miR-375 Inhibits Proliferation of Mouse Pancreatic Progenitor Cells by Targeting YAP1

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Key Words
YAP1 • Pancreas • Progenitor cells • miR-375 • Proliferation

Abstract

Background/Aims: The Hippo signaling pathway regulates expansion and differentiation of stem cells and tissue progenitor cells during organ development and tissue regeneration. Previous studies have shown that YAP1, a potent effector of the Hippo signaling pathway, plays a crucial role in pancreas development, but the function of YAP1 in pancreatic progenitor cells is less known. Methods: The spatio-temporal expression pattern of YAP1 in mouse developing pancreata was detected by in situ hybridization. The effect of silencing YAP1 on the proliferation of pancreatic progenitor cells was analyzed by CCK-8 assay and Ki67 immunostaining. The regulation of miR-375 on YAP1 expression was determined by dual luciferase reporter assay, QRT-PCR and western blot. Finally, the influence of miR-375 on proliferation of pancreatic progenitor cells was analyzed by CCK-8 assay and Ki67 immunostaining. Results: We found that YAP1 was highly expressed in embryonic and adult pancreatic progenitor cells. Knocking down YAP1 by siRNA inhibited the proliferation of pancreatic progenitor cells. The mouse YAP1 was a target gene of miR-375, and miR-375 could target the 3’ UTR of YAP1 mRNA to decrease its protein and mRNA levels. Similar to silencing YAP1 by siRNA, the proliferation of pancreatic progenitor cells was inhibited significantly by miR-375. Conclusion: Our results indicate that YAP1 is necessary for the proliferation of pancreatic progenitor cells and miR-375 participates in regulating YAP1 expression during pancreatic progenitor cells differentiation.
Introduction

The development of organs and tissues is dependent on the balance of proliferation and differentiation of stem/progenitor cells. The proliferation and differentiation of tissue stem/progenitor cells are regulated by a complex process containing crosstalk and interplay of multiple signaling pathways [1-3]. By constitutively activating or inactivating the transcriptional co-activator Yki/YAP1 of the Hippo signaling pathway in Drosophila and mammals, researchers have uncovered a key function of Hippo signaling pathway in the regulation of the expansion and differentiation of stem cells and organ size [4-6]. For instance, YAP1 depletion in mouse embryonic stem cells (ESCs) results in loss of pluripotency, whereas overexpression of YAP1 prevents ESCs differentiation [7]. In mouse intestine, YAP1 expression is restricted to the stem/progenitor cell compartment. Transgenic expression of YAP1 expands intestine multipotent progenitor cells. In contrast, the deletion of YAP1 in mouse intestine results in a reduced number of intestinal progenitor cells, thinner intestinal epithelium and deficiency of the regeneration of intestine [8]. Similarly, forced expression of YAP1 thickens the epidermal basal layer that harbors the epidermal progenitor cells and inhibits the epidermal progenitor cells differentiation into other epidermal cell lineages in mouse skin [9]. Conditional knockout of YAP1 in mouse epidermis leads to decreasing proliferation of basal cells, thinner epidermis and failure of skin expansion [10].

In recent years, a few reports have shown the Hippo signaling pathway exerts a potent function during pancreas development. Pancreas consists of two types of gland tissue: endocrine islets and exocrine acini, which control glucose homeostasis and produce digestive enzymes, respectively [11]. During embryonic pancreas development, all the endocrine and exocrine cells are generated from a common pool of multipotential progenitor cells [12, 13]. It has been found that YAP1 is highly expressed in the multipotent progenitor cells of mouse embryonic 12.5 (E12.5) pancreas [14], and overexpression of YAP1 during the secondary transition of pancreas development blocks differentiation of the endocrine and exocrine compartments [15]. Moreover, in mice where YAP1 overexpression is induced by doxycycline, the pancreas increases in total size and has more acinar cells [8]. The above findings suggest that YAP1 may play a special role in proliferation and differentiation of pancreatic progenitor cells.

In adult mouse pancreas, the expression of YAP1 disappears in pancreatic islets but largely confines to the ductal network and terminal duct centroacinar cells [14, 15], which have long been assumed to harbor pancreatic progenitor cells [16, 17]. These findings reveal that YAP1 is repressed in pancreatic progenitor cells which differentiate towards endocrine or exocrine cells. However, less is known about regulating factors for mouse YAP1. It is reported that human YAP1 is a target of miR-375, overexpression of miR-375 inhibits YAP1 expression at post-transcriptional level in human liver cancer cells [18]. Furthermore, it has been found that miR-375 is expressed highly in pancreatic islets [19], indicating that miR-375 may participate in regulating YAP1 during pancreatic progenitor cells differentiation. Here, we analyzed the role of YAP1 in mouse pancreatic progenitor cells and explored the regulation of miR-375 on YAP1 in pancreatic progenitor cells differentiation.

Materials and Methods

Experimental animals

Outbred strain CD1 mice were obtained from Beijing Laboratory Animals Research Centre, and kept in compliance with the protocols established and approved by the Animal Care and Ethics Committee of Northeast Forestry University.

Cells Culture

3T3 cells were maintained in Dulbecco’s modified Eagle’s medium (DMEM) containing 25 mmol/L glucose supplemented with 10% heat-inactivated fetal bovine serum (FBS). The MIN6 insulinoma cells
were maintained in DMEM containing 25 mmol/L glucose supplemented with 15% FBS and 50 μmol/L 2-mercaptoethanol. Isolation of adult pancreatic progenitor cells was described in our previous report [20]. Pancreatic progenitor cells were cultured with DMEM/F12 medium containing 2% FBS and supplemented with 1×B27 (Invitrogen), 20 ng/mL EGF (R&D), 10 μg/mL insulin (Sigma-Aldrich) and 50 μmol/L 2-mercaptoethanol (Sigma-Aldrich).

miR-375 target genes prediction

miR-375 target genes were predicted using the TargetScan algorithm from TargetScan Version 6.0 (http://www.targetscan.org/).

DNA construct

A 572-bp fragment from the 3’ UTR of YAP1 (position 2846–3417, NM_001171147) containing the predicted miR-375 binding site was cloned into the SpeI and MluI sites of pMIR-report vector. The corresponding mutant construct was created by mutating the seed region of the miR-375 binding site. All constructs were confirmed by DNA sequencing.

Cells transfection

100 nmol/L (final concentration) mimic miRNA (Table 1), 100 nmol/L siRNA or 120 nmol/L miRNA inhibitor (2’-O-Me modified antisense oligonucleotides) was transfected into pancreatic progenitor cells or MIN6 cells with Lipofectamine RNAiMAX (Invitrogen) according to the manufacturer’s instructions. 48 h after transfection, cells were lysed for western blot, and total RNA was isolated for real time quantitative PCR (QRT-PCR).

Dual luciferase reporter assay

3T3 cells were plated into 24-well plates 24 h before transfection, 200ng pMIR-report containing the wild type or mutated potential target gene 3’ UTRs, 2.5ng pRL-SV40 and 30 nmol/L mimic miRNA were cotransfected into 3T3 cells by Lipofectamine 2000. Relative firefly luciferase activity (normalized to Renilla luciferase activity) was measured 48 h after transfection by using the Dual-Luciferase Reporter Assay Kit (Promega) on GloMaxTM 20/20 Luminometer (Promega).

QRT-PCR and RT-PCR

QRT-PCR was performed using the ABI 7500 sequence detection system (Applied BioSystems). β-actin was used for mRNA. All reactions were run in triplicate and included no template controls for each gene. The relative amount of each mRNA to β-actin was calculated using the 2^−ΔΔCt method. The level of significance was determined by Student’s t-test, all quantitative data presented were the mean ± SEM. Sequences of primers used for QRT-PCR and RT-PCR in this study were described in Table 2.

Western blotting

Cellular total proteins were extracted with cell lysis buffer. Protein extracts were then centrifuged at 12,000×g for 5 min at 4 °C and the supernatants were harvested for research. Proteins were separated on 10% SDS-PAGE gels and electroblotted onto a nitrocellulose membrane. The membranes were incubated with primary antibodies against YAP1 (Cell Signaling Technology), and β-actin (Boisynthesis). The antigen-antibody complex was detected using Super ECL Detection Reagent (APPLYGEN).
Table 2. Primers used to perform PCR of mRNA and miRNA

| Primer Name | Sequence |
|-------------|----------|
| Oligodt-Ambion | GCCAGCACAGAAATACACCTAAGATTTAGTTTGTGTTTGTGTTT |
| Reverse-Ambion | GCCAGCACAGAAATACACATATAC |
| u6 forward | GCCATGCTCACAGCACAGATATAC |
| u6 reverse | TTAGAGTCAGTCCGCTGTGTTT |
| mmu-miR-375 | TTAGAGTCAGTCCGCTGTGTTT |
| β-actin forward | GACGAGAGGCAGGAGGCTG |
| β-actin reverse | TCAGAGGTACGAGGAGGTCG |
| YAP1 forward | GACGAGAGGCAGGAGGCTG |
| YAP1 reverse | TCAGAGGTACGAGGAGGTCG |

**Immunofluorescent staining**

Cells were fixed in 4% fresh paraformaldehyde for 30 min followed by permeabilization using 0.3% triton X-100. After blocking for 1 h, cells were incubated in primary antibody anti-ki67 (1:500, novocastra) overnight at 4 °C. Incubation with secondary antibody FITC-conjugated IgG (1:200, Jackson Immunoresearch) was conducted for 1 h at room temperature. The cell nucleus was detected by propidium iodide (PI). Images were acquired using a fluorescent microscope equipped with a cooled three-chip charge-coupled-device camera (Carl Zeiss).

**CCK-8 assay**

For CCK-8 (Dojindo Molecular Technologies) assay, pancreatic progenitor cells were plated into 96-well plates. 72h after transfection, cells were incubated in CCK-8 solution for 2 h at 37 °C. The amount of formazan dye was measured by absorbance at 450 nm with a microplate reader.

**In situ hybridization**

The spatio-temporal expression pattern of YAP1 mRNA was analyzed by in situ hybridization. The embryonic pancreas sections were hybridized with the YAP1 probe (1 ng/μL) for 20 h at 55 °C. Post-hybridization, the sections were incubated by anti-Dig antibody for overnight at 4 °C. 20 h after immunoreactivity, the sections were placed in 125 mg/mL BCIP and 250 mg/mL NBT which are the substrate of alkaline phosphatase conjugated the anti-Dig with keeping out of sun at temperature.

**Results**

**YAP1 is expressed in embryonic and adult pancreatic progenitor cells**

Previous studies have shown that the expression and cellular localization of YAP1 overlaps with defined stem cell compartments in the single basal layer of the skin and the crypt of the intestine [8, 9]. Here, we determined the spatio-temporal expression pattern of YAP1 in the mouse embryonic pancreas (E12.5 to E17.5) by in situ hybridization. As shown in Fig. 1A, at E12.5, YAP1 mRNA was expressed highly in pancreatic epithelial cells, which contain a population of multipotent progenitor cells. This observation was consistent with the previous finding that YAP1 protein is expressed highly in E12.5 mouse pancreas [14]. Along with the pancreas development, YAP1 expression was gradually decreased during the secondary transition (from E13.5 to E15.5), in which massive cells proliferate and differentiate rapidly (Fig. 1A). The mRNA expression of YAP1 was almost undetectable at E17.5 (Fig. 1A). To further explore the expression of YAP1 in pancreatic progenitor cells, we detected YAP1 mRNA and protein in isolated mouse adult pancreatic progenitor cells. The results show that YAP1 is expressed highly in adult pancreatic progenitor cells but not seen in β-cell line MIN6 (Fig. 1B and 1C).

**Silencing YAP1 inhibits the proliferation of pancreatic progenitor cells**

Previous studies have shown that the Hippo signaling pathway is required to maintain tissue-specific progenitor cell populations in the skin, liver, and intestine, the deletion of YAP1 in these progenitor cells inhibited their proliferation [8, 9, 21]. Since our findings showed that
YAP1 was highly expressed in mouse embryonic and adult pancreatic progenitor cells, we estimated that YAP1 may participate in regulating the proliferation of pancreatic progenitor cells. To address this hypothesis, siRNA which targeted YAP1 (si-YAP1), was transfected into adult pancreatic progenitor cells. As shown in Fig. 2A and Fig. 2B, si-YAP1 significantly decreased endogenic YAP1 expression at both mRNA and protein level. CCK-8 assay results showed that YAP1 knockdown inhibited the proliferation of pancreatic progenitor cells (Fig. 2C).

Fig 1. Expression of YAP1 in early mouse embryonic pancreas and adult pancreatic progenitor cells. In situ hybridization of YAP1 mRNA during early mouse pancreas development. The time points were indicated. Scale bar, 50 μm (A); Expression of YAP1 mRNA in pancreatic progenitor cells and MIN6 cells was determined by QRT-PCR assay. Expression of mRNA was normalized to β-actin (B); Expression of YAP1 protein in pancreatic progenitor cells and MIN6 cells was determined by western blot assay. β-actin was used as a loading control (C).

Fig 2. Knocking down YAP1 inhibits the proliferation of pancreatic progenitor cells. Pancreatic progenitor cells were transfected with si-YAP1 or NC. 48 h after transfection, RNA was extracted and the expression of YAP1 was determined by QRT-PCR assay. Expression of mRNA was normalized to β-actin (A); 48 h after transfection, the total proteins were obtained and YAP1 expression was analyzed by western blot. β-actin was used as a loading control (B); 72 h after transfection, cells proliferation was evaluated by CCK-8 assay (C) and Ki67 immunostaining assay (D). Single and double stars indicated P < 0.05 and P < 0.01, respectively. Scale bar, 50 μm.
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Fig 3. Identification of mouse YAP1 as a target gene of miR-375. A schematic representation of the luciferase reporter constructs used in this study (up). Location and sequence of miR-375 target site in the mouse YAP1 3’ UTR. The sequence of mouse miR-375 was indicated, alongside the mutation introduced in the target site (underlined nucleotides) for generating mutated reporter construct (middle). Luciferase activity was determined in 3T3 cells, 48 h after transfection. All luciferase values were normalized to Renilla luciferase (down) (A). Pancreatic progenitor cells were transfected with miR-375 or NC. 48 h after transfection, the total proteins were obtained from the lysis cells and western blot determined the expression of YAP1. β-actin was used as a loading control (B); QRT-PCR assay determined the expression of YAP1, expression of mRNA was normalized to β-actin (C). The expression of miR-375 in pancreatic progenitor cells and MIN6 cells were determined by RT-PCR. U6 was used as a loading control (D). MIN6 cells were transfected with miR-375 inhibitor or NC inhibitor; 48 h after transfection, YAP1 expression was analyzed by western blot. Pancreatic progenitor cell protein was used as positive control and β-actin was used as a loading control (E); miR-375 and/or miR-375 inhibitor were transfected into pancreatic progenitor cells. 48 h after transfection, QRT-PCR assay determined the expression of YAP1, expression of mRNA was normalized to β-actin (F). Single star indicated p < 0.05.

2C). To gain deep insight, we examined the proliferation activity of pancreatic progenitor cells by Ki67 immunostaining analysis. In contrast to negative control, the ratio of Ki67 positive cells was reduced when YAP1 was repressed (Fig 2D). Taken together, these results demonstrate that YAP1 is an essential factor for maintaining proliferation of pancreatic progenitor cells.
**YAP1 is a direct target of miR-375**

Using bioinformatics predicting software Targetscan [22], we found that mouse YAP1 was a potential target gene of miR-375. To test the effect of miR-375 on the YAP1 expression, a fragment of mouse YAP1 3' UTR containing the putative target site was cloned into the downstream of the firefly luciferase stop codon of pMIR-report vector. After the recombinant vector was co-transfected with synthetic miR-375 into 3T3 cells, the luciferase activity was significantly inhibited than negative control (Fig. 3A). Then we mutated the targeting site of YAP1 mRNA 3'UTR in the pMIR-report vector. When the targeting site was mutated, the luciferase activity was rescued (Fig. 3A).

In order to validate whether miR-375 was capable of regulating mouse YAP1 expression, miR-375 or control miRNA was transfected into pancreatic progenitor cells. Western blotting results showed that the YAP1 protein was significantly inhibited by miR-375 (Fig. 3B). miRNAs can direct the RISC to downregulate target gene expression by either of two posttranscriptional mechanisms: mRNA cleavage or translational repression [23]. To verify the regulation mechanism of miR-375, we carried out QRT-PCR analysis to detect the expression of YAP1 mRNA. As shown in Fig. 3C, ectopic expression of miR-375 decreased YAP1 mRNA level. These results indicate that miR-375 regulates the expression of YAP1 by mRNA cleavage.

To further explore miR-375 repressing YAP1 in pancreatic progenitor cells differentiation. We carried out RT-PCR assay to detect the expression of miR-375 in pancreatic progenitor cells and MIN6 cells. As shown in Fig. 3D, miR-375 was expressed highly in MIN6 cells, but was undetectable in pancreatic progenitor cells. Conversely, YAP1 was highly expressed in pancreatic progenitor cells and undetected in MIN6 cells (Fig. 1B).

miR-375 inhibitor was transfected into MIN6 cells, we carried out western blot analysis to detect YAP1 expression. However, as shown in Fig. 3E, YAP1 was still undetectable when miR-375 was inhibited. The inhibitional effect on miR-375 of miR-375 inhibitor was confirmed in pancreatic progenitor cells. As shown in Fig. 3F, when miR-375 and miR-375 inhibitor were co-transfected into pancreatic progenitor cells, the expression of YAP1 was rescued.

**miR-375 inhibits the proliferation of pancreatic progenitor cells by targeting YAP1**

It has been demonstrated that miR-375 is required for normal glucose homeostasis [19]. In addition, miR-375 is involved in maintaining mouse pancreatic α and β cell mass
Previous studies indicated that the expression of miR-375 is down-regulated in human pancreatic cancer, squamous cell carcinoma, liver cancer, adenocarcinoma of the esophagus and gastric carcinomas, ectopic expression of miR-375 disturbed the proliferation of these cancer cells [25-27]. Based on the above findings that YAP1 was the target of miR-375 (Fig. 3), and silencing YAP1 inhibited the proliferation of pancreatic progenitor cells (Fig. 2C and Fig. 2D), we hypothesized that miR-375 could influence the proliferation of pancreatic progenitor cells. To address this question, miR-375 and si-YAP1 was transfected separately into pancreatic progenitor cells. As shown in Fig. 4A, the expression of YAP1 was repressed by both miR-375 and si-YAP1. Similar to silencing YAP1, CCK-8 assay showed that the proliferation of pancreatic progenitor cells was inhibited significantly by miR-375 (Fig. 4B). The inhibitional effect was further confirmed by Ki67 immunostaining analysis (Fig. 4C). Altogether, these results confirm that miR-375 inhibits the proliferation of pancreatic progenitor cells by targeting YAP1.

Discussion

YAP1 has been known to be highly expressed in ESCs, intestine progenitor cells and epidermal progenitor cells, thus regulating the organ development and tissue growth [7-9]. Both we and George et al. have found that YAP1 is expressed highly in pancreatic epithelial cells at E12.5, and then decreased along with their differentiation towards the endocrine and exocrine cells [14]. At E16.5, YAP1 expression becomes gradually limited to prospective pancreatic ductal and acinar regions, but prospective endocrine cells lack detectible YAP1 expression. Moreover, nuclear YAP1 expression remains high in the productal cells, whereas YAP1 expression in acinus-fated cells is mostly within the cytoplasm [14]. Gao et al. have reported that forced expression of YAP1 during the secondary transition of pancreas development results in a marked expansion of the ductal compartment, whereas the differentiation of acinar cells and endocrine cells is inhibited [15]. Our results also found that silencing of YAP1 by siRNA inhibited the proliferation of pancreatic progenitor cells. These findings suggest that YAP1 also plays key role for maintaining stemness in pancreatic progenitor cells, just as in ESCs, intestinal and epidermal progenitor cells.

YAP1 was highly expressed in pancreatic progenitor cells, down-regulated in differentiated pancreatic epithelia, and undetected in pancreatic β-cells indicating that YAP1 expression was repressed during pancreatic progenitor cells differentiation into β-cells. In the Hippo signaling pathway, Mst1/2 phosphorylates and activates Lats1/2. Subsequently, YAP1 is phosphorylated by activated Lats1/2, leading to cytosolic sequestration and proteasome-mediated degradation [28, 29]. It is reported that the active phosphorylated form of Mst1/2 is most readily detectable in pancreatic islets whereas YAP1 expression in pancreatic islets is undetectable [14]. Unexpectedly, YAP1 remains undetectable within pancreatic islets when Mst1/2 is Knockout [14]. So there may be other mechanism by which the expression of YAP1 is regulated in pancreatic islets besides the Hippo signaling pathway. It has been found that miR-375 is highly expressed in pancreatic islets [19]. And miR-375 is demonstrated to be located in the pancreatic ductal epithelium at E14.5, also YAP1 is found co-localized in ductal epithelium during the secondary transition [14, 30]. Both our results and Liu et al. illustrated that YAP1 was negatively regulated by miR-375 at post-transcriptional level through a specific target site within the 3' UTR in human and mouse [18]. Ectopic expression of miR-375 in pancreatic progenitor cells, not only reduced the YAP1 mRNA level, but also protein level. Based on statements above, we considered that miR-375 participated in the suppression of YAP1 expression during pancreatic progenitor cells differentiation to mature cells. However, when miR-375 was inhibited in MIN6 cells, YAP1 was still undetectable (Fig. 3E). Previous studies have shown that there are other mechanisms regulating YAP1 expression besides Hippo signaling pathway, for example, YAP1 is repressed by miR-200a in breast cancer and deltaNp63 bound to the YAP1 promoter to suppress its expression in head and neck squamous cell carcinomas [31, 32]. It is possible that multiple signaling pathways
participate collectively in repressing YAP1 in pancreatic islets.

In this study, we found that ectopic expression of miR-375 reduced the proliferation of pancreatic progenitor cells through targeting suppression of YAP1. Recent studies have shown that solid tumors including pancreatic cancer are organized and sustained by a distinct subpopulation of cancer stem cells [33]. Moreover, YAP1 overexpression has been found in many human tumors [34]. High YAP1 expression is also associated with poor prognosis of several human cancers, including non-small cell lung cancer, hepatocellular carcinomas, esophageal squamous cell carcinomas and ovarian cancer [35-38], indicating that the potential application of miR-375 in cancer therapy.

Conflict of Interests

The authors have declared that no conflict of interest exists.

Acknowledgements

This work was supported by the National Science Foundation of China (No. 31272520) and the National Science Foundation of Heilongjiang Province, China (No. C201215). The authors thank Professor Heng-Yu Fan for supplying YAP1 antibody.

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