 in IPMNs suggests a high likelihood of progression to invasive cancer [3,5–7].

MicroRNAs (miRNAs) play an important role in the regulation of crucial cell processes, such as proliferation, differentiation, and development, by causing translational repression or transcript degradation [8,9].

Despite the many demonstrated roles of MUC1 in pancreatic ductal lesions, highly specific diagnostic markers and therapeutically effective molecular targeted drugs for PDAC have not been fully commercialized, and MUC1, despite its status as a key molecule for PDAC, is also not recognized as clinically practical for diagnosis [3,4,7,10–12]. This is due to the fact that MUC1 has also been important in its physiological role and thus has not achieved the status of a cancer-specific marker. Searching for useful markers for diagnosis and treatment of PDAC from miRNAs associated with this important molecule, MUC1, and its target molecules will help in molecular diagnosis and molecular targeted therapy of PDAC.

In this study, we demonstrated that MUC1, which has been used as a biomarker for predicting tumor capacity and prognosis in pancreatic cancer, acts as a key molecule in maintaining the cell proliferation and invasive capacity of pancreatic carcinomas. Additionally, miR-345 regulates the expression of MUC1 and tight junction protein 2 (TJP2), a putative target of MUC1. Therefore, miR-345 regulates cell proliferation, through apoptosis and the production of reactive oxygen species, and invasive capacity in pancreatic cancer cells.

2. Materials and Methods

2.1. Cell Lines

We obtained three human PDAC cell lines (Panc1, Panc0203, and PL45) from the American Type Culture Collection (Manassas, VA, USA). Panc1 was cultured in RPMI-1640 medium (nakalai tesque, Kyoto, Japan). supplemented with 10% fetal bovine serum and 50 U/mL penicillin-streptomycin (Nakalai Tesque, Kyoto, Japan), Panc0203 was cultured in RPMI-1640 medium supplemented with 15% fetal bovine serum, 50 U/mL penicillin-streptomycin and 10 U/mL insulin, and PL45 was cultured in DMEM medium (nakalai tesque, Kyoto, Japan) supplemented with 10% fetal bovine serum and 50 U/mL penicillin-streptomycin. All cell lines were cultured at 37 °C in 5% CO_2.

2.2. Tissue Samples and Immunohistochemistry

The present study was approved by the ethics committee of Nara Medical University (IRB3041), and informed consent was obtained from all patients. Seventy-one formalin-fixed paraffin-embedded tissues surgically resected between 2018 and 2021 were selected. Pancreatic ductal lesions included 26 cases of PanIN/IPMN, 12 cases of adenocarcinoma in situ, and 33 cases of PDAC. These specimens were pathologically diagnosed and verified by visual inspection of hematoxylin-eosin stained sections by at least two experienced pathologists. Sections were incubated with the primary antibodies for 15 min at room temperature (Table S1). Reactions were visualized using BOND-III Full Automatic Immunohistochemical Staining System (Leika, Wetzlar, Germany) according to the manufacturer's protocol, with diaminobenzidine as the chromogen and light hematoxylin counterstaining.

2.3. Genetic Mutation Analysis in PDAC Cell Lines

The extracted DNA from each PDAC cell line was analyzed by next-generation sequencing (NGS) using MiniSeq (Illumina, Inc., San Diego, CA, USA). Library construction was performed using AmpliSeq for Illumina Library Plus (Illumina, Inc., San Diego, CA, USA). The index adapter for the AmpliSeq for Illumina Cancer HotSpot Panel v2 assay (50 target genes and 207 amplicons) was used following the AmpliSeq for Illumina CD Indexes Set A (Illumina, Inc., San Diego, CA, USA). After the first PCR step, the AmpliSeq for Illumina Cancer HotSpot Panel v2 protocol (Illumina, Inc., San Diego, CA, USA) was applied. The library size and concentration were confirmed with 4200 TapeStation using the Genomic DNA ScreenTape Assay (Agilent Technologies, Santa Clara, CA, USA). NGS analyses were conducted using a MiniSeq sequencing platform with 2 × 150 bp paired-end runs using Mid

Output v2 (Illumina, Inc., San Diego, CA, USA) chemistry. FASTQ files were uploaded to the BaseSpace Sequencing Hub and analyzed using DNA amplicon v1.1.0.

2.4. miRNA Precursor and siRNA Transfection in PDAC Cell Lines

For transfection, the PDAC cell lines were seeded at a density of 1.5×10^5 cells/well in a 6-well dish and were transfected with 100 ng/L of siRNA against MUC1 or tight junction protein 2 (TJP2) for 72 h. Transfection with each siRNA and Ambion® Pre-miR™ miRNA Precursor (hsa-miR-345-5p; Thermo Fisher Scientific, Waltham, MA, USA) was carried out using Lipofectamine RNAiMAX (Thermo Fisher Scientific, Waltham, MA, USA), in accordance with the manufacturer's protocol. The following MUC1 and TJP2 siRNA sequences were designed after selecting the appropriate DNA target sequences: 5′-GCAGCCT-CTCTTACACAAA-3′ and 5′- CTACCTGTTAGAAATCCCTAA -3′, respectively.

2.5. Quantitative RT-PCR (qRT-PCR) Analysis of miRNA and mRNA

For purification of total RNA, including miRNA from cells, we used the miRNeasy Mini kit (QIAGEN, Hilden, Germany). To conduct qRT-PCR, cDNA was synthesized from 1 μg of total RNA using PrimeScript RT Master Mix (Perfect Real Time) and SYBR Premix Ex Taq II, and Tli RNaseH Plus (Takara, Otsu, Japan). The qRT-PCR conditions were set at 95 °C for 30 s, followed by 55–63 °C for 30 s for a total of 35–45 cycles. The primer sequences are listed in Table S2.

2.6. Cell Proliferation Assay

The CellTiter 96 AQueous One Solution Cell Proliferation Assay (Promega, Madison, WI, USA) was used for the MTS assay, in accordance with the manufacturer's protocol, to measure cell proliferation. Data were collected from quintuplicate measurements.

2.7. Cell Viability and Reactive Oxygen Species (ROS) Assay

Following transfection with MUC1 or TJP2 siRNA or miR-345 precursor (miR-345pre), the treated cells were washed with phosphate-buffered saline. Annexin V and ROS measurements were performed using the Muse® Annexin V & Dead Cell Kit and Muse® Oxidative Stress Kit (Luminex, Austin, TX, USA), respectively, according to the manufacturer's protocols.

2.8. Invasion Assay

In vitro invasion assays were performed using Corning Biocoat Matrigel Invasion Chambers (Corning, Bedford, MA, USA) according to the manufacturer's instructions. In short, the PDAC cell lines were seeded at a density of 2.5×10^4 cells/well in a 24-well dish and transfected with 100 nmol/L MUC1 or TJP2 siRNA or miR-345pre for 72 h using Lipofectamine RNAiMAX (Life Technologies, Carlsbad, CA, USA). After culturing for the indicated time, the samples were removed and re-plated in Matrigel chambers. After culturing for 72 h, invading cells were stained and counted under a light microscope in accordance with the manufacturer's protocol. The experiment was repeated three times.

2.9. Statistical Analysis

Statistical analysis was performed with GraphPad Prism 8.0 (GraphPad Software, Inc., La Jolla, CA, USA) using the one-way ANOVA to compare three groups and two-tailed Student's *t*-test to compare two groups. Graphical data are presented as mean ± SEM. Results were considered significant at $p < 0.05$. All experiments were performed with $n \geq 3$.

3. Results

3.1. Evaluation of MUC1 Expression in Pancreatic Duct Lesions by Immunohistochemical Staining

The 26 PanIN/IPMN tissues had absent or weak intensities of MUC1 protein expression (Figure 1A). Similarly, in the non-invasive adenocarcinoma tissue samples, the majority (10 of 12) showed negative or weak intensities (Figure 1A). In contrast, 27 PDAC samples showed intermediate or strong intensities (Figure 1A). This indicates that MUC1 tends to be highly expressed in invasive carcinomas. However, the ANOVA test did not yield significant differences among the three groups evaluated.

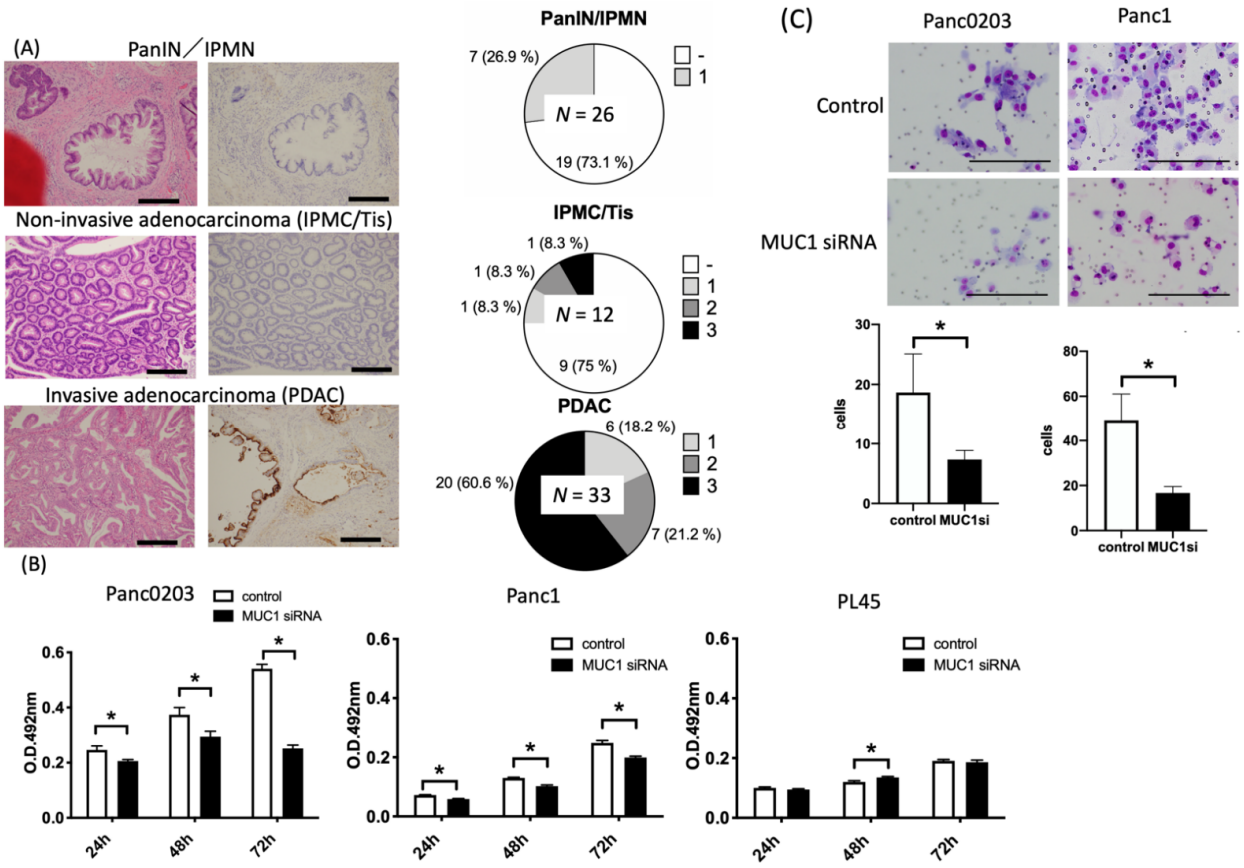

Figure 1. Expression of MUC1 in pancreatic ductal lesion tissues and its involvement in cell proliferation and invasion. (**A**) MUC1 expression in pancreatic intraepithelial neoplasia/intraductal papillary mucinous neoplasm (PanIN/IPMN); adenocarcinoma in situ; and PDAC samples are shown. The left panel is hematoxylin-eosin stained and the right panel anti-MUC1 immuno-stained. The degree of MUC1 expression at the cell membrane was assessed semi-quantitatively as 0 (negative), 1 (weak intensity), 2 (intermediate intensity), and 3 (strong intensity). (×100; scale bar; 250 μm) A one-way ANOVA comparison of the three groups showed no statistically significant differences ($p = 0.69$). (**B**) The MTS assay under transfection with MUC1 siRNA at 24, 48, and 72 h in PDAC cell lines (* $p < 0.05$). (**C**) Matrigel invasion assay showing the promotion of invasive capability in Panc0203 and Panc1 cells transfected with MUC1 siRNA. (×100; scale bar; 100 μm).

The suppression of MUC1 expression significantly decreased cell proliferation 72 h after transfection with MUC1 siRNA in Panc0203 and Panc1 (Figure 1B). The cell proliferation rate of PL45 was low, and its MUC1 inhibitory effect could not be fully evaluated (Figure 1B). Therefore, Panc0203 and Panc1 were used in the subsequent experiments. In the Matrigel assay, both Panc0203 and Panc1 showed decreased invasive capacity due to the suppression of MUC1 expression (Figure 1C). These results indicate that MUC1 has the potential to contribute to cell proliferation and invasion in PDAC cells.

3.2. Characterization of Panc0203 and Panc1 and Analysis of the Mechanism of Regulation of Cell Proliferation by MUC1 in PDAC Cells

In order to use the two types of cells for subsequent experimentation, we confirmed the immunophenotype of Panc0203 and Panc1 by immunocytochemical staining with mucin molecular markers and epithelial markers. Panc0203 was positive for MUC4, CAM5.2, CK7, CK19, and MUC1/EMA, while Panc1 was weakly positive for MUC1/EMA and positive for CAM5.2 and CK19 (Figure 2A). This suggests that Panc1 may be a poorly differentiated adenocarcinoma compared to Panc0203. Evaluation of MUC1 mRNA in the two cell types by Ct value using quantitative RT-PCR showed that the Ct value of MUC1 was above 20 in both cases, and even higher in Panc1, suggesting that MUC1 mRNA expression is also low (Figure 2A). Therefore, since evaluation at the protein level is not sensitive enough for the following experiments using these two cells, mRNA was used to evaluate MUC1 expression levels. Furthermore, PL45, Panc0203, and Panc1 were genetically characterized by NGS analysis using a panel of 50 cancer-related genes. Although each cell line had different genetic variants, the KRAS mutation (G12V), a characteristic of PDA, was observed in all cell lines (Figure S1). This confirmed that the cells were PDACs, although they differed in origin and degree of differentiation.

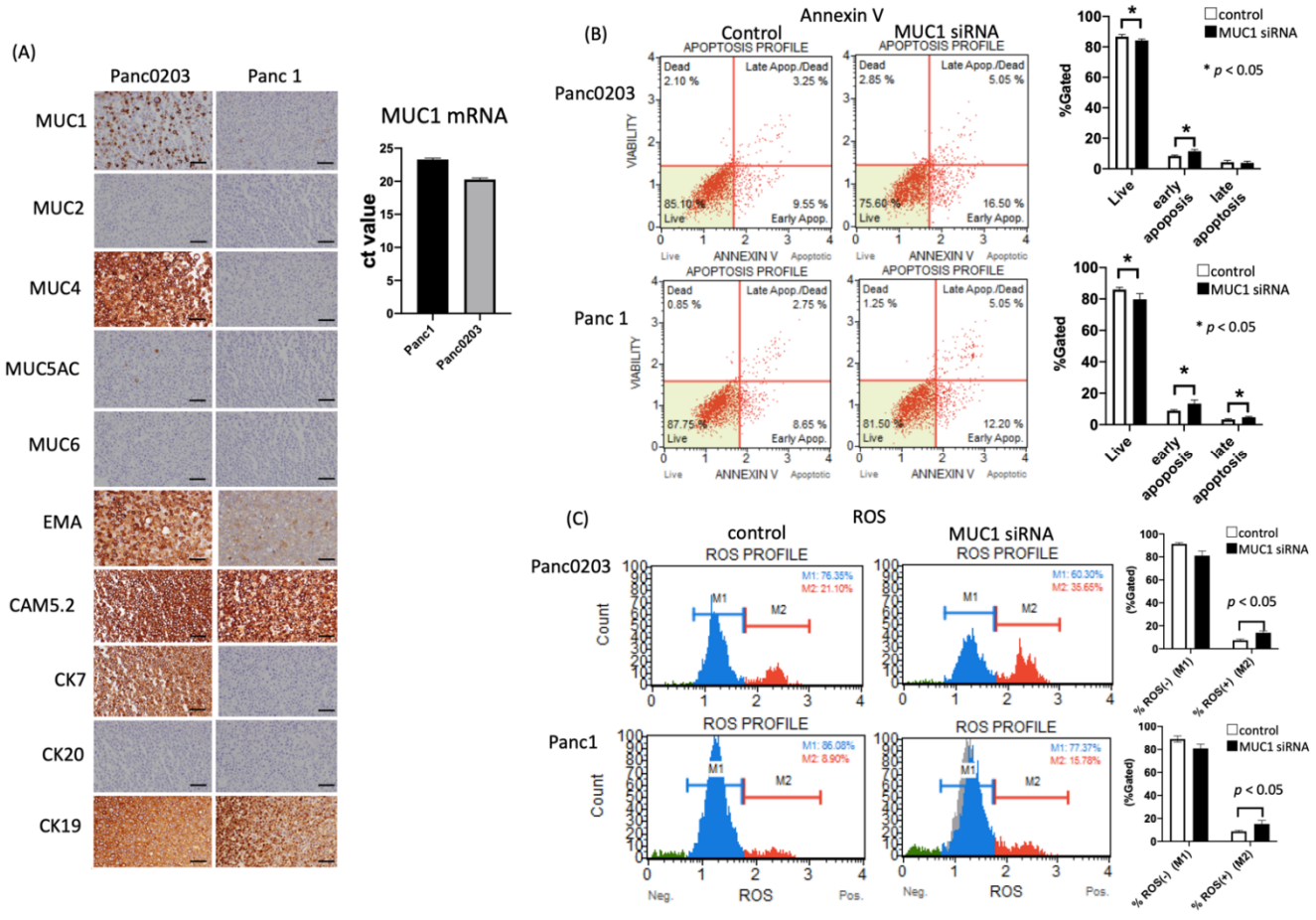

Figure 2. Characterization and function analysis under MUC1 suppression in Panc0203 and Panc1. (**A**) (**Left**) Immunocytochemical staining of various epithelial markers. Panc0203 and Panc1 cell blocks were prepared and immunohistochemically stained. (×100; scale bar; 100 μm) (**Right**) Evaluation of Ct value of MUC1 mRNA by quantitative RT-PCR method. (**B**) Annexin V analysis for the detection of apoptosis in Panc0203 and Panc1 cells, (* $p < 0.05$). (**C**) Dynamics of reactive oxygen species in Panc0203 and Panc1 transfected with MUC1 siRNA. Left panel: Blue peaks indicate ROS negative (M1 peak) and red peaks indicate ROS positive (M2 peak). Gray shadows indicate negative controls.

To elucidate the mechanism of the proliferative potential of MUC1 in PDAC cells, we measured Annexin V and reactive oxygen species. In both Panc0203 and Panc1, the Annexin V assay revealed that MUC1 suppression promoted early apoptosis (Figure 2B). Previous studies have shown that suppression of MUC1 results in altered ROS production in fetal cells and various tumors [13–15]. We investigated this possibility in PDAC cells and found that the suppression of MUC1 increased ROS production (Figure 2C). These results suggest that the proliferative potential of MUC1 is related to the regulation of apoptosis and ROS production.

3.3. MiR-345 Regulates the Expression of MUC1 in PDAC Cell Lines

Overexpression of miR-345 significantly suppressed the expression of MUC1 in Panc0203 (Figure 3A). Although miR-345 suppressed the expression of MUC1 in Panc1, it was not significant (Figure 3A). A possible reason for this is that Panc1 is poorly differentiated compared to Panc0203 and exhibits lower MUC1/EMA protein levels. Although no significant difference was observed, a trend was observed, and therefore, we continued to use Panc1 cells for subsequent experiments.

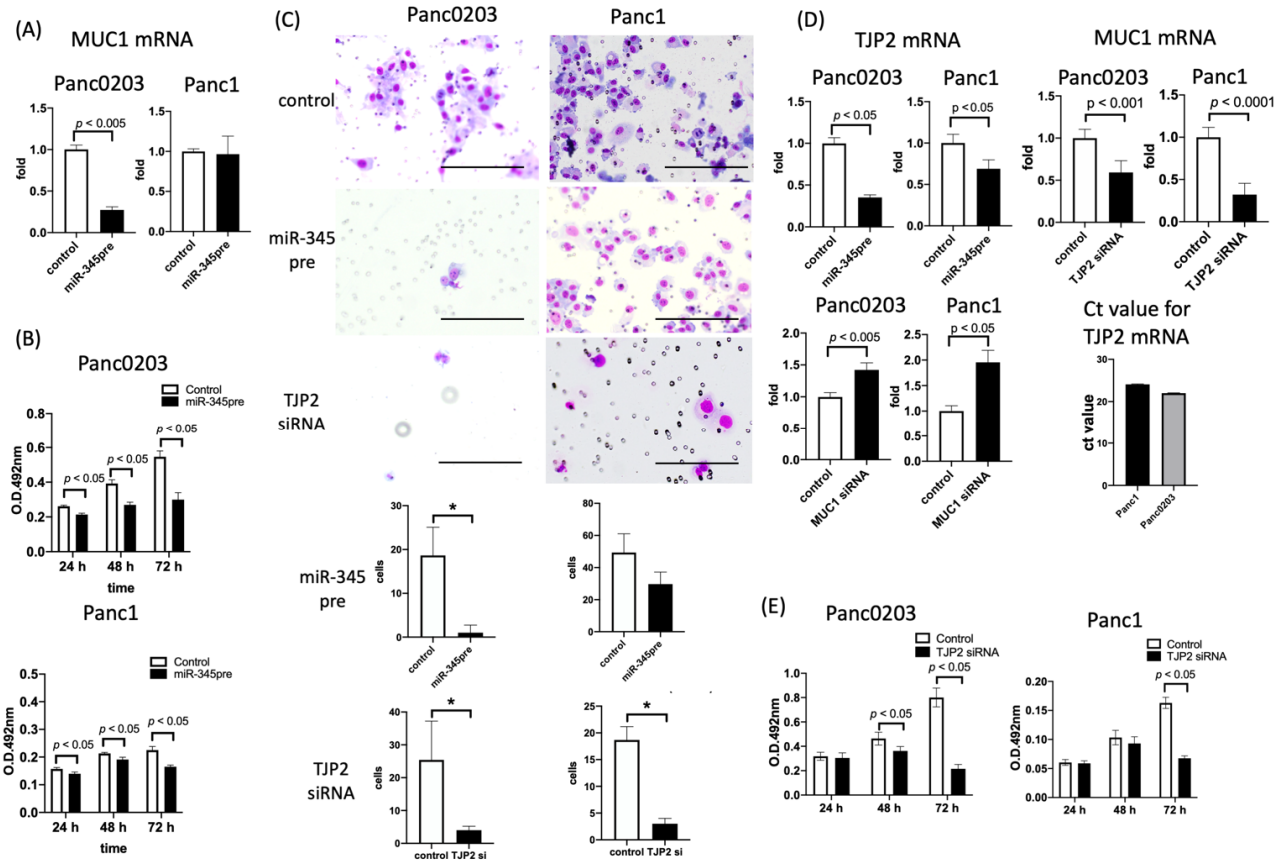

Figure 3. Changes in MUC1 and TJP2 expression, cell proliferation and invasion under miR-345pre or TJP2 or MUCI siRNA transfection. (**A**) Dynamics of MUC1 mRNA expression in Panc0203 and Panc1 under miR-345pre transduction. (**B**) The MTS assay under transfection with miR-345pre at 24, 48, and 72 h in Panc0203 and Panc1 cells. (**C**) Matrigel invasion assay showing the promotion of invasive capability of Panc0203 and Panc1 cells transfected with miR-345 and TJP2 siRNA. * $p < 0.05$. (×100; scale bar; 250 μm) (**D**) (**Left**) Dynamics of TJP2 mRNA expression in Panc0203 and Panc1 under miR-345pre or MUC1 siRNA transduction. (**Right/upper**) Dynamics of MUC1 expression under TJP2 suppression. (**Right/lower**) Evaluation of Ct value of TJP2 mRNA by quantitative RT-PCR method. (**E**) The MTS assay under transfection with TJP2 siRNA at 24, 48, and 72 h in Panc0203 and Panc1 cells.

Overexpression of miR-345 suppressed cell proliferation and invasive capacity in both Panc0203 and Panc1 cells (Figure 3B,C). Similar to that observed by the suppression of MUC1, induction of early apoptosis and production of ROS was observed after miR-345 was overexpressed, in both Panc0203 and Panc1 cells (Figure 4A,B). These results suggest that miR-345-mediated suppression of MUC1 expression contributes to the suppression of cell proliferation and invasion.

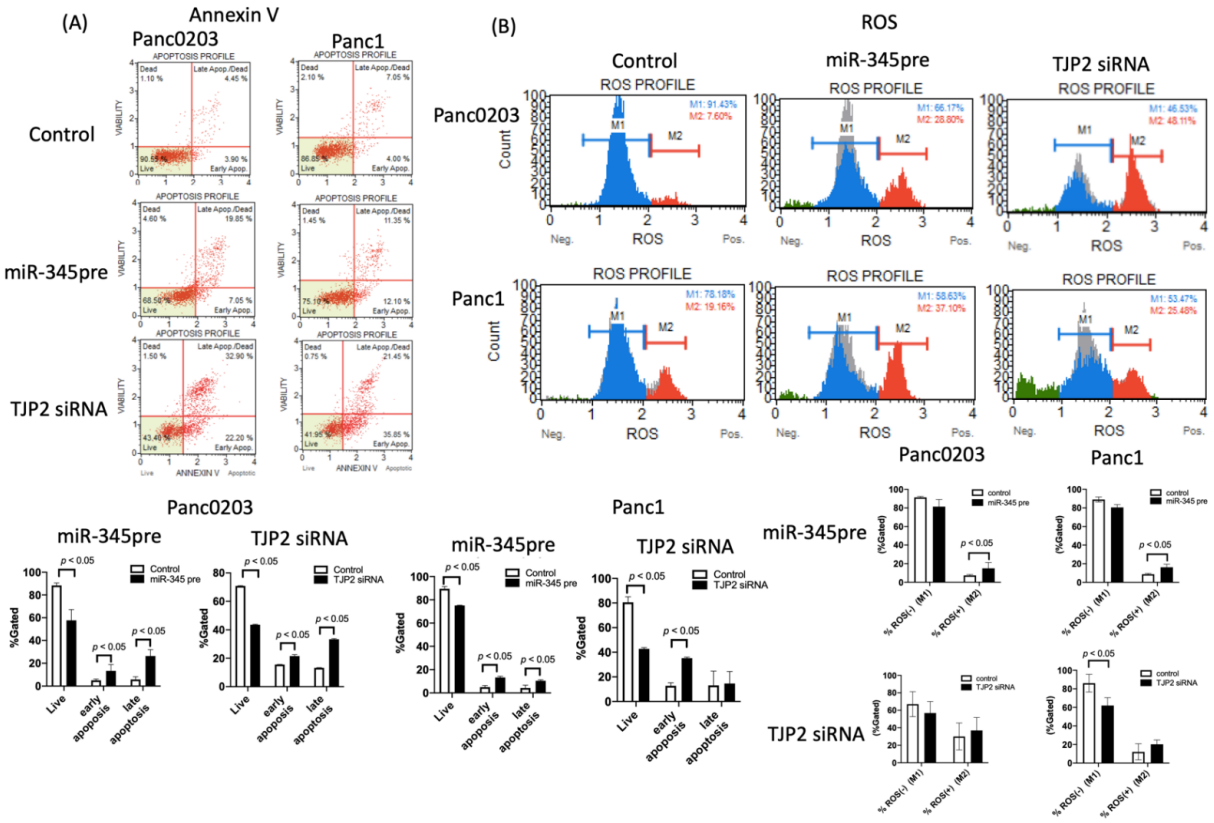

Figure 4. Function analysis under miR-345 overexpression or TJP2 suppression in Panc0203 and Panc1 cells. (**A**) Annexin V analysis for the detection of apoptosis in Panc0203 and Panc1 cells under transfected with miR-345pre and TJP2 siRNA. (**B**) Dynamics of reactive oxygen species in Panc0203 and Panc1 under transfected with miR-345pre and TJP2 siRNA. Blue peaks indicate ROS negative (M1 peak) and red peaks indicate ROS positive (M2 peak). Gray shadows indicate negative controls.

3.4. TJP2 Is Responsible for Cell Proliferation and Invasive Potential of PDAC Cell Lines

We screened several putative targets of miR-345 using in silico TargetScan analysis (release 7.1, June 2016), and identified SAT1, TJP2, CCL8, HABP4, and KISS1 as possible targets of miR-345. SAT1, TJP2, and HABP4 expressions significantly decreased after miR-345 overexpression in Panc0203 and Panc1 cells (Figure S3); Only TJP2 mRNA expression was significantly increased after MUC1 suppression, indicating that MUC1 negatively regulates TJP2 (Figures 3D and S3). The mRNA expression level of TJP2 was similar to that of MUC1, and the Ct value by quantitative RT-PCR method was as low as 20–25. mRNA expression level was used to evaluate the function of TJP2 (Figure 3D). MUC1 mRNA expression significantly decreased by TJP2 suppression (Figure 3D). These reactions indicate that MUC1 is linked TJP2, but MUC1 negatively regulates TJP2. Several putative binding sites for miR-345 were found in the TJP2 gene (Figure S2). Suppression of cell proliferation and invasive capacity was observed in PDAC cell lines after suppressing TJP2 expression (Figure 3C,E). Furthermore, suppression of TJP2 expression promoted early apoptosis, and the production of ROS increased, but not significantly (Figure 4A,B). Therefore, TJP2, a putative target of miR-345, partially contributes to cell proliferation and invasion in PDAC cells.

4. Discussion

Although it is well known that MUC1 is highly expressed in invasive pancreatic ductal carcinoma, its role in the progression of the disease is unknown. This study revealed that MUC1 is a key molecule for cell proliferation and invasion in PDACs, and that TJP2, a target of MUC1 and miR-345, contributes to PDAC cell proliferation. Additionally, miR-345 can suppress MUC1 and TJP2 expression to promote apoptosis and increase ROS in the cells.

MUCs are high molecular weight glycoproteins that play an important role in carcinogenesis and tumor invasion. Some MUCs—MUC1, MUC2, MUC4, and MUC5AC—are thought to be useful for the early detection and malignant evaluation of pancreas bile duct neoplasms [3,5–7,16,17]. Epigenetic regulation, such as DNA methylation and histone modification mechanisms, is also considered an important mechanism in carcinogenesis [11]. This study found that MUC1 is a key molecule for cell proliferation and invasion of PDAC by regulating apoptosis and ROS production. The significance of MUC1 in PDAC has been reported that its increased expression in cancer is an indicator of malignancy, and that it is involved in cell proliferation, angiogenesis, changes in life expectancy due to methylation, and enhanced efficacy of drugs, making it valuable as a diagnostic marker and therapeutic target [11,12,18–20]. However, no treatment has been established that directly targets MUC1. The reason is that MUC1 is likely to be important for the maintenance of normal cellular functions. Therefore, miRNAs and their target molecules that regulate the expression of MUC1 while avoiding direct targeting of MUC1 may be of value as alternative diagnostic markers and therapeutic targets for MUC1. We emphasize that finding miRNAs and their candidate targets involved in the expression of MUC1 may also contribute to the diagnosis and treatment of PDAC from this point of view.

In various cancers, miRNAs play important roles in functions related to cancer progression, such as cell proliferation, invasion, epithelial-mesenchymal transition, metabolism, immune response, and metastasis. Several miRNAs have been reported to be involved in molecular biological functions in PDAC cells. The expression of miRNAs in PDAC cells was comprehensively analyzed by array analysis, and the involvement of a significant number of miRNAs was presumed [21–27]. In particular, miR-100/miR-125b is induced by TGF-β and produces various physiological effects [28]. Several miRNAs have been implicated in lymph node metastasis [26]. MiR-34a targets TP53 and functions in a suppressive manner against PDA [29]. Array analyses revealed that miR-196a-2 may be a poor prognostic factor for PDA and miR-192 is involved in cancer growth [21,27]. It has been shown that miR-454 acts on SDF-1 and is involved in PDA proliferation [23]. MiR-7-5p, let-7d, and miR-135b were revealed to inhibit cancer stem cell growth [30]. MiR-141 was shown to inhibit PDA proliferation and colony formation by targeting YAP-1 [31]. In this study, Panc0203 and Panc1, human PDA cell lines, were used to examine the expression of cancer-related miRNAs that have been previously studied [32–37]. We examined miR-345, which has relatively low expression levels and partial gene sequence complementarity with MUC1 (Figure S2). miR-345 has been reported to suppress cell function in various cancers [38–41]. In our previous experiments, we found that miR-345 expression was maintained at low levels in tumor cell lines, such as bladder and prostate cancers (not published). This study found that miR-345 was also expressed at low levels in PDA cell lines, and its overexpression suppressed cell proliferation and invasive ability.

TJP2 has been reported to be involved in congenital or juvenile hepatobiliary carcinogenesis due to a defect in the protein caused by a genetic abnormality [42,43]. In the present study, TJP2 was suppressed by miR-345, while suppression of TJP2 expression also reduced MUC1 expression. This suggests that TJP2 is a target molecule of miR-345 and also regulates the expression of MUC1 to suppress tumor proliferation and invasion, suggesting that TJP2 may provide functions different from those of carcinogenesis caused by genetic abnormalities.

The function at the gene level in cancer cells has been reported in various cancers, and bioinformatics analysis has shown that DNA methylation of cancer-related genes can predict life expectancy due to differences in tumor growth potential and drug

sensitivity [44–46]. In addition to MUC1, which plays an important role in PDAC, miR-345 and its target molecule TJP2 were picked up by this study. They were shown to function similarly to MUC1 and to be involved in PDAC proliferation and invasive capacity, suggesting that both MUC1 and TJP2 are involved in PDAC progression. We plan to validate the expression of these two molecules using circulating tumor cells and tissues and perform bioinformatics analysis on tissue differentiation, tumor behavior, prediction of recurrent metastasis, and estimation of prognosis in the near future.

In this study, we found a new insight into the role of MUC1, as the increased expression of MUC1 in PDAC can be confirmed histopathologically by immunohistochemical staining, and we hope that this important molecule will lead to the development of molecular diagnostic markers and molecular targeted therapy for PDAC. We expect that this important molecule will lead to the development of molecular diagnostic markers for PDAC and molecular targeted therapy. On the other hand, the expression of MUC1 was not sensitive enough to be visualized at the protein level in PDAC cell lines, so we only examined it at the mRNA expression level, and based on this study for TJP2, we will carefully examine methods to detect it at the mRNA or protein level that are close to the expression level of MUC1 and have high sensitivity. We would like to investigate the possibility of detecting circulating tumor cells or using TJP2 as a serum tumor marker using clinical specimens.

5. Conclusions

In conclusion, miR-345 was found as a miRNA involved in MUC1 which is upregulated in invasive pancreatic cancer. In vitro functional analysis using PDAC cell lines showed that miR-345 targets MUC1 and TJP2, and that TJP2 regulates cell proliferation and invasiveness of PDA by also modulating MUC1 expression (Figure 5). The in vitro results obtained in this study may provide an opportunity to identify the utility of miR-345 and its targets as promising biomarkers and therapeutic targets for the prevention of progression to invasive cancer.

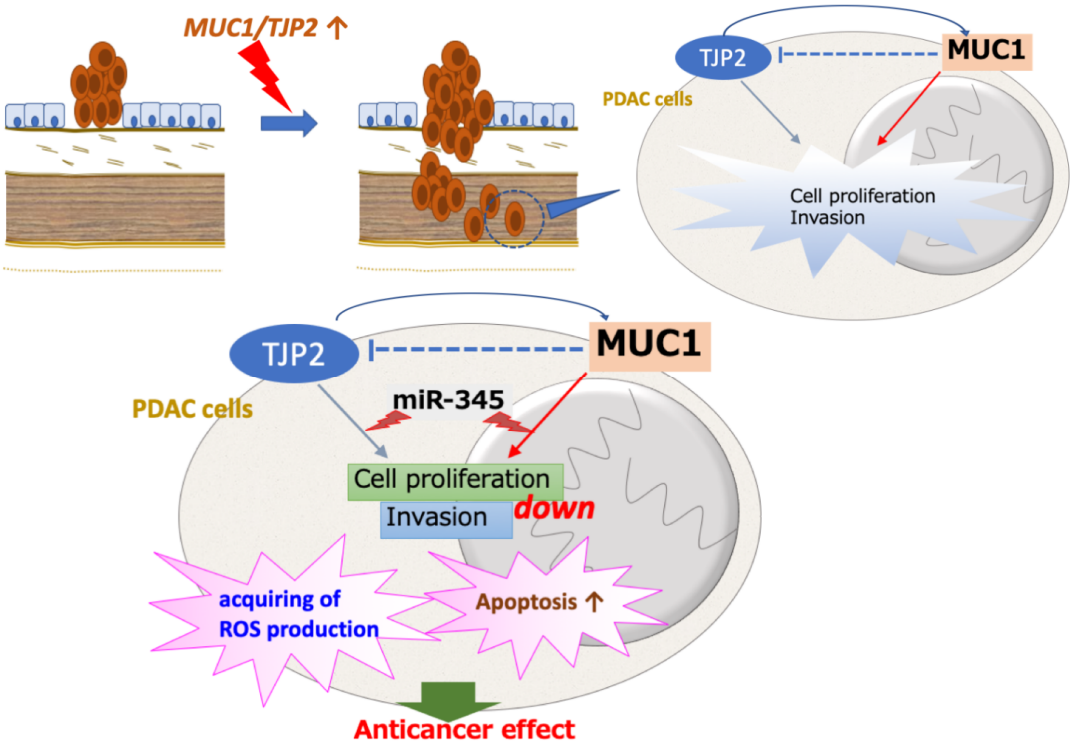

Figure 5. Function of MUC1 and TJP2 in PDAC (Panc0203 and Panc1 cells). MUC1 and TJP2 are putative target molecules of miR-345. miR-345 regulates cell proliferative and invasive potential and ROS production in PDAC.

6. Patents

No patents are available.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/app12115351/s1, Figure S1: Disease type and character of PDAC cell lines. The upper panel shows the origin and character descriptions of PL45, Panc0203, and Panc1. The lower panel shows genetic mutations in PL45, Panc0203, and Panc1. Figure S2: Putative binding site of miR-345 on MUC1 and TJP2 mRNA. The left panel shows the putative miR-345 binding site on MUC1. The right panel shows the putative miR-345 binding site on TJP2. Figure S3: In silico evaluation of mRNA expression of five putative target molecules of miR-345. MiR-345pre or MUC1 siRNA was transfected into Panc0203 and Panc1, and the expression changes of SAT1, TJP2, CCL8, HABP4, and KISS1 were evaluated. Table S1: Immunohistochemical analysis of the prostatic tissue. Table S2: Primer sequences.

Author Contributions: Conceptualization, T.F., M.T., M.S. and C.O.; data curation, S.T. and T.U.; formal analysis, S.T., Y.S.-H., K.M. and H.I.; funding acquisition, T.F., M.T.; investigation, T.F., S.T., M.T., A.S., Y.S.-H., K.M., T.U., H.I. and M.T.; methodology, S.T., A.S., Y.S.-H., K.M., T.U., H.I. and M.T.; project administration, T.F., M.T., M.Y. and C.O.; supervision, T.F., M.T., M.S. and C.O.; validation, T.F., S.T., M.T., M.Y., M.S. and C.O.; visualization, T.F., M.T. and M.Y.; writing—original draft, T.F., S.T. and M.T.; writing—review and editing, T.F., M.T., T.U., M.T., M.Y., M.S. and C.O. All authors have read and agreed to the published version of the manuscript.

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Institutional Review Board Statement: I The study was conducted according to the guidelines of the Declaration of Helsinki, and approved by the Institutional Review Board of Nara Medical University (#253-6) and the ethics committee of Nara Medical University (IRB3041). Informed consent was obtained from all patients.

Informed Consent Statement: Patient consent was waived by disclosing the opt-out statement on our institution's website and on the bulletin board of the institution's affiliated hospital.

Data Availability Statement: The data that support the findings of this study are available from the corresponding author upon reasonable request.

Conflicts of Interest: The authors declare no conflict of interest.

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