Regulatory mechanism on expression of stem cell markers in parotid acinar cells after tissue injuries

(組織傷害後の耳下腺腺房細胞における幹細胞マーカーの発現調節機構)

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Abstract

Since saliva is essential for maintenance of healthy oral environment, secretory function of salivary gland is important for clinical dentistry. Although tissue injuries result in decrease in acinar cells and consequent dysfunction of saliva secretion, the number of acinar cells is recovered if the damages are not too severe. The origin of the regenerated acinar cells is unclear. One hypothesis is that acinar cells that are atrophied in response to tissue injuries can redifferentiate. We have previously established a primary culture of parotid acinar cells to study the mechanism of dysfunction and regeneration of salivary glands. During the culture, the expression levels of acinar markers decreased whereas immature ductal markers increased, suggesting that the cells changed to immature duct-like cells.

In this study, to clarify the properties of the cultured cells, we examined the expression of nestin, a stem cell marker of pancreatic exocrine cells. We found that nestin began to be expressed and increased during the culture. Immunofluorescence microscopy revealed that nestin-expressed cells had secretory granules, indicating that the cells were derived from acinar cells. The Src family kinase inhibitor PP1 suppressed the expression of nestin. There is a possibility that nestin expression is a programmed response to survive cellular stresses and to acquire redifferentiation potential.

To study the mechanism for change in characteristics of parotid acinar cells, we examined changes in expression pattern of micro RNAs (miRNAs) in parotid acinar cells by microarray analysis. MiRNAs are considered to regulate the development and dysfunction of salivary glands. Analysis on expression values after normalized with robust multi-array averaging method identified that 18 miRNAs were up-regulated and 52 miRNAs were down-regulated after 1-day culture compared with just after isolation. Pathway analysis on the

expression-altered miRNAs suggested that they were involved in the regulation of multipotent stem cells. Among the down-regulated miRNAs that were highly expressed, miR-3473, whose fold change was the largest, significantly decreased during the culture for 2 days. Inversely, Sox10, a candidate of target genes for miR-3473, increased during the culture, which was confirmed by real time RT-PCR and immunoblot analyses. Inhibition of Src kinase activity suppressed the changes in expression levels of miR-3473 and Sox10. Since Sox10 is involved in maintenance of stemness and promotes nestin expression, there is a possibility that the miRNAs regulate acquisition of stemness in parotid acinar cells.

Introduction

Irradiation treatment for head and neck cancer frequently causes dysfunction of salivary glands and consequent reduction of quality of life after the amelioration. Decrease in saliva makes patients easy to suffer serious dental caries, periodontitis and mucus infections and also causes difficulties in mastication and swallowing (1). Thus, it is important in clinical dentistry to clarify the mechanism to protect and recover the salivary gland function against tissue injuries such as γ-irradiation.

In irradiated salivary glands, the number of acinar cells decreases and ducts become prominent, which leads to hyposecretion of saliva. If the damage is not too severe, acinar cells increase again and their function to generate saliva is recovered after the irradiation (2). The origin of regenerated acinar cells is still under debate. The presence of adult stem cells that reside in intercalated ducts has been proposed (3). The stem cells are activated and increase in response to tissue injuries and differentiate both into acinar and duct cells. In contrast, acinar cells was reported to have a capacity of self duplication (4). Tissue injuries changed the morphology and gene expression patterns of acinar cells into duct-like cells and the cells redifferentiate to acinar cells after the injuries (5, 6).

We have established a system of primary culture of parotid acinar cells in order to study the mechanism of salivary gland dysfunction induced by tissue injuries (7). Although the cultured acinar cells have maintained capacities of granule generation and stimulus-dependent exocytosis at least for three days, the functions declined gradually. The expression of acinar cells such as aquaporin-5 and amylase decreased and ductal markers such as claudin-4 began to be expressed (8). These changes were induced by the process of cell isolation, which triggers cellular stress signals. During the cell isolation process, activation of

Src family kinases (SFK) and p38 MAP kinase (MAPK) occurred, which is essential for the changes of gene expression pattern (9, 10). Therefore, these changes are considered as a programmed response to tissue injuries. The cultured acinar cells also expressed claudin-6, which is transiently expressed during salivary gland development, and cytokeratin-14, which is expressed in the basal cells of excretory ducts, suggesting that the character of the cultured cells is similar to that of undifferentiated duct cells (9). There is a possibility that the changes of acinar cells induced by tissue injuries imply not just dysfunction, but the acquisition of redifferentiation potential.

Research 1: Expression of the stem cell marker nestin in response to tissue injuries of parotid acinar cells

Introduction

In the first study, as a candidate of stem cell marker of parotid acinar cells, we focused nestin. Nestin was first identified as a stem cell marker of neuron (11) and was also reported to be expressed in other developing organs (12, 13). Pancreatic exocrine cells, which have a similarity to parotid acinar cells, are differentiated from nestin-positive precursor cells (14). To investigate the characters of the cultured cells, we examined the expression of nestin in the primary culture.

Materials and methods

Primary culture of rat parotid acinar cells

Parotid glands were taken from male Sprague-Dawley rats (150 - 200 g each) under anesthesia. Acinar cells were isolated and were cultured as described previously (7). Isolated acinar cells were cultured in the absence and presence of SFK inhibitor PP1 (Calbiochem, La Jolla, CA, USA; final concentration of 10 μM). Media were changed at 1 day and at 4 days after the cell isolation and PP1 was added to the fresh medium.

RNA preparation and real time RT-PCR analysis of mRNA expression

Total RNA was isolated from the parotid glands, cells immediately after isolation or culture for 1-7 days, using the TRIzol reagent (Invitrogen, Carlsbad, CA) and RNeasy Mini kits

(Qiagen, Hilden, Germany). Amounts of RNA were quantified by measuring the absorbance at 260 nm. Expression level of nestin mRNA was determined with the QuantiTect® SYBR® RT-PCR kit (Qiagen) using a thermal cycler (Thermal Cycler Dice, Takara Bio, Kusatsu, Japan). Amplification of rat GAPDH was performed as according to a previous study (8). Primer pair for the amplification of rat nestin was as follows: forward 5'-GCT ACA TAC AGG ACT CTG CTG-3', reverse 5'-GAG CAC AGA TCC CAG GTA CT-3' with a predicted size of 147 bp. PCR products were evaluated by melting curve analysis and by examining the sizes of the PCR products separated on 2.0% agarose gels. Relative RNA expression level for each sample was obtained by normalizing to GAPDH.

Immunoblotting analysis

Glands and cultured cells were lysed with 20 mM HEPES (pH7.4) containing 0.1% Triton X-100 and 1 × Complete Protease Inhibitor Cocktail (Roche Diagnostics, Basel, Switzerland). Proteins were separated by SDS-PAGE and were transferred to Hybond-LFP membranes (GE Healthcare, Buckinghamshire, UK). Each membrane was blocked in ECL Prime Blocking Reagent (GE Healthcare), and was probed with mouse monoclonal anti-nestin antibody (Cell Signaling). Immunoreactivity was determined by using ECL-Plex (GE Healthcare) and images were acquired using an Image analyzer Typhoon Trio (GE Healthcare). Intensities of immunoreactivities were quantified using ImageQuantTL software (GE Healthcare).

Immunofluorescence microscopy of cultured acinar cells

Isolated acinar cells were cultured in collagen I-coated glass-base dishes (AGC Techno Glass, Haibara, Japan). Cells were fixed with 10% formalin in phosphate-buffered saline (PBS) and

were permeabilized with 0.2% Triton X-100/PBS. After blocking with 1% bovine serum albumin and 0.05% preimmuned goat IgG in PBS, the cells were labeled with mouse monoclonal anti-nestin and rabbit polyclonal anti-amylase (Sigma-Aldrich, St. Louis, MO, USA) antibodies, followed by Alexa Fluor 568-conjugated anti-mouse IgG and Alexa Fluor 647-conjugated anti-rabbit IgG antibodies. Images were acquired using LSM-510 confocal microscope.

Statistical analysis

Statistical testing was performed using paired *t*-test. Values are shown as mean ± SEM. $P < 0.05$ were considered to indicate significance.

Results

Expression of stem cell marker nestin in primary culture of parotid acinar cells

Real time RT-PCR analysis revealed that the expression level of nestin mRNA in parotid acinar cells increased during the culture and reached the peak at 3 days. After that, its expression slightly decreased (Fig. 1.1). The nestin protein was faintly detected in the homogenates of parotid glands, but not in the isolated acinar cells. Nestin was detected in the cell lysates at 1 day and its amount increased over the culture (Fig. 1.2).

Because the parotid gland consists of several types of cells such as acinar, duct, myoepithelial and mesenchymal cells, there is a possibility that nestin is expressed in other than acinar cells in the culture. Although we confirmed that more than 90% cells are acinar

cells after the cell isolation, some cells other than acinar cells were contaminated in the culture (9). To assign the nestin-positive cells in the culture, we performed immunofluorescence microscopy by using anti-nestin and anti-amylase antibodies. Amylase is a good marker of acinar cells and its antibody labels secretory granules. At 3 days after the cell isolation, secretory granules were observed in the cultured cells, indicating that the cells were derived from acinar cells. The same cells that had secretory granules were also labeled with anti-nestin antibody (Fig. 1.3). Nestin signals were observed as filament meshwork. Nestin is an intermediate filament and forms network structures in the cytosol. These results showed that nestin is expressed in acinar cells and increased during the culture.

SFK signaling is involved in expression of nestin

In the previous studies, we reported that tissue injuries triggered SFK-p38 MAPK signal via generation of reactive oxygen species (10). Inhibition of SFK and p38 MAPK suppressed the changes of gene expression pattern of cultured acinar cells (9). To clarify whether the same signaling pathway is involved in the nestin expression, we examined the effect of PP1, a SFK inhibitor. Addition of PP1 suppressed the increase of nestin, which was shown by both real time RT-PCR and immunoblotting analysis. The expression level of nestin mRNA in the cells cultured in the presence of PP1 was significantly less than in its absence (**$P < 0.01$; ***$P < 0.001$, paired *t*-test; Fig. 1.1). The nestin protein was also decreased by addition of PP1 (Fig. 1.2). These results indicate that the expression of nestin is mediated by activation of SFK.

Discussion

In this study, we found that nestin, which is considered as a stem cell marker of pancreas,

began to be expressed in the cultured parotid acinar cells. Its expression may be a programmed response to tissue injuries, which is mediated by SFK-p38 MAPK signaling pathway. It was reported that nestin-positive cells in pancreas can differentiate into not only exocrine but also endocrine cells, which indicates that the nestin-positive cells have a multipotency (15). It is likely that the changes of gene expression in the cultured parotid acinar cells mean not just dysfunction, but a process to revert to a primitive phenotype. There is a possibility that parotid acinar cells in the primary culture also maintain the potential for redifferentiation into acinar cells.

It was reported that nestin transiently increased in response to EGF, which causes pancreatic acinar-ductal metaplasia (16). Since metaplastic ductal epithelium is considered a premalignant lesion in pancreas, the expression of nestin is likely involved in tumorigenesis. Nestin was reported to be observed in human salivary gland tumor whereas it was rarely expressed in non-neoplastic glands (17). The possibility is raised that the dedifferentiated acinar cells induced by tissue injuries is a source of salivary gland tumors. Further studies are required to uncover the involvement of oncogenic transformation and alteration of acinar cells in salivary glands.

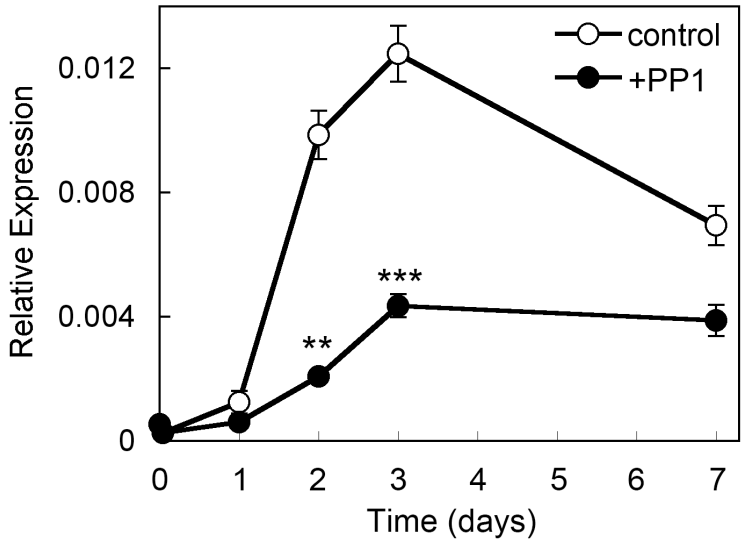

Fig. 1.1 Expression of nestin mRNA in primary culture of parotid acinar cells.

Acinar cells were isolated from rat parotid glands and were cultured up to 7 days in the absence or presence of 10 μM PP1. Total RNA was isolated at each time point for real time RT-PCR analysis. The expression levels of nestin mRNA were shown as relative values to that of GAPDH. The values in the homogenates of intact parotid glands and the cells just after isolation were plotted at 0 and 1 h, respectively. The expression levels of nestin mRNA in the PP1-treated culture for 2 (**$P < 0.01$) and 3 days (***$P < 0.001$) were significantly lower than those of the control culture.

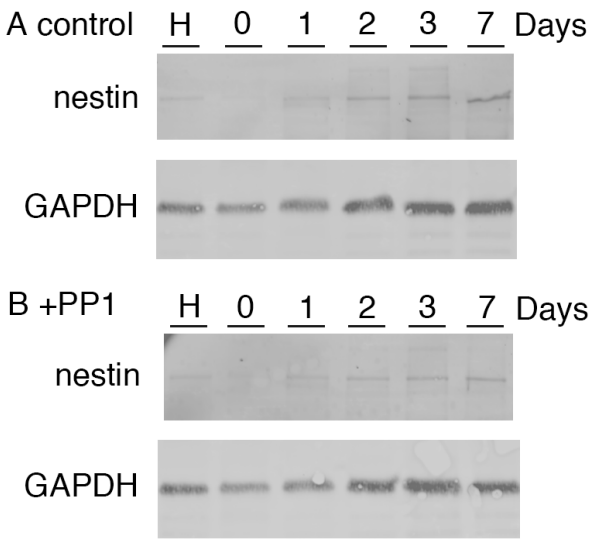

Fig. 1.2 Expression of the nestin protein in primary culture of parotid acinar cells

Homogenates of the parotid glands (H), cell lysates just after isolation (0) and after culture for 1-7 days in the absence (A) and presence (B) of PP1 were prepared. The same amounts of proteins were used for immunoblotting analysis with anti-nestin and anti-GAPDH antibodies.

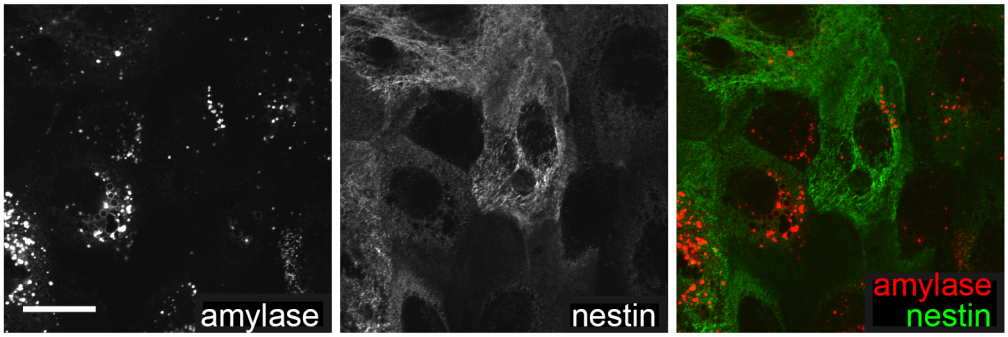

Fig. 1.3 Expression of nestin in acinar-derived cells in the culture

The cells were fixed after 3-days culture and were labeled with anti-amylase and ant-nestin antibodies. Nestin signals are observed in the cells that have secretory granules, which are expected to be derived from acinar cells. Bar, 20 μm.

Research 2: Change in expression patterns of micro RNAs in the primary culture of parotid acinar cells

Introduction

Micro RNAs（miRNAs）are small non-coding RNAs consisting of 20-25 nucleotides. They are not used as templates for protein synthesis and regulate the expression of other genes by degradation of mRNAs or suppression of translation. They are involved in the development, differentiation, cell proliferation and apoptosis. In the salivary glands, it was reported that miRNAs secreted from the mesenchyme reacted with the epithelium and modulated branching morphogenesis (18). In addition, different miRNAs are detected in the saliva from patients with a parotid gland neoplasm compared with the healthy control (19). Therefore, miRNAs may regulate development and function of salivary glands. In the second study, we examined changes in expression pattern of miRNAs in the primary culture of parotid acinar cells by microarray analysis.

Materials and Methods

Comparison of the expression patterns of the total miRNAs by microarray analysis

Total RNAs were purified from the cells just after isolation (Day 0) and after 1-day culture (Day 1) with miRNeasy mini kit (Qiagen, Hilden, Germany). We used each one sample of Day 0 and Day 1 to microarray analysis. The quality of RNAs was confirmed by Agilent 2100 (Agilent Technology, Palo Alto, CA, USA). The expression patterns of miRNAs were analyzed by GeneChip® miRNA 4.0 Array (Affymetrix, Santa Clara, CA, USA), and the

signal intensities were normalized with robust multi-array average (RMA) method by Affymetrix Expression Console Software (Affymetrix). The ratio of the normalized values of Day 1 to those of Day 0 was calculated, and expression-altered miRNAs were identified GeneSpring software (Agilent Technologies). The values of weighted average difference between Day 0 and Day 1 were calculated as previously reported (20). The threshold value of up-regulated or down-regulated miRNA was a fold change greater than 2.0. The analysis protocol was shown in Fig. 2.1.

Prediction of target genes for miRNAs

The expression-altered miRNAs were further filtered based on their expression values. After identification of altered miRNAs from the microarray data, the predicted targets for the miRNAs were identified using TargetScan 7.2 (http://www.targetscan.org), miRDB (http://mirdb.org), miRSearch 3.0 (https://www.exiqon.com/mirsearch) and DIANA-microT-CDS 5.0 (http://www.microrna.gr/microT-CDS) software tools. We selected genes that were commonly predicted by two or more prediction tools.

Pathway analysis

The Kyoto encyclopedia of genes and genomes (KEGG) database provides searchable pathways for molecular interactions and cellular processes. DIANA-mirPath 3.0 (http://snf-515788.vm.okeanos.grnet.gr) was used to search KEGG pathways related to the set of expression-altered miRNAs. The set of target gene candidates for the chosen miRNAs was analyzed by DAVID functional annotation tool 6.8 (https://david.ncifcrf.gov).

Real time RT-PCR of miRNA and mRNA

The expression level of miR-3473 was determined by miRCURY LNA micro RNA PCR kit (Takara). U6 snRNA was used as a reference. The expression level of Sox10 was determined with One Step TB Green PrimeScript PLUS kit (Takara Bio, Kusatsu, Japan). Rps18 was used as a reference. The sequence of primer pair for Sox10 was forward: 5'-TCA CTA CAA GAG TGC CCA C-3' and reverse: 5'-CAC ATT GCC GAA GTC GAT G-3'. The predicted size of PCR fragment was 215 bp. The primer pair for Rps18 was purchased from Takara Bio.

Antibodies and immunoblot analysis

Rabbit monoclonal anti-Sox10 antibody was purchased from Abcam (Cambridge, UK). Cells were lysed with 20 mM HEPES (pH7.4) containing 0.1% Triton X-100 and 1 × Complete Protease Inhibitor Cocktail (Roche, Basel, Switzerland). The proteins were separated by SDS-PAGE and transferred to Hybond-LFP membranes (GE healthcare, Chicago, IL, USA). The membranes were blocked at room temperature for 1 h in Blocking reagent (GE Healthcare), and probed with anti-Sox10 antibody. Immunoreactivity was determined using ECL-Plex (GE Healthcare) and images were acquired using Typhoon Trio (GE Healthcare). Intensities of immunoreactivities are quantified using ImageQuantTL software (GE Healthcare).

Statistical analysis

Values are shown as mean ± SEM. Statistical testing was performed using ANOVA and Bonferroni's test. $P < 0.05$ were considered to indicate significance.

Results

Expression profiles of miRNAs in parotid acinar cells

Among the 728 mature miRNAs on the array, 377 miRNAs were absent both just after isolation (Day 0) and after 1-days culture (Day 1), and were excluded from further analysis. The expression levels of 18 mature miRNAs were up-regulated and 52 mature miRNAs were down-regulated from Day 0 to Day 1 (Table 1). To analyze the role of these miRNAs, KEGG pathway enrichment analysis was performed. Candidates of target genes were predicted by microT-CDS 5.0 and pathways were searched by mirPath 3.0. We excluded two miRNAs from the analysis because the two were not assigned by miRBase (ver. 21). From 68 miRNAs, 43 pathways were identified (Table 2).

We have already performed the comprehensive analysis on the expression patterns of mRNAs of parotid acinar cells in the primary culture (21). Thus, we compared the expression patterns of miRNAs and mRNAs. Since the expression levels of miRNAs were various, we selected seven miRNAs (miR-375-3p, miR-3473, miR-429, miR-30b-5p, miR-15b-5p, let-7f-5p and miR-25-3p) whose normalized expression values were more than 1000 at Day 0 from the 52 down-regulated miRNAs. As target genes for the seven miRNAs, 1088 genes were predicted. Among them, we selected 202 genes whose mRNAs were up-regulated more than two-fold after 1- or 2-day culture. From the 202 genes, 21 pathways were identified (Table 3). Among them, 13 pathways were common to the result of direct KEGG pathway analysis from expression-altered miRNA (compare Tables 2 and 3). The down-regulation of miRNAs in the culture may be involved in the 13 pathways.

Change in the expression level of rno-miR-3473 in parotid acinar cells during the culture

Among the seven down-regulated miRNAs that were highly expressed, the fold change of miR-3473 was the largest (Table 1 and Fig. 2.2). We also calculated values of weighted average difference in order to rank differentially expressed miRNAs (20). The weighted average difference of miR-3473 was the largest among all rat miRNAs in the array. We confirmed the change in expression of miR-3473 by real time RT-PCR. The expression level of miR-3473 was significantly decreased during the culture (Fig. 2.3). In contrast, in the presence of a Src kinase inhibitor PP1, its expression was gradually declined but the difference was not significant.

Change in the expression level of Sox10 mRNA in parotid acinar cells during the culture

We searched target gene candidates for miR-3473 by TargetScan 7.2. Among them, we focused on Sox10. Sox10 is known as a transcription factor that regulates stem/progenitor cell states in mammary epithelial cells. In Research 1, we found that stem/progenitor cell marker nestin began to be expressed and increased during the culture. The expression of nestin has been reported to be regulated by Sox10 (22, 23). Thus, we examined the expression of Sox10 in parotid acinar cells by real time RT-PCR. As a result, the expression level of Sox10 mRNA was increased and was sustained for 3 days (Fig. 2.4A). Although Sox10 in the PP1-treated cells was increased at Day 1 as well as in the control, it decreased later. The expression level of Sox10 mRNA after 3-day culture with PP1 became almost the same level as before culture. The Sox10 protein in the control cells also increased during the culture and addition of PP1 suppressed its expression (Fig. 2.4B).

Discussion

First, we performed KEGG pathway analysis on 68 expression-altered miRNAs. Next, we performed pathway analysis on the genes extracted by combination of miRNA and mRNA expression array analyses. As a result, the pathways that are involved in the stemness were commonly identified (map04550: Signaling pathways regulating pluripotency of stem cells). In addition, signaling of Wnt, Hippo and PI3K-Akt, which have been reported to regulate maintenance of stemness (24-28), were identified. Pathways related to cancers were also found by the analysis. Cancer cells frequently have properties of stem cells and the stemness sustains cancer progression. These results indicate that the expression levels of stemness-related genes and miRNAs changed in the primary culture of parotid acinar cells. In the previous reports, miR-375, miR-30b, miR-29c and miR-148b increased during the development of parotid glands and are considered to be involved in stemness (29). Thus, their decrease in the primary culture may be related to change to undifferentiated state and acquisition of stemness.

Nestin has been shown to be associated with stemness of several types of cell (30). We have previously reported that expression of nestin and cytokeratin-14, stem/progenitor markers, increased during the culture of parotid acinar cells (9, 31). In addition, ligation of parotid excretory duct induced expression of nestin and cytokeratin-5 (32, 33). After injury, various epithelial cells begin to express nestin, which may indicate a reversion to an immature phenotype. Pancreatic acinar cells express nestin by stimulation with growth factors and trans-differentiate to duct cells (16, 34). Salivary acinar cells and pancreatic exocrine cells

have been reported to have a capacity of self-renewal and trans-differentiation to duct-like cells (6, 9, 16, 34, 35). These studies suggest that even fully-differentiated exocrine cells maintain multipotency. The ability allows exocrine acinar cells to dedifferentiate under cellular stress and re-differentiate to acinar cells after that.

In this study, we searched target gene candidates for miR-3473, which has been rarely studied so far. Among candidate genes, Sox10 was increased during the culture in an inverse manner. Sox10 is a member of the SRY-related HMG box family that function as transcription factors. It regulates embryonic development and maintains multipotency of neural crest cells (36, 37). Since Sox10 promotes expression of nestin (22, 23), down-regulation of miR-3473 may be one of the mechanisms to induce nestin expression via increase of Sox10.

The most up-regulated genes identified by mRNA expression analysis included inflammation markers and cell-cell attachment molecules (9, 21). Because cells were isolated from the parotid glands by digestion with enzymes, the tissue organization was destructed. This is the reason that the expression of such proteins remarkably changed. In contrast, combination analysis with miRNA array revealed that the expression of genes related to stemness also changed. There is a possibility that changes in miRNA expression in the primary culture of parotid acinar cells is involved in acquisition of stemness.

Table 1 Microarray analysis of miRNAs whose expression was changed during the culture

Up-regulated	Fold Change	Down-regulated	Fold Change
rno-miR-6216	2.3	rno-miR-375-3p	0.38
rno-miR-17-5p	2.0	rno-miR-3473	0.30
rno-miR-146b-5p	3.4	rno-miR-429	0.48
rno-miR-31a-5p	3.1	rno-miR-30b-5p	0.47
rno-miR-1224	2.3	rno-miR-15b-5p	0.40
rno-miR-18a-5p	2.5	rno-let-7f-5p	0.44
rno-miR-21-5p	2.9	rno-miR-25-3p	0.44
rno-miR-92b-5p	2.7	rno-miR-352	0.46
rno-miR-874-3p	2.6	rno-miR-6215	0.15
rno-miR-503-5p	4.0	rno-miR-1949	0.33
rno-miR-31a-3p	3.6	rno-miR-29c-3p	0.48
rno-miR-326-5p	2.8	rno-miR-30e-3p	0.40
rno-miR-92a-1-5p	2.1	rno-miR-672-3p	0.41
rno-let-7b-3p	2.5	rno-miR-181c-5p	0.48
rno-miR-150-3p	2.1	rno-miR-26b-5p	0.43
rno-miR-207	2.1	rno-miR-148b-3p	0.20
rno-miR-327	2.5	rno-miR-29b-2-5p	0.32
rno-miR-365-5p	4.5	rno-miR-30b-3p	0.43
		rno-miR-7a-1-3p	0.33
		rno-miR-382-5p	0.42
		rno-miR-466b-5p	0.33
		rno-miR-664-1-5p	0.43
		rno-miR-344g	0.29
		rno-miR-19a-3p	0.45
		rno-miR-134-5p	0.48
		rno-miR-204-5p	0.38
		rno-miR-128-3p	0.41
		rno-miR-1843-5p	0.26
		rno-miR-674-3p	0.44
		rno-miR-487b-3p	0.28
		rno-miR-872-3p	0.50
		rno-miR-29b-3p	0.25
		rno-miR-350	0.42
		rno-miR-101b-3p	0.46
		rno-miR-24-1-5p	0.14
		rno-miR-34c-5p	0.48
		rno-miR-138-5p	0.47
		rno-miR-195-3p	0.40
		rno-miR-130b-3p	0.42
		rno-miR-326-3p	0.42
		rno-miR-3068-5p	0.44
		rno-miR-204-3p	0.39
		rno-miR-29c-5p	0.25
		rno-miR-362-3p	0.25
		rno-miR-329-3p	0.48
		rno-miR-322-3p	0.30
		rno-miR-297	0.22
		rno-miR-376b-3p	0.37
		rno-miR-493-3p	0.44
		rno-miR-338-5p	0.33
		rno-miR-221-5p	0.38
		rno-miR-299a-5p	0.46

The values of Fold change were shown as ratio (Day 1/Day 0) of expression values. Up-regulated miRNAs and down-regulated miRNAs were sorted in order of normalized expression values at Day 1 and Day 0, respectively.

Table 2 KEGG pathways that were identified from 68 expression-altered miRNAs

KEGG pathway	*p*-value	Number of genes	Number of miRNAs
Metabolism			
N-Glycan biosynthesis	0.002	17	25
Valine, leucine and isoleucine biosynthesis	0.031	2	3
Mucin type O-Glycan biosynthesis	0.004	11	15
2-Oxocarboxylic acid metabolism	0.050	7	9
Genetic Information Processing			
Protein processing in endoplasmic reticulum	< 0.001	62	46
Ubiquitin mediated proteolysis	0.002	53	54
Environmental Information Processing			
MAPK signaling pathway	< 0.001	100	63
Ras signaling pathway	< 0.001	78	54
Wnt signaling pathway	< 0.001	59	45
TNF signaling pathway	0.007	41	46
Hippo signaling pathway	0.011	56	45
Rap1 signaling pathway	0.013	70	56
FoxO signaling pathway	0.022	47	44
TGF-beta signaling pathway	0.031	33	34
PI3K-Akt signaling pathway	0.040	102	57
Cellular Process			
Gap junction	0.001	34	36
Signaling pathways regulating pluripotency of stem cells	0.001	51	43
Endocytosis	0.001	78	49
Focal adhesion	0.028	70	57
Organismal Systems			
Endocrine and other factor-regulated calcium reabsorption	< 0.001	19	30
Neurotrophin signaling pathway	< 0.001	51	43
Circadian rhythm	< 0.001	16	28
Synaptic vesicle cycle	0.001	25	35
Estrogen signaling pathway	0.001	36	39
Axon guidance	0.010	49	47
Adrenergic signaling in cardiomyocytes	0.011	50	50
Dorso-ventral axis formation	0.026	13	25
Melanogenesis	0.036	37	38
Oxytocin signaling pathway	0.049	53	53
Vasopressin-regulated water reabsorption	0.049	19	31
Human Diseases			
Prion diseases	< 0.001	12	22
MicroRNAs in cancer	< 0.001	66	46
Proteoglycans in cancer	< 0.001	83	52
Transcriptional misregulation in cancer	< 0.001	64	50
Pathways in cancer	< 0.001	130	54
Glioma	< 0.001	28	34
Renal cell carcinoma	< 0.001	29	37
Chronic myeloid leukemia	< 0.001	33	37
Hepatitis B	0.001	48	50
Prostate cancer	0.004	37	45
Cocaine addiction	0.007	19	30
Pancreatic cancer	0.036	26	33
Acute myeloid leukemia	0.044	22	32

Table 3 KEGG pathways that were identified from combination analysis of 7 down-regulated miRNAs and mRNA expression microarray

KEGG pathway	*p*-value	Number of genes
Metabolism		
Amino sugar and nucleotide sugar metabolism	0.027	4
Genetic Information Processing		
Ubiquitin mediated proteolysis	0.003	8
Environmental Information Processing		
PI3K-Akt signaling pathway	< 0.001	14
HIF-1 signaling pathway	< 0.001	8
Hippo signaling pathway	0.001	9
Wnt signaling pathway	0.011	7
VEGF signaling pathway	0.045	4
Cellular Process		
Oocyte meiosis	< 0.001	9
Signaling pathways regulating pluripotency of stem cells	0.041	6
Organismal Systems		
Melanogenesis	< 0.001	8
Estrogen signaling pathway	0.010	6
Neurotrophin signaling pathway	0.028	6
GnRH signaling pathway	0.036	5
Insulin signaling pathway	0.041	6
Human Diseases		
MicroRNAs in cancer	< 0.001	9
Renal cell carcinoma	0.002	6
Pathways in cancer	0.007	13
Choline metabolism in cancer	0.012	6
Viral carcinogenesis	0.040	8
Hepatitis B	0.040	6
HTLV-I infection	0.042	9

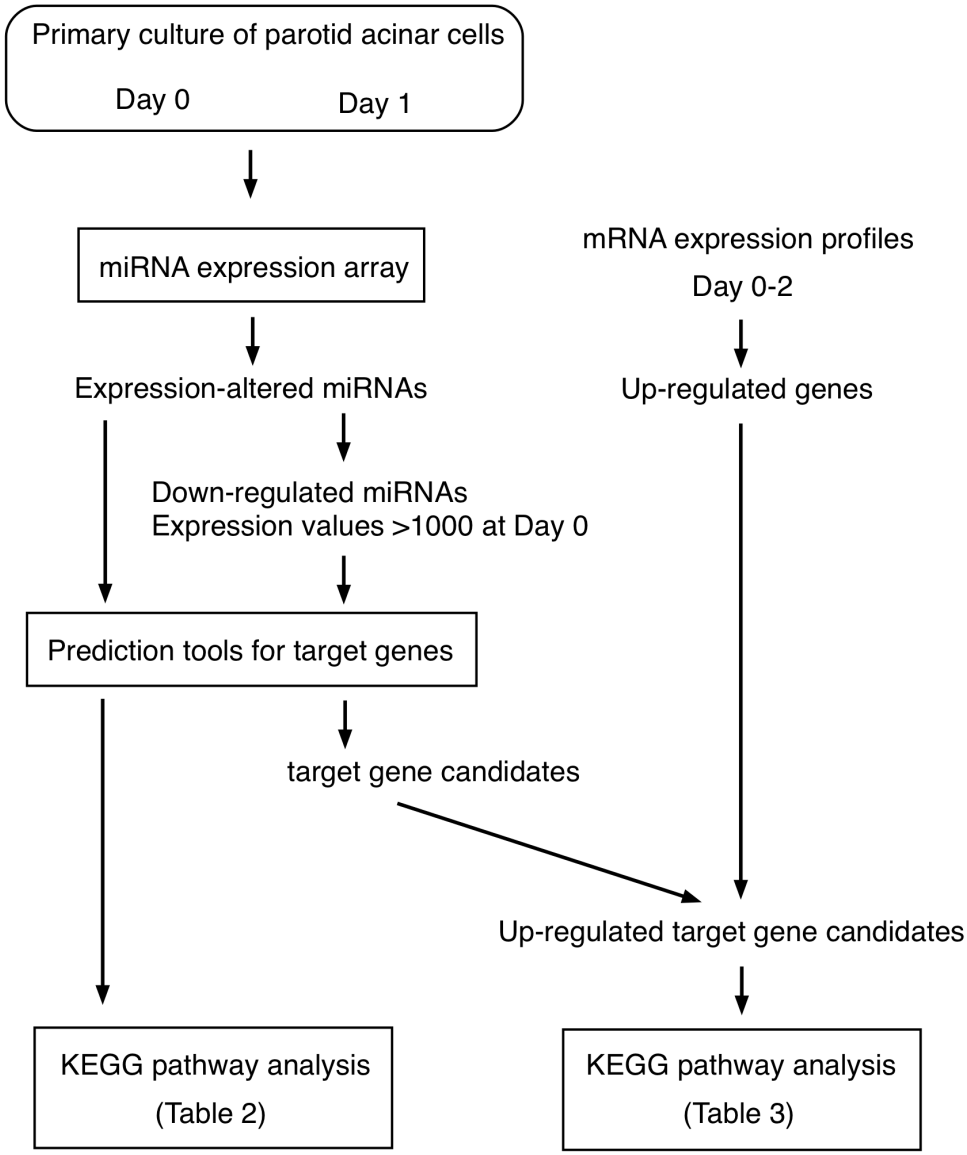

Fig. 2.1 Experimental procedure for miRNA expression analysis.

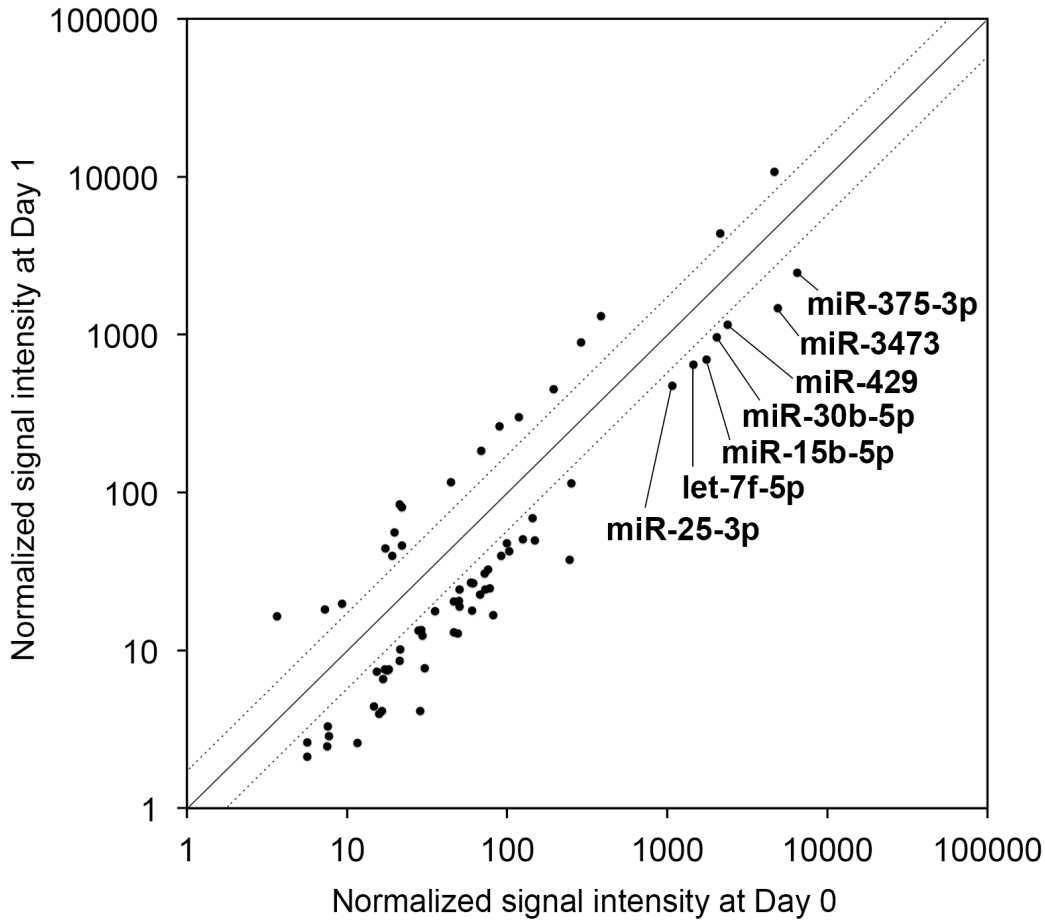

Fig. 2.2 Scatter plot of the miRNA microarray data.

The data from microarray are shown as a scatter plot to visualize differences in miRNA expression between Day 0 and Day 1. Both axes indicate expression values after normalization with RMA method (x-axis: Day 0; y-axis: Day 1). Only up-regulated and down-regulated miRNAs are shown. The dotted lines indicate 2-fold change. The expression of the miRNAs above the top line or below bottom line changed more than two-fold during the 1-day culture. The spot corresponding to expression values of rno-miR-3473 at Day 0 and Day 1 was indicated.

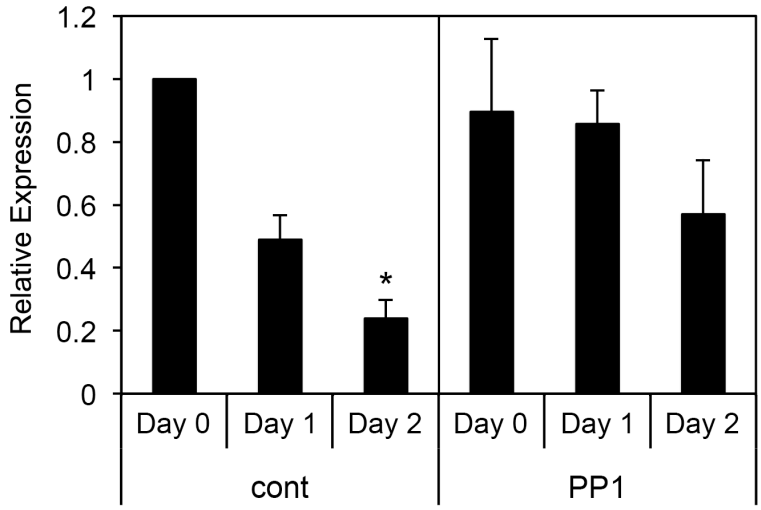

Fig. 2.3 Change in the expression level of miR-3473 during the culture.

The expression level of miR-3473 after 2-day culture (Day 2) was significantly decreased compared to that of Day 0 (*$P < 0.05$, ANOVA and Bonferroni's test). In the presence of PP1, its expression was also decreased but there was no significant difference between the expression levels of Day 0 and Day 1 or 2.

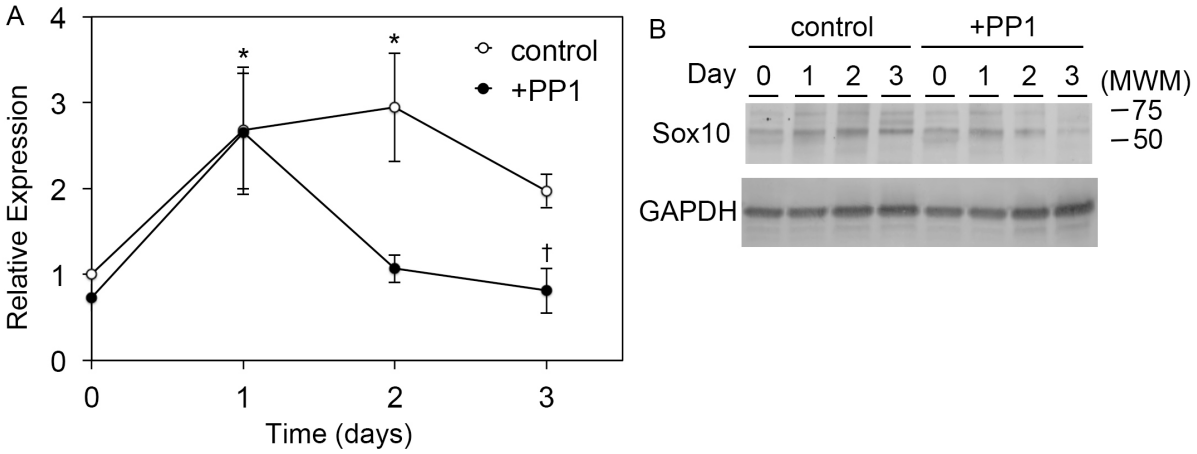

Fig. 2.4 Change in the expression level of Sox10 during the culture.

A. Change in expression level of Sox10 mRNA. The expression level of Sox10 at Day 1 and 2 was significantly higher than Day 0 (*$P < 0.05$, ANOVA and Bonferroni's test). Addition of PP1 suppressed its expression and the expression level of Sox10 after 3-day culture with PP1 was significantly lower than the control culture without PP1 (†$P < 0.05$, paired *t*-test). B. Immunoblot analysis of the Sox10 protein. Electrophoretic mobility of molecular weight markers (MWM) is shown in the left side of the panel.

Conclusion

It has been reported that tissue injuries induced increase in stem cell markers in salivary glands, which may be activation and proliferation of stem/progenitor cells. In this study, we found that the stem cell marker nestin was expressed in response to tissue injury in parotid acinar cells. We also showed that the changes in expression pattern of miRNAs, which may be involved in regulation of stem cells. One of the expression-altered miRNA, miR-3473 decreased, and reversely Sox10, a candidate of target genes of miR-3473, increased during the culture. Since Sox10 is a transcription factor to regulate nestin expression, the decrease in miR-3473 may be one of the causes of nestin expression in response to tissue injuries. There is a possibility that the change in expression patterns of genes and miRNAs induced by tissue injuries in parotid acinar cells is a programmed response for cell survival and acquisition of stemness to redifferentiate.

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